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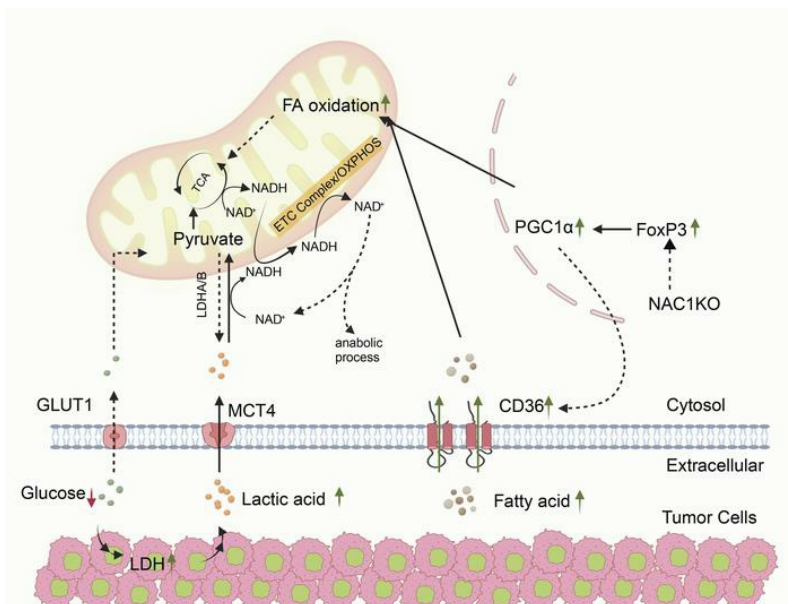
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Graphical abstract

Metabolic Fitness of NAC1-Deficient Regulatory T Cells in the Tumor Microenvironment Fuels Tumor Growth

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Running Title: Effect of NAC1-deficient Tregs on tumor growth

31   **Abstract**

32   The nucleus accumbens-associated protein-1 (NAC1) has recently emerged as a pivotal factor in

33   oncogenesis by promoting glycolysis. Deletion of NAC1 in regulatory T cells (Tregs) has been

34   shown to enhance FoxP3 stability, a suppressor of glycolysis. This study delves into the intriguing

35   dual role of NAC1, uncovering that Tregs-specific deletion of NAC1 fosters metabolic fitness in

36   Tregs, thereby promoting tumorigenesis. Our results unveil that NAC1-deficient Tregs exhibit

37   prolonged survival and heightened function, particularly in acidic environments. Mechanistically,

38   we find that NAC1-deficient Tregs adapt to adverse conditions by upregulating FoxP3 expression,

39   engaging in CD36-mediated lipid metabolism, and enhancing PGC-1α-regulated mitochondrial

40   function. In mouse tumor xenograft models, NAC1-deficient mice demonstrate increased

41   susceptibility to tumor growth. Notably, Tregs lacking NAC1 not only display elevated lipid

42   metabolism and mitochondrial fitness but also exhibit enhanced tumoral infiltration. Adoptive

43   Treg transfer experiments further underscore the supportive role of NAC1-deficient Tregs in tumor

44   growth. These findings suggest that modulating NAC1 expression in FoxP3^{+} Tregs could serve as

45   a promising approach to augment antitumor immunity. Understanding the intricate interplay

46   between NAC1 and Tregs opens avenues for potential therapeutic strategies targeting the tumor

47   microenvironment (TME).

48
49   **Keywords:** NAC1; Tregs; Cell metabolism; TME; Tumor growth

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Introduction

Nucleus accumbens-1 (NAC1), a transcription co-factor associated with cancer, is a member of the BTB/POZ (bric-a-brac tramtrack broad complex/poxvirus and zn-finger) family of nuclear proteins. NAC1 overexpression is a feature of several cancer types including ovarian, cervical and uterine cancers (1-3), and is believed to promote tumor initiation and progression (4, 5). Furthermore, High expression of NAC1 is closely associated with recurrent ovarian serous carcinoma and contributes to cancer drug resistance (6).

NAC1 is a critical regulator of glycolysis in ovarian cancer development through stabilization of HIF-1α, direct regulation of c-myc in tumor cells and subsequent regulation of cellular metabolism (7). NAC1 enhances the expression of LDHA in tumor cells leading to higher accumulation of lactic acid (LA) which resulted in altered cellular metabolism of cytotoxic T cells (CTLs) in the tumor micro environment (TME) (8). It has been shown that T-cells can switch to lactate uptake in a glucose starved TME. However, the reversal of the LDH reaction to generate pyruvate drains the cellular NAD^+ pools, effectively inhibiting GAPDH activity and glycolytic flux. The nutrient deficient tumor environment along with LDHA mediated lactic acid production create hostile condition for TILs leading to immune evasion (8-10). $FoxP3^+$ regulatory T cells (Tregs), a distinct and dynamic subset of $CD4^+$ T cells, are an essential for maintenance of immune cell homeostasis (11). Elevated levels of $FoxP3^+$ Tregs within the tumor microenvironment (TME) showed a positive correlation with poor prognosis in various cancer patients (12). Foxp3 reprograms T cell metabolism by suppressing c-myc and glycolysis, enhancing oxidative phosphorylation, and increasing NAD oxidation (13). Foxp3 drives upregulation of components of all the electron transport complexes, increasing their activity and ATP generation by oxidative phosphorylation. These adaptations provide a survival and metabolic advantage to the Tregs over effector T cells, by generating energy through FA metabolism (OXPHOS) in glucose deficient and

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74   lactate rich TME (14-17). We recently reported that NAC1 acts as a negative regulator of FoxP3

75   in Tregs, and that NAC1-deficient (KO) mice are resistant to autoimmunity and exhibit strong

76   immunosuppressive activity as compared to wild-type (WT) mice (10). We show that NAC1 KO

77   mice generate higher frequency of $CD4^+$ Tregs that exhibit a higher metabolic profile and immune

78   suppressive activity, increased acetylation and expression of FoxP3, and slower turnover of this

79   transcriptional factor (10). Because $FoxP3^+$ Tregs are critically involved in tumor development and

80   progression *via* suppressing antitumor immunity and the elevated amount of $FoxP3^+$ Tregs in

81   tumor microenvironment (TME) is positively correlated with poor prognosis in patients with

82   cancer (12), we intended to know whether and how NAC1-mediated control of Tregs and their

83   function impacts tumorigenesis. Our study found that Tregs with NAC1 deficiency are

84   metabolically more robust and functionally stronger than WT Tregs in TME, with upregulation of

85   the CD36 - PGC-1α pathway and promotes tumor growth through increasing the infiltration of

86   FoxP3 Tregs to tumors. This FoxP3 and CD36 upregulation plays a pivotal role in supporting

87   enhanced lipid metabolism, mitochondrial fitness, and biogenesis within the tumor

88   microenvironment, all through a mechanism dependent on PGC-1α. In adoptive Tregs transfer

89   experiment we confirmed that Treg specific NAC1 deficiency is sufficient to support tumor

90   initiation and growth. These results underscore the potential of targeting metabolic adaptation in

91   intratumoral Treg cells as a promising strategy for metabolic reprogramming of the TME.

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Results

Syngeneic NAC1 KO mice are prone to tumor growth

In our previous study, we reported the inhibitory role of NAC1 in FoxP3 expression within Tregs. Given FoxP3's pivotal role in Treg function and its regulatory nexus with NAC1, we set out to investigate whether NAC1 has any impact on tumor growth. To explore the role of NAC1 in tumor development, we subcutaneously inoculated 3×10^6 B16-F10 or MC38 tumor cells into the right flank of both wild-type (WT) and NAC1 knockout (NAC1 KO) mice, monitoring the progression of the tumors. Remarkably, we observed that B16-F10 cells grew significantly faster and formed larger tumors in NAC1 KO mice than in the age-matched WT mice (**Figure 1A**), leading to shorter survival of the tumor-bearing mice (**Figure 1B**). We further examined tumor tissue sections (**Figure 1C**) for lymphocyte infiltration using hematoxylin and eosin (H&E) staining (**Figure 1D**), immunofluorescence staining (**Figure 1E**), and imaging mass cytometry (IMC) analysis (**Figure 1F, 1G**). H&E-stained tissue sections demonstrated an increased lymphocyte infiltration in the MC38 tumors grown in NAC1 KO mice, as compared to that grown in WT animals (**Figure 1D**). *t*-distributed stochastic neighbor embedding (*t*-SNE) plots of the imaging mass cytometry (IMC) data confirmed that the total tumoral infiltration of FoxP3$^+$ Tregs was significantly higher in NAC1 KO mice than that in WT mice, but the infiltration of total CD4$^+$ T cells was significantly higher in WT mice than in NAC1 KO mice (**Figure 1G**). Also, the infiltration of CD8$^+$ and CD8$^+$PD1$^+$ T cells was significantly higher in tumors grown in NAC1 KO mice than in tumors grown in WT animals (**Figure 1G**), suggesting that exhaustion and reduced survival of CD8$^+$ T cells may contribute to the enhanced functional fitness of the tumor-infiltrating Tregs in NAC1 KO mice.

NAC1 negatively affects the survival and polarization of Tregs in acidic environments

117   To demonstrate the effect of NAC1 on Tregs in harsh environment, we first compared the survival

118   of NAC1 KO Tregs with that of WT Tregs. CD4$^+$CD25$^-$ conventional T cells (Tconv) were isolated

119   from FOXP3-IRES-mRFP (FIR) reporter mice, cultured under polarizing conditions to generate

120   RFP$^+$ induced Tregs (iTregs), and the optimal polarization efficiency was confirmed on various

121   days using flow cytometry (**Supplemental Figure 1A, 1B**). We also confirmed significant

122   difference of FoxP3 expression (**Supplemental Figure 1C**) in iTregs and similar IL-10

123   (**Supplemental Figure 1D**) and TGF-β (**Supplemental Figure 1E**) secretion profile in nTregs

124   and iTregs, Subsequently, we isolated CD4$^+$CD25$^-$ Tconv cells from both WT and NAC1 KO

125   mice, cultured them under identical polarizing conditions to generate iTregs. On day 5, WT and

126   NAC1 KO iTregs were cultured in media treated with 10 mM lactic acid (LA) or B16-F10

127   conditioned media (CM) for 48 hours. As NAC1 deficiency promotes FoxP3 expression (10), we

128   assessed FoxP3 expression in these cells. Notably, we observed that the untreated (70.1%), LA-

129   treated (72.2%), and CM-treated (75.1%) NAC1 KO iTregs displayed a higher polarization

130   efficiency than the untreated (57.0%), LA-treated (63.6%), and CM-treated (65.7%) WT iTregs

131   (**Figure 2A, 2B**). This observation aligns with the increased frequency of Tregs in the peripheral

132   tissues of NAC1 KO mice as compared to WT mice (10). We further examined whether expression

133   of NAC1 affects Treg proliferation. Using carboxyfluorescein succinimidyl ester (CFSE)-labeled

134   WT and NAC1 KO iTregs, we cultured them in LA-treated and CM-treated media and assessed

135   their proliferation using CFSE dilution assay. Interestingly, we found that the proliferation of both

136   WT and NAC1 KO iTregs was unaffected by LA treatment or CM treatment, suggesting that the

137   higher infiltration of Tregs in NAC1 KO mice is not associated with altered proliferation of Tregs

138   (**Figure 2E**). We also determined whether NAC1 deficiency has any effect on apoptosis, and found

139   that the untreated (15.6%), LA-treated (25.8%), and CM-treated (30.1%) NAC1 KO iTregs

140   exhibited significantly lower levels of apoptosis than the WT iTregs subjected to the same

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141 treatment (22.0%, 37.1% and 45.7% respectively) (**Figure 2C, 2D**). These results indicate that

142 NAC1 KO iTregs are significantly less apoptotic and more resistant to the stress caused by acidic

143 environment and suggest that deficiency of NAC1 may promotes the survival of Tregs in the TME,

144 thereby increasing infiltration of Tregs to tumors.

145

146 ***Loss of NAC1 leads to enhanced functional activity of Tregs***

147 We next compared the functional activity of NAC1 KO Tregs with that of WT Tregs using an *ex-*

148 *vivo* suppression assay. In this assay, we stimulated the CFSE-labeled CD8$^+$ effector T cells and

149 then co-cultured them in control media or CM with FACS-sorted Tregs isolated from the spleen

150 and lymph nodes (LN) of WT or NAC1 KO mice. The suppressive function was assessed by

151 analyzing CFSE dilution using flow cytometry. Our findings revealed that NAC1 KO Tregs were

152 significantly more suppressive compared to WT Tregs in both untreated and CM treated conditions

153 (**Figure 3A, 3B, Supplemental Figure 4A**). Further, the expression of Granzyme B (GzmB), an

154 enzyme that is highly expressed in tumor-infiltrating Tregs (18) and plays an important role in

155 increasing the metastatic burden in the lungs and eliminating conventional T cells in colorectal

156 cancer (19, 20), was significantly higher in NAC1 KO iTregs that in WT iTregs in the control

157 media, LA-containing media or CM (**Figure 3C, 3D, Supplemental Figure 3B**), suggesting that

158 GzmB is an important mediator of the suppressive capacity of NAC1 KO Tregs. Also, NAC1 KO

159 iTregs produced significantly higher amounts of suppressive cytokines, TGF-β and IL-10, than

160 WT iTregs, as evidenced by intracellular staining (**Figure 3E, 3F**). We also conducted

161 bioinformatics analysis in human tumor samples submitted in The Cancer Genome Atlas (TCGA)

162 database and we found that higher FoxP3 regulatory T cells are correlated with lower survival in

163 Kidney Renal Clear Cell Carcinoma (KIRC) (**Supplemental Figure 5A, 5B**) and Glioblastoma

164 Multiforme (GBM) (**Supplemental Figure 5C, 5D**). Furthermore, lower expression of NAC1 in

165   intra-tumoral Tregs also correlated with lower survival (**Supplemental Figure 5E**). These results

166   indicate that NAC1 has negative effects on the suppressive function of Tregs.

167

168   ***Loss of NAC1 results in upregulation of CD36 expression and elevation of lipid metabolism of***

169   ***Tregs in acidic environments.***

170   Since we observed that deletion of NAC1 prolonged the survival and enhanced the function of

171   Tregs under acidic condition (**Figure 2** and **Figure 3**) and NAC1 has a critical role in metabolic

172   reprogramming (3), we quired whether the effects of NAC1 on fitness and function of Tregs are

173   mediated through altered metabolism in Tregs. Because CD36, phagocytic receptor that mediates

174   fatty acid-induced metastasis via regulating fatty acid intake and metabolism, is selectively

175   upregulated in the intratumoral Tregs and functions as a central metabolic modulator that fine-

176   tunes mitochondrial fitness in lactic acid-rich TME (16, 21-23), we then examined the expression

177   of CD36 in the tumor-infiltrating Tregs. Tregs were isolated from the spleen (SPL), lymph nodes

178   (LN), and tumors of the B16-F10 melanoma bearing-NAC1 KO mice or WT mice, and the

179   expression of CD36 on WT and NAC1 KO iTregs was analyzed at 24 hours, 48 hours, and 72

180   hours following treatment with 10 mM of LA. Interestingly, NAC1 KO iTregs exhibited

181   significantly higher CD36 expression than WT iTregs at 48 hours (1.09% *vs*. 0.43%) and 72 hours

182   (3.79% *vs*. 1.16%) following the treatment, respectively, although the expression was similar at 24

183   hours after treatment. **(Supplemental Figure 2A, 2B).** Notably, tumor associated Tregs showed

184   significantly higher expression of CD36 than normal Tregs from the SPL and LN (**Supplemental**

185   **Figure 2C, 2D**). These results indicate that the deletion of NAC1 causes an increased CD36

186   expression under acidic conditions. Because NAC1 has a role in promoting expression of fatty

187   acid synthase (24) and CD36 participates in regulation of lipid metabolism (25), we next

188   determined whether the increased expression of CD36 in NAC1 KO Tregs was associated with

8

189   altered lipid metabolism. We examined lipid uptake through BODIPY FL C12 staining (BODIPY

190   500/510) and neutral lipid content through BODIPY (BODIPY 493/503) staining in iTregs treated

191   with LA or CM. Our experiments found a significantly higher level of neutral lipids in NAC1 KO

192   iTregs treated with CM than that in WT iTregs treated with CM, as evidenced by the staining of

193   BODIPY 493/503 (**Figure 4A**). Similarly, NAC1 KO iTregs internalized a significantly higher

194   amount of BODIPY 500/510 than WT iTregs under all the conditions (untreated, LA-treated, and

195   CM-treated (**Figure 4B**), with the most pronounced increase observed in the CM-treated cells

196   (**Figure 4C, 4D**). Furthermore, we analyzed the expression of the transcription coactivator

197   peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A, also known

198   as PGC-1α), which is a master regulator of lipid metabolism and fatty acid transporters (such as

199   FAT/CD36 and FABP3). and mitochondrial biogenesis and coordinates with enhanced oxidative

200   phosphorylation and the electron transport chain (26, 27). Our analysis showed that compared with

201   the untreated, lactic acid-treated, or conditioned media-treated WT iTregs with NAC1 KO iTregs,

202   the level of PGC-1α in the CM-treated NAC1 KO iTregs was significantly higher than that in the

203   corresponding WT iTregs, although both WT and NAC1 KO iTregs exhibited a significantly

204   increased PGC-1α expression when treated with CM (**Figure 4E, 4F**). These results indicate that

205   NAC1 depletion is associated with increased PGC-1α expression, CD36 expression, and enhanced

206   fatty acid transport and metabolism, rendering NAC1 KO Tregs metabolically more active and

207   better adapted to the environmental stress. These results demonstrate the role of NAC1 in

208   modulating Treg metabolism, especially in acidic environments, and imply the metabolic

209   adaptability of NAC1 KO Tregs in an unfavorable TME.

210

211   ***Loss of NAC1 improves mitochondrial fitness of Tregs***

212   PGC-1α is a key regulator of mitochondrial biogenesis and coordinates with enhanced oxidative

213   phosphorylation and the electron transport chain, playing a crucial role in cellular energy and

214   metabolism (26, 27). Our observation of the upregulated PGC-1α and FoxP3 in NAC1 KO iTregs

215   prompted us to determine the effects of NAC1 deficiency on energy metabolism and mitochondrial

216   fitness in Tregs following LA or CM treatment. We found that mitochondrial respiration, as

217   indicated by the oxygen consumption rate (OCR) or oxidative phosphorylation (OXPHOS), was

218   significantly higher in NAC1 KO iTregs following treatment with LA or CM for 48 hours, as

219   analyzed by the mitochondrial stress test using oligomycin, FCCP and rotenone/antimycin A in a

220   Seahorse bioanalyzer (**Figure 5A**). We also examined different components of mitochondrial

221   respiration, including basal respiration, ATP-linked respiration, maximal respiration, and spare

222   respiratory capacity, which reflect mitochondrial and cellular fitness (28, 29). We did not observe

223   any significant difference in basal respiration between the untreated-NAC1 KO iTregs and WT

224   iTregs (**Figure 5B**); however, both the spare respiratory capacity (the ability of cells to respond

225   to changes in energetic demand) (**Figure 5C**) and maximal respiration (reflecting maximum

226   capacity of the electron respiratory chain) (**Figure 5D**) were significantly higher in NAC1 KO

227   iTregs than that in WT iTregs treated with LA or CM. These results imply that NAC1 KO Tregs

228   are more capable of meeting high energy demands in the acidic environment. Furthermore, we

229   examined the mitochondrial membrane potential of the Tregs using JC-1 staining. We observed

230   that the mitochondrial membrane potential of NAC1 KO iTregs was significantly higher than that

231   of WT iTregs in the presence of LA or CM (**Figure 5E-5H**). These results provide additional

232   evidence that NAC1-deficiency in Tregs appears to contribute to increased mitochondrial fitness

233   and biogenesis, enabling them to better meet the energy demands within the TME.

234

235   **Loss of NAC1 in Tregs facilitates their tumor infiltration and supports tumor progression**

236   Considering the crucial impact of NAC1 deletion on mitochondrial respiration (OXPHOS) and the

237   observed increases in OCR, FoxP3 expression, PGC1-α expression, lipid metabolism, and overall

238   metabolic fitness in NAC1-deficient Tregs in acidic conditions, we further validated our findings

239   by examining CD36 expression, lipid uptake, PGC-1α expression, GzmB expression, and Treg

240   infiltration in B16-F10 tumors isolated from WT and NAC1KO mice. To assess lipid uptake and

241   CD36 expression, we first digested the tumor tissue using a tumor dissociation kit (Milteni Biotec),

242   isolated CD3 cells using a CD3 selection kit (BioLegend), and then stained these isolated CD3

243   cells with BODIPY 500/510 and CD36. lipid uptake (BODIPY 500/510) was significantly higher

244   in the intratumoral NAC1 KO Tregs than that in the intratumoral WT Tregs (**Figure 6A, 6B**).

245   Similarly, CD36 expression of the intratumor NAC1 KO Tregs was significantly higher than that

246   of WT Tregs (**Figure 6C, 6D**). Interestingly, both the intratumoral WT Tregs and NAC1 KO Tregs

247   had significantly higher CD36 expression than the splenic Tregs (**Figure 6C, 6D**). Moreover, the

248   infiltration of Tregs into the tumors was significantly higher in NAC1 KO mice (52.1%) than in

249   WT mice (31.4%) (**Figure 6E**). This observation was further confirmed by immune staining of

250   tumor tissue sections, which revealed a higher infiltration of CD36-expressing Tregs in NAC1 KO

251   mice (**Figure 1E**). Additionally, NAC1 KO intratumoral Tregs displayed higher expression of

252   FoxP3 (**Figure 6G, 6J**), GzmB (**Figure 6I, 6L**), and PGC-1α (**Figure 6H, 6K**) compared to WT

253   intratumoral Tregs. These results confirmed that NAC1 deletion in Tregs lead to enhanced lipid

254   metabolism and mitochondrial biogenesis. Additionally, the enhanced infiltration and suppressive

255   effects of NAC1-deficient Tregs in the TME suggest that NAC1 deficiency in Tregs may

256   contribute to faster tumor initiation and growth in NAC1 KO mice compared to WT mice.

257

258   ***Specific depletion of NAC1 in Tregs is a critical factor that supports tumor progression***

259   To further substantiates the role NAC1-deficient Tregs and its suppressive function of in tumor

260   progression, we conducted an adoptive transfer experiment in which the WT iTregs or NAC1

261   iTregs (Thy1.2$^+$) were transferred into Thy1.1$^+$ congenic recipient mice on day 1, and on the

262   following day, B16-F10 melanoma cells were *s.c.* injected into the flank of the recipient mice

263   (**Figure 7A**). Remarkably, the tumors in Thy1.1$^+$ congenic recipient mice that received NAC1 KO

264   iTregs grew significantly faster than the tumors in mice that received WT iTregs (**Figure 7B**),

265   leading to shorter survival (**Figure 7C**). Similarly, MC38 tumors also exhibited significantly faster

266   growth in mice receiving NAC1 KO iTregs than tumors in mice receiving WT iTregs

267   (**Supplemental Figure 3A**). In another experiment, WT iTregs or NAC1 KO iTregs (Thy1.2$^+$)

268   were adoptively transferred into Thy1.1$^+$ congenic recipient mice following B16-F10 tumor

269   engraftment. The results demonstrated that NAC1 KO iTregs promoted tumor growth.

270   (**Supplemental Figure** 7). These experiments demonstrate that loss of NAC1 in Tregs causes their

271   metabolic reprogramming and enhances robustness of mitochondria in an acidic TME, leading to

272   increases of Treg infiltration. The enhanced PGC-1α expression leads to increased lipid

273   metabolism, and mitochondrial fitness within the TME and contributing to tumor progression

274   (**Figure 7D**).

Discussion

NAC1 promotes glycolysis and the survival of hypoxic tumor cells, possibly through the direct regulation of c-Myc. Deletion of NAC1 in tumor cells leads to oxidative stress, reduced LDHA activity, and enhanced infiltration of cytotoxic T lymphocytes (CTLs) within the tumor mass (8). Compared to Tconv, Tregs have a significantly reduced NAC1 expression. Deletion of NAC1 results in increased acetylation of FoxP3, leading to enhanced FoxP3 expression and the suppressive function of Tregs. NAC1 deletion was found to impair T cell development in the thymus. However, in peripheral blood and secondary lymphoid organs, Treg function is primarily regulated by FoxP3, which is upregulated in the absence of NAC1. These findings suggest that while NAC1 influences broader aspects of T cell biology, its effects on Tregs are largely FoxP3-dependent (10). Additionally, the critical role of NAC1 in memory T cell development had been also recently reported (4, 9). Prompted by our recent findings that NAC1 is a critical suppressor of Treg development and function, and this role of NAC1 is mediated through epigenetic regulation of FoxP3 expression and Treg stability (10), in the current study we investigated the implications of the NAC1-mediated control of Tregs in tumor progression. We show here that Tregs with deficiency in NAC1 have enhanced metabolic capacity to adapt to the acidic TME, primarily through CD36/PGC-1α-driven enhancement of mitochondrial fitness and lipid metabolism, and NAC1-deficient Tregs promote tumor growth by increasing their tumor infiltration and strengthening their suppressive function within the TME (**Figure 7D**).

Our analysis of Tregs from WT and NAC1 KO mice found that in the LA-containing media or CM, the survival of NAC1 KO Tregs is prolonged as compared with that of WT Tregs (**Figure 2C, 2D**), suggesting that NAC1 deficiency confers a survival advantage to Tregs, allowing them to thrive in the TME. This is likely due to the upregulation of FoxP3 expression in NAC1 KO Tregs which reprograms T cell metabolism by suppressing glycolysis but promoting OXPHOS,

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299 rendering Tregs resistant to lactate inhibition. The increased NAD:NADH ratio in Tregs, driven

300 by FoxP3-mediated metabolic changes, may enable them to effectively utilize lactate and convert

301 it into pyruvate and favor Tregs to survive in the TME where lactate is abundant. Indeed, we show

302 that lipid metabolism and mitochondria activity are enhanced in Tregs deficient in NAC1 when

303 cultured in the LA-rich media or CM (**Figure 3**). We also found that loss of NAC1 in Tregs results

304 in a significant increase in CD36 expression, a key fatty acid receptor, in Tregs cultured in CM

305 (**Supplemental Figure 2**) or on the tumoral Tregs (**Figure 6**). Selective upregulation of CD36 in

306 intratumoral Tregs and its role as a central metabolic modulator that finely tunes mitochondrial

307 fitness in the context of lactic acid-rich TME were reported previously (16, 21-23). This

308 upregulation was accompanied by an increased fatty acid uptake, as evidenced by BODIPY

309 500/510 staining (**Figure 4A**) and by an increase in neutral fat content, as indicated by BODIPY

310 493/503 staining, particularly in CN-treated or intratumoral NAC1 KO Tregs (**Figure 6A, 6B**).

311 Consistent with these findings, we also observed the upregulation of PGC-1α, a critical regulator

312 of mitochondrial biogenesis in NAC1 KO Tregs. In Seahorse analysis, we found that NAC1 KO

313 Tregs display significantly higher levels of maximal respiration and spare respiratory capacity

314 cultured in CM (**Figure 5**). These observations imply that depletion of NAC1 leads to

315 upregulations of expressions of PGC-1α, CD36, and fatty acid transport and metabolism,

316 collectively endowing NAC1 KO Tregs with increased metabolic vigor and adaptability within the

317 acidic TME.

318   Furthermore, NAC1 KO Tregs show a significant enhancement in their suppressive

319 function, as demonstrated by *in vitro* suppression assays (**Figure 3**) and evidenced by a substantial

320 increase in GzmB and TGF-β in NAC1 KO Tregs, particularly when cultured in CM (**Figure 3C,**

321 **3D**) or in the tumoral NAC1 KO Tregs (**Figure 3E, 3F**), implying that NAC1-deficient Tregs

322 possess higher suppressive activity over CTLs within the acidic TME. These results may explain

323 our *in vivo* experiments showing that absence of NAC1 in Tregs supports tumor growth in B16

324 melanoma (**Figure 7, Supplemental Figure 7**) and MC38 colon carcinoma models

325 (**Supplemental Figure 3A**). Additionally, IMC analysis of the tumor tissue sections shows that

326 the increased tumor infiltration of Tregs is associated with an increase in apoptotic CD8$^+$ cells

327 expressing the PD1 marker (CD8$^+$ PD1$^+$) (**Figure 1F, 1G**). Together, these experiments pinpoint

328 the role of NAC1 in controlling the survival, metabolic fitness, and suppressive function of Tregs,

329 all are causally associated with immune evasion and the TME (14-17).

330   It is worth noting that the mice employed in this study were subjected to complete knockout

331 of the *NACC1* gene, therefor, the deficiency of NAC1 in other types of cells may also affect tumor

332 progression. In particular, the precise roles of NAC1 in other T cell subtypes, including CD8$^+$ T

333 cells and conventional CD4$^+$ T cells (e.g., Th1, Th2, Tfh, and Th17), and in innate immune cells

334 like macrophages and dendritic cells, are relatively unexplored. Thus, the exact impact of NAC1

335 on tumor development and progression remains to be further delineated.

336

Materials and Methods

Sex as a biological variable

Sex was not considered as a biological variable; both female and male mice were used.

Cell lines and mice

The B16-F10 (CRL-6475) melanoma cell line and MC38 CEA colon adenocarcinoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and used for experiments when in the exponential growth phase. All reagents were from Sigma-Aldrich (St Louis, MI). C57BL/6 (B6), Foxp3-IRES-mRFP (FIR) reporter and Thy 1.1[+] congenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME). NAC1 KO mice were generated by Dr. Jianlong Wang and crossed in the B6 background for more 10 generations (10). All the animal experiments were performed in compliance with the regulations of The Texas A&M University Animal Care Committee (IACUC no. 2018-0065) and in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

T cell culture and proliferation/cell division

WT and NAC1 KO T cells isolated from mice using T cell isolation kits including mouse CD4^{+} (no. 130-104-454), CD8a^{+} (no. 130-104-075) and CD4^{+}CD25^{+} Treg (no. 130-091-041) and were activated by anti-mouse CD3 antibody (clone 2C11; BioLegend, San Diego, CA) plus anti-mouse CD28 antibody (clone 37.51; BioLegend, San Diego, CA) in RPMI 1640 media with 10% FBS (Omega Scientific, CA) and monitored for their survival by trypan blue cell exclusion method using a TC20 automated cell counter (Bio-Rad, USA). *In vitro* T cell survival was determined

360 using trypan blue exclusion. Proliferation/division of T cells was measured using the CellTrace

361 CFSE Cell Proliferation Kit (no. C34554, Invitrogen).

362

363 *Cancer cell-conditioned medium and iTreg cell culture*

364 iTreg cells were generated by activating naïve CD4$^+$ T cells with anti-CD3 (Clone#145-2C11) and

365 anti-CD28 (Clone #37.51) monoclonal antibodies (BioLegend) in RPMI 1640 media

366 supplemented with 10% FBS, 5 ng/ml transforming growth factor-β (TGF-β) and 5 ng/ml

367 interleukin-2 (r-IL-2) for 3 days. Then, activated CD4$^+$ T cells were maintained in RPMI media

368 plus 10% FBS and 10 ng/ml for another 2 d. Differentiated iTreg cells were first sorted using a

369 fluorescence-activated cell sorting (FACS) cell sorter and then incubated in cancer cell-

370 conditioned medium and under the indicated culture conditions for 48 hours. Control RPMI 1640

371 for the treatment of iTreg cells in vitro was prepared with RPMI 1640 medium (Biological

372 Industries) supplemented with 2 mM glucose, 10 mM glutamine, 10% dialyzed FBS, 0.1% β-

373 mercaptoethanol and the indicated concentrations of lactic acids as we previously described (8).

374 B16-F10 cancer cell-conditioned medium was collected by incubating B16-F10 cells (70–80%

375 confluent) with the control RPMI 1640 described above for 18 hours. Then, the culture medium

376 was collected and centrifuged at 200 × g. for 15 minutes to remove debris and cancer cells as

377 cancer cell-conditioned medium. B16-F10 cancer cell conditioned medium collected as described

378 above was passed through with 0.2 μm membrane filter before Treg cell culture at a volume ratio

379 of 1:3.

380

381 *In vitro mouse Treg generation*

382 CD4$^+$CD25$^-$ naive CD4 T cells sorted from spleen and LNs of WT or NAC1 KO or FIR mice from

383 CD4 cells enriched using a negative selection kit (MojoSort Mouse naive CD4 T Cell Isolation

17

384   Kit; BioLegend). iTreg cells were generated by activating naive CD4$^+$ T cells isolated from spleen

385   and LNs of WT or NAC1 KO mice with anti-CD3 plus anti-CD28 monoclonal antibodies

386   (BioLegend) in RPMI 1640 media supplemented with 10% FBS, 5 ng/ml TGF-β and 5 ng/ml rIL-

387   2 for 3 days. Then, activated CD4$^+$ T cells were maintained in RPMI 1640 media plus 10% FBS

388   and 10 ng/ml for another 48 hours. Efficiency of iTreg differentiation was determined by FACS

389   analysis.

390

391   *Flow cytometry, cell sorting and antibodies*

392   Single-cell suspensions were incubated with TruStain FcX™ (anti-mouse CD16/32) antibodies

393   (BioLegend) on ice for 10 minutes before staining. Cell suspensions were first stained using a

394   LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Thermo Fisher Scientific) or Zombie NIR™

395   Fixable Viability Kit (BioLegend) or Zombie Aqua™ Fixable Viability Kit (BioLegend) at 37 °C

396   for 10 min. After washing, surface markers were stained for 30 minutes at 4 °C. Intracellular

397   staining was performed after incubation of single-cell suspensions with BD GolgiStop from BD

398   Biosciences (#AB_2869012, San Diego, CA, USA) in medium for 4 hours using Intracellular

399   Staining Permeabilization Wash Buffer and Fixation Buffer from BioLegend (#421002, San

400   Diego, CA, USA). Apoptosis was determined by staining with Apotracker™ Green (#427402).

401   Samples were analyzed on BD Fortessa X-20 flow cytometers (BD Biosciences) and data were

402   analyzed with FlowJo as gating strategy shown in **Supplemental Figure** 6. Cells were sorted on

403   FACSAria III sorter (BD Biosciences). Tregs were defined by the following staining:

404   Live/Dead$^-$CD45$^-$CD3$^+$CD4$^+$CD8$^-$CD25$^+$ FoxP3$^+$. CD8 T cells were defined by the following

405   staining: CD45$^+$CD3$^+$CD8$^+$CD4$^-$. The following antibodies against mouse proteins were used:

406   anti-CD45 (Clone #30-F11), anti-CD3ε (Clone #17A2), anti-CD4 (Clone #RM4-5), anti-CD8α

407   (Clone #53.6.7), anti-CD44 (Clone #IM7), anti-CD4 (Clone #GK1.5), anti-CD8 (Clone #53-6.7),

408   anti-CD25 (Clone #3C7), anti-CD36 (Clone #HM36), anti-FoxP3 (Clone #MF-14), anti-GFP/YFP

409   (Clone #FM264G), Thy1.2 (Clone #140331), anti-PGC-1α, and anti-a. These antibodies were

410   purchased from BioLegend, Thermo Scientific, eBiosciences or Cell Signaling.

411

412   *Tumor engraftment and Murine melanoma models*

413   Before tumor induction, 8–10-week-old mice were shaved on back on the skin surface, to induce

414   tumor formation. For tumor engraftment, 3×10^6 cells B16-F10 or 3×10^6 MC38 tumor cells in 100

415   μl phosphate-buffered saline were injected subcutaneously into the right flank of B6. Thy1.1 or

416   WT or NAC1KO mice. Tumors were measured every 2–3 days after tumor engraftment and

417   calculated. Tumor volumes were calculated by volume = (length × width^2)/2 for engrafted tumors

418   or volume = (length × width × height) for inducible tumors. When mice-bearing tumors reach a

419   maximum size of 2,000 mm^3, tumors were prepared for analysis. All experiments were conducted

420   according to Swiss federal regulations.

421

422   *Tumor digestion and cell isolation*

423   Tumors were washed with PBS and minced into small pieces in RPMI containing the enzyme mix

424   of mouse tumor dissociation kit (Miltenyi Biotec; #130-096-730). Tumor digestion was done

425   according to the manufacturer protocol. Single cell suspension was filtered through a 100 μm cell

426   strainer and washed with PBS. Tumor cells were incubated with RBC Lysing Buffer (BD) to lyse

427   red blood cells and then washed with FACS buffer (phosphate-buffered saline with 2% FBS and

428   2 mM EDTA). Tumor-infiltrating T cells were further enriched by CD3 cells selection kit

429   (BioLegend) as described by manufacturer. Cells were further stained and analyzed by FACS

430   analysis.

431

432   *Imaging mass cytometry (IMC) analysis*

433   IMC uses heavy metal label–conjugated antibodies, greatly enhancing the deep

434   immunophenotyping analysis of tumor samples. A dimensionality reduction technique, *t*-SNE,

435   was used to analyze several tumor-associated immune cell markers in the WT *vs*. NAC1 KO

436   groups of mice. A bar graph of the number of cells per neighborhood across the imaged tumor

437   samples were constructed to analyze the local cell densities within individual neighborhoods as

438   described previously (30). Ir191, Er167, Dy162, Er170Sm149, and Yb176 were used for staining

439   DNA, Ki-67 Ag, $CD8^+$ T cells, B220 (B cells), CD11b (dendritic cells), and F4/80 (macrophages),

440   respectively.

441

442   *Mitochondrion, fatty acid uptake and lipid content assay*

443   To measure mitochondrial membrane potential, cells were washed and incubated with prewarmed

444   (37 °C) staining solution (DPBS with 1% FBS) and stained with JC-1 assay kit (M34152; Thermo

445   Scientific) at working concentrations of 1 μM for 30 minutes as suggested by manufacturer. After

446   staining, the cells were washed and resuspended in fresh FACS buffer for surface marker staining,

447   as described above. To measure fatty acid uptake, cells were incubated in RPMI 1640 medium (or

448   human T cell culture medium) containing C1-BODIPY 500/510 C12 (Life Technologies) at a final

449   concentration of 0.5 μM for 15 minutes at 37°C. After incubation, cells were washed with FACS

450   buffer for surface staining. For lipid content detection, after permeabilization and fixation, cells

451   were stained using BODIPY 493/503 (Life Technologies) at a final concentration of 500 ng/ml

452   together with other intracellular markers.

453

454   *Adoptive T cell transfer*

455   Naive CD4$^+$ T cells were harvested using a combination of negative magnetic selection (MojoSort

456   Mouse CD4 T Cell Isolation Kit; BioLegend) and FACS sorting (>98% purity) from spleen and

457   LNs of WT or NAC1 KO mice and cultured in polarizing condition to Treg as mentioned above.

458   On day 0, approximately 3 x 10^6 WT and NAC1 KO iTreg (day 5) were *i.p.* injected into two

459   separate groups of Thy1.1 mice (n=5) prepared for tumor engraftment. On day 1, 3 x 10^6 B16-F10

460   melanoma or MC32 CEA tumor were *s.c.* injected into each of the recipient mice of both groups

461   and tumor progression were monitored. The mice were monitored for survival and tumor size up

462   to day 28 after tumor induction. The experiment was terminated on day 28, and the explanted

463   tumor was analyzed by flow cytometry, image mass cytometry, and confocal microscopy as

464   described (30).

465

466   *Tumor imaging and immunohistochemistry*

467   Tumor tissues were fixed with 10% neutral formalin solution (VWR, West Chester, PA, USA) for

468   24 hours. and placed in the labeled cassettes for further embedding in molten paraffin wax. From

469   the fixed samples in paraffin block tissue sections at a thickness of 3–5 μm were prepared and

470   stained with H&E as described. For immunofluorescence microscopy, the tissues were frozen in

471   cryovials on dry ice immediately following resection. Cryo-sectioning and immunofluorescence

472   staining were performed as described (30). Alexa Fluor 488-CD36 (Abcam), Alexa Fluor 647-

473   FoxP3 (BioLegend) and eFluor 570-Thy1.1 (Thermo) from BioLegend (San Diego, CA) were used

474   to detect the tumor-infiltrating Tregs.

475

476   *Ex vivo Treg suppression assay*

477   CD8$^+$ T cells from the spleens and LNs of Thy1.1 mice were enriched using a negative selection

478   kit (MojoSort Mouse CD8 T Cell Isolation Kit; BioLegend) and stained with a CellTrace CFSE

479 Cell Proliferation Kit (Thermo Fisher Scientific) for 10 minutes at RT. A total of 2.5×10^4 CD8$^+$

480 cells were seeded into a CD3 (4 μg/ml) coated 96-well round plate in RPMI 1640 medium

481 containing of 4 μg/ml CD28. CD4$^+$CD25$^+$ Tregs sorted from spleen and LNs of WT and NAC1

482 KO mice. CD4 T cells enriched using a negative selection kit (MojoSort Mouse CD4 T Cell

483 Isolation Kit; BioLegend) were added according to the indicated ratios for Treg to effector T cell

484 (Teff). Then 5 ng/ml rIL2 were supplemented into cultures media. As negative controls,

485 CD4$^+$CD25$^+$ Tregs and CD4$^+$CD25$^-$ responder T cells were cultured without any stimulus. Cells

486 were incubated at 37 °C under 5% CO2 for 72 hours and then the proliferation of CD8$^+$ T cells

487 was determined by CFSE dilution with flow cytometry analysis and Suppression of responder T

488 cells was determined.

489

490 *Metabolic profiling: Seahorse Assay*

491 Seahorse XF Cell mito stress test was performed with a Seahorse XF96 Extracellular Flux

492 Analyzer using Seahorse XF Cell mito stress test kit (#103010-100; Agilent) according to the user

493 guides provided with kit. Approximately 2×10^5 cells iTregs were plated in the Cell-Tak–coated

494 Seahorse Bioanalyzer XFe96 culture plates in assay medium consisting of minimal RPMI 1640

495 medium supplemented with 1% bovine serum albumin (BSA) and 25 mM glucose, 2 mM

496 glutamine and 1 mM sodium pyruvate. Basal rates were taken for 30 min and a set of drugs,

497 oligomycin (2 mM), FCCP (0.5 mM), and rotenone/antimycin A (0.5 mM) were injected into each

498 sample at different times and readings were measured at every 3 minutes for 1 to 2 hours. Each

499 condition was analyzed, with 6-12 replicates in each single experiment. The Extra Cellular

500 Acidification Rate (ECAR) was measured in the glycolytic rate and the Oxygen Consumption Rate

501 (OCR) was tested to indicate oxidative phosphorylation.

502

503   *TCGA bioinformatics analysis*
504

505   The bioinformatic analysis was conducted using data collected from The Cancer Genome Atlas

506   (TCGA) database, accessed through the GEPIA2 web server (http://gepia2.cancer-pku.cn/). Two

507   specific TCGA subtypes were analyzed: Kidney Renal Clear Cell Carcinoma (KIRC) and

508   Glioblastoma Multiforme (GBM). The focus of the analysis was on the expression of regulatory T

509   cells (Tregs), annotated by the markers FOXP3, CTLA4, CCR8, and TNFRSF9. These curves

510   were generated to compare high versus low expression groups for each marker, providing insights

511   into the prognostic significance of Treg expression in KIRC and GBM. The overall survival (OS)

512   and disease-free survival (DFS) rates associated with the expression levels of these Treg markers

513   were illustrated using Kaplan-Meier survival curves. Statistical analyses were performed to assess

514   the significance of survival differences between the high and low expression groups. The log-rank

515   test was employed to determine the p-values, and a p-value of less than 0.05 was considered

516   statistically significant.

517

518   *Statistical analysis*

519   Paired or unpaired Student's t-test or one-way or two-way ANOVA was performed to analyze the

520   differences between the groups, using GraphPad Prism (GraphPad Software, San Diego, CA). For

521   mice survival curve analysis, the Kaplan-Meier method was adopted and compared statistically

522   using the log-rank test in GraphPad Prism. Each point represented a biological replicate, and all

523   data are presented as means ± s.d. or means ± s.e.m., as indicated. *P* values (***$P < 0.001$, **$P <$

524   0.01$ and *$P < 0.05$) are labeled in the figures. *$P < 0.05$ was considered statistically significant.

525   The illustrations and schematic representations in figures are created by using the BioRender

526   software.

527

528 *Data availability*

529   The datasets shown in all the figures are listed in an associated spreadsheet of Supporting Data

530   Values.

531

562

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647

Figure Legends

649 **Figure 1. NAC1 deficiency supports tumor growth in mice.** B16F10 melanoma cells (3 x 10^6

650 cells/mouse) were *s.c.* inoculated into WT and NAC1 KO mice and the tumors were harvested for

651 histology examination. (**A**) Tumor growth curves (n=5). (**B**) Representative survival curve was

652 plotted. (**C-E**) Tumors were harvested and examined for the infiltration of lymphocytes by H&E

653 staining (**D**) and) FoxP3-expressing Tregs by IHC staining (**E**). (**F**) *t*-SNE plot showing

654 quantification of infiltrating immune cells population in WT *vs*. NAC1 KO generated by IMC

655 analysis was performed with the tumor tissue section. (**G**) Quantification of infiltrating immune

656 cell population analyzed by IMC analysis.

657

658 **Figure 2. NAC1 KO Tregs show prolonged survival and enhanced polarization in acidic**

659 **environments.** (**A**) Representative flow cytometry shows FoxP3$^+$CD25$^+$ Treg frequency

660 following LA and CM treatment. (**B**) Proliferation of Tregs as determined by CFSE staining (n=3).

661 (**C**) Representative plots of Apotracker green and Live-Dead expression on the WT and NAC1

662 KO Tregs treated with LA or CM for 48 hours (n=3).

663

664 **Figure 3. NAC1 KO Tregs show enhanced functional activity of Tregs in acidic**

665 **environments.** (**A**) CD8 cells were labelled with CFSE and co-cultured with WT or NAC1 KO

666 Tregs (1:1) in the presence of anti-CD3 and CD28 antibodies. Histogram of representative

667 experiment showing the proliferation CD8 cells in the CM-treated culture. (**B**) Quantification

668 analysis of the in vitro suppression assay (n=3). (**C**) Representative histogram of the expression of

669 GrazB in WT and NAC1 KO Tregs after 48-hour treatment with LA or CM (n=5). (**D**)

670 Quantification of differential expression of GrazB in WT *vs*. NAC1 KO Tregs after the indicated

671 treatment for 48 hours (n=5). (**E**) Representative histogram of TGF-β expression in WT Tregs and

672   NAC1 KO Tregs after 48-hour treatment with LA or CM (n=5). (**F**) Quantification of differential

673   expression of TGF-β in WT Tregs *vs.* NAC1 KO Tregs after the indicated treatment for 48 hours

674   (n=5).

675

676   **Figure 4. NAC1 KO Tregs have elevated lipid uptake and neutral lipid content in an acidic**

677   **condition.** (**A**) Representative histogram of lipid content measured by BODIPY 493/503 staining

678   in Tregs in the indicated culture conditions. (**B**) Representative histogram of fatty acid uptake

679   measured by C1-BODIPY 500/510 C12 staining in Tregs in the indicated culture conditions. (**C**)

680   Quantification of fatty acid uptake (C1-BODIPY 500/510 C12; n=5). (**D**) Quantification of lipid

681   content (BODIPY 493/503; n=5). (**E**) Representative histogram of PGC-1α expression in WT and

682   NAC1 KO Tregs in the indicated culture conditions. (**F**) Quantification of PGC-1α expression in

683   WT and NAC1 KO Tregs in the indicated culture conditions. The differences were analyzed by

684   Two-way ANOVA with multiple comparisons correction using GraphPad prism software. ***:

685   *p*≤0.001.

686

687   **Figure 5. NAC1 KO Tregs show enhanced mitochondrial respiration in an acidic**

688   **environment.** (**A**) Effect of LA or CM treatment on mitochondrial respiration of WT and NAC1

689   KO Tregs, as measured by Seahorse XFe96 Metabolic Analyzer. Data are represented as mean ±

690   SEM; n=6 per condition from two independent experiments. (**B**) Basal respiration. (**C**) Spare

691   respiratory capacity. (**D**) Maximum respiration quantified by seahorse wave 3.0 software. The

692   differences were analyzed by Two-way ANOVA with multiple comparisons correction using

693   GraphPad prism software. ***: *p*≤0.001. (**E – H)** The mitochondrial membrane potential of WT

694   *vs.* NAC1 KO Tregs measured by JC-1 staining. (**E**) Untreated. (**F**) 10mM LA. (**G**) CM. (**H**)

695   Quantification of mitochondrial membrane potential (n=5. The differences were analyzed by Two-

696 way ANOVA with multiple comparisons correction using GraphPad prism software. ***:
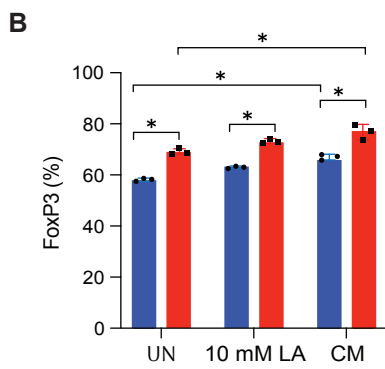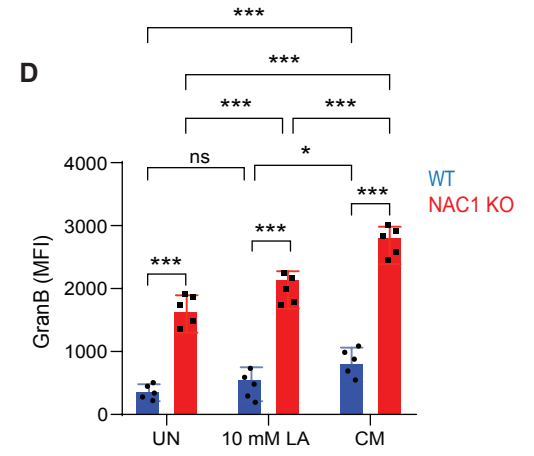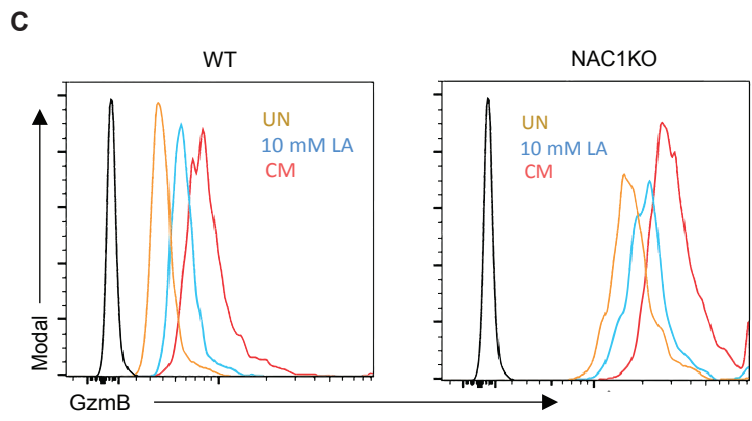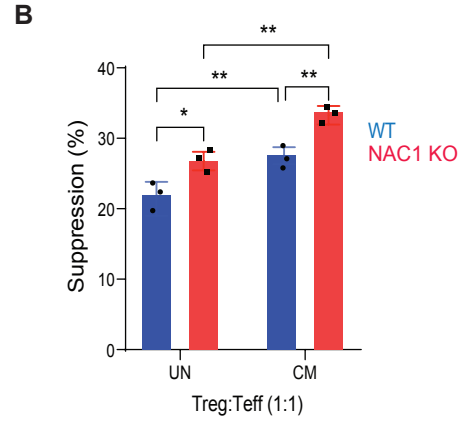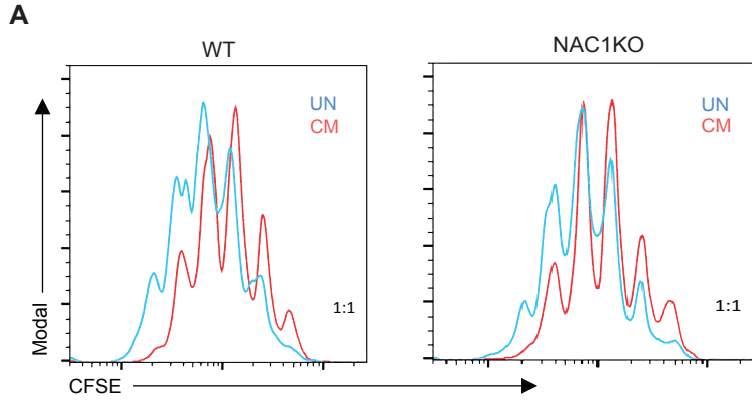
697 $p \leq 0.001$.

698

699 **Figure 6. NAC1 KO Tregs are robust in the TME.** B16F10 tumors from WT or NAC1 KO mice

700 were harvested, dissociated, and the tumoral Tregs were stained and analyzed by Flow cytometry.

701 (**A, B**) Fatty acid uptake of intratumoral Tregs and splenic Tregs as measured by C1-BODIPY

702 500/510 C12. (**A**) Representative histogram; (**B**) Quantification of fatty acid uptake. (**C, D**) CD36

703 expression in intratumoral Tregs and splenic Tregs. (**C**) Representative histogram; (**D**)

704 Quantification of CD36 expression (n=3). The differences were analyzed by Two-way ANOVA

705 with multiple comparisons correction using GraphPad prism software. ***: $p \leq 0.001$. (**E**)

706 FoxP3^{+}CD4^{+} Treg frequency in tumors isolated from WT or NAC1KO mice. Representative

707 histogram of expression of FoxP3 (**F**), PGC-1α (**G**) and GrazB (**H**), and their quantification:

708 FoxP3 (**I**), PGC-1α (**J**), and GrazB (**K**). (n=4). The differences were analyzed by Two-way

709 ANOVA with multiple comparisons correction using GraphPad prism software. ***: $p \leq 0.001$.
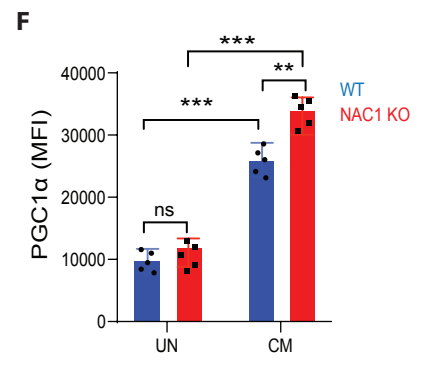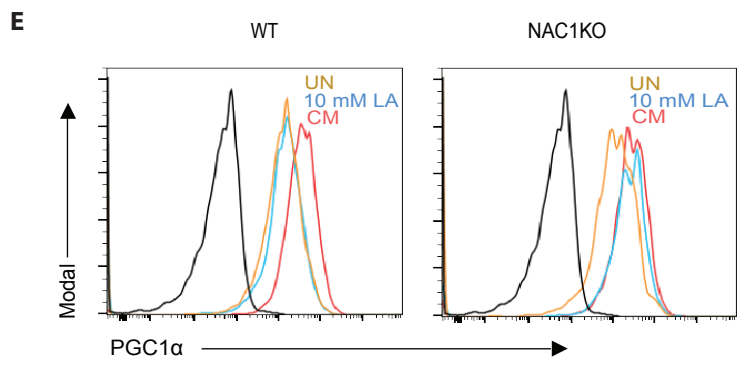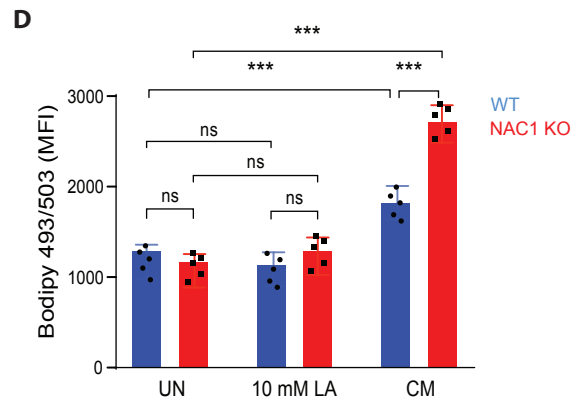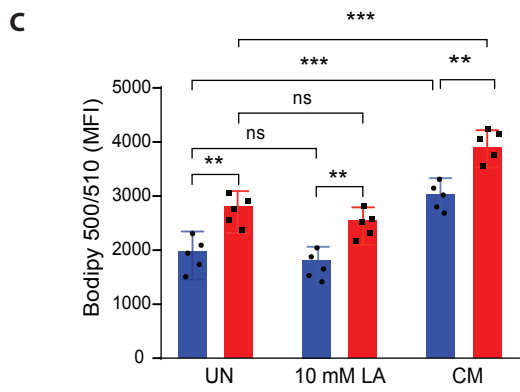
710

711 **Figure 7. NAC1 KO Tregs support tumor growth.** Three million Tregs were *i.p.* injected in

712 each of the recipient Thy1.1^{+} congenic mice, followed by tumor engraftment. (**A**) Schematic

713 representation of adoptive Treg transfer and tumor engraftment. (**B**) Tumor growth curve of

714 B16F10 melanoma (n=5). (**C**) Representative survival curve. (**D**) Illustration of the proposed and

715 the rationale pathway regulated by NAC1-FoxP3, leading to mitochondrial fitness in the TME.

A

Tumor size (mm³)

NAC1KO WT

ns * * **

Days after tumor engraftment

B

Survival

WT
NAC1 KO

Days

C

WT   NAC1 KO

D

WT   NAC1 KO

E

WT   NAC1 KO

DAPI
CD36
FoxP3

F

WT   NAC1 KO

tSNE2

tSNE1

G

Cluster cell density

WT
NAC1 KO

CD11b+ CD11b+F480+ B220+ CD4+ Foxp3+ CD8a+ CD8a+PD1+

ns ns ns * * * **

A

UN   10 mM LA   CM

WT

60.3   i   63.6   ii   63.7   iii

NAC1 KO

71.3   iv   74.2   v   72.0   vi

CD25

FoxP3

B

FoxP3 (%)

WT
NAC1 KO

UN   10 mM LA   CM

C

Apoptosis (%)

WT
NAC1 KO

UN   10 mM LA   CM

D

UN   10 mM LA   CM

WT

22.0   i   37.1   ii   45.7   iii

NAC1 KO

15.6   iv   25.8   v   30.1   vi

Apotracker Green

Live/Dead Voilet

E

UN   10mM LA   CM

WT
NAC1 KO

Modal

CFSE

A

WT | NAC1KO

Modal / CFSE

UN / CM / 1:1

B

Suppression (%)

UN / CM

Treg:Teff (1:1)

WT / NAC1 KO

C

WT | NAC1KO

Modal / GzmB

UN / 10 mM LA / CM

D

GranB (MFI)

UN / 10 mM LA / CM

WT / NAC1 KO

E

WT | NAC1KO

Modal / TGFβ

UN / 10 mM LA / CM

F

TGFβ (MFI)

UN / CM

WT / NAC1 KO

A

OCR (pmol/min) vs Time (minutes)

Oligomycin   FCCP   Rotenone

- WT UN
- WT LA10mM
- WT CM
- NAC1KO UN
- NAC1KO LA10mM
- NAC1KO CM

B

Basal Res

UN   10 mM LA   CM

- WT
- NAC1 KO

ns / ns / * / ns / * / ns

C

Spare Res Capacity

UN   10 mM LA   CM

ns / ** / ns / * / **

D

Maximum Res

UN   10 mM LA   CM

ns / ** / ns / **** / **

E

UN   WT / NAC1 KO

F

10 mM LA   WT / NAC1 KO

Modal / JC-1 Red

G

CM   WT / NAC1 KO

Modal / JC-1 Red

H

JC-1 Red (MFI)

UN   CM

- WT
- NAC1 KO

ns / *** / *** / ***

A

Day 0 — Prepare mice

Day 1 — iTregs Injection

Day 2 — Tumor Injection

Day 10 — Tumor growth

Day 20 — Euthanize mice & Tumor disection

B

Tumor size (mm³)

NAC1 KO iTegs

WT iTregs

3×10³

2×10³

1×10³

***

***

**

*

Days after tumor engraftment

8   10   12   14   16   18

C

Survival

100

50

0

WT iTregs

NAC1 KO iTregs

Days

0   5   10   15   20   25

D

FA oxidation ↑

TCA

NADH

NAD⁺

Pyruvate

ETC Complex/OXPHOS

NADH

NAD⁺

NADH

LDHA/B

NAD⁺

anabolic process

PGC1α ↑ ← FoxP3 ↑

NAC1 KO

GLUT1

MCT4

CD36 ↑

Cytosol

Extracellular

Glucose ↓

Lactic acid ↑

Fatty acid ↑

Tumor Cells

LDH ↑