Modulation of glucose metabolism by 2-Deoxy-D-Glucose (2DG) promotes IL-17 producing human T cell subsets

Xin Chen[1,2,3], Lina Kozhaya[1], Chuxi Wang[1,2], Lindsey Placek[1], Ece Karhan[1], Derya Unutmaz[1,2]

[1] Jackson Laboratory for Genomic Medicine, Farmington, Connecticut, USA.

[2] Department of Immunology, University of Connecticut School of Medicine, Farmington, CT USA.

[3] Institute for Immunity, Transplantation, and Infection, Stanford University, Stanford, CA USA.

* Corresponding author E-mail: derya@mac.com

Abstract

Activation and differentiation of T cells are closely linked to their cellular metabolic programs. Glycolysis and mitochondrial metabolism are thought to be critical in modulating T cell function. Here we asked to what extent inhibition of glycolysis, using 2-Deoxy-D-Glucose (2DG), regulate activation, effector function, or differentiation of human T cell subsets. We found that glycolysis is required for T cell receptor (TCR) -mediated activation and proliferation of human naive CD4+ T cells but had less of an impact on memory subsets. CD4+ T cells cultured in the presence of 2DG displayed higher level of IL-17-secreting cells (Th17) from memory or *in vitro* differentiated naive regulatory T cell (Tregs) subsets. Moreover, the mucosal associated invariant T (MAIT) cell subset survived or expanded better and secreted higher IL-17 in the presence of 2DG. Remarkably, we found that the 2DG effect was reversed by mannose but not by glucose. Collectively, these findings suggest that 2DG could enrich IL-17 secreting human effector T cell subsets and their cellular functions. Our finding provides a framework to manipulate glycolytic pathways in human T cells in infectious diseases such as COVID19 and in enhancing cancer immunotherapy.

31 ## Introduction

32 Metabolism has been recognized as an important regulator in T cell activation and lineage
33 differentiation (Araki et al., 2010; Chapman et al., 2020; Jacobs et al., 2008; MacIver et al., 2013;
34 Palmer et al., 2015; van der Windt & Pearce, 2012). Upon T cell activation, TCR signals and co-
35 stimulation activate the phosphatidtyl-inositide-3 kinase (PI3K)/Akt/mTORC1 signaling pathway
36 for the increased anabolic needs of effector T cells, which become more sensitive to metabolic
37 regulation (Ho et al., 2015; Palmer et al., 2015; Sena et al., 2013). Importantly, an efficient immune
38 response involves various T cell subsets, which have different metabolic requirements for
39 development and effector functions (MacIver et al., 2013; Pearce et al., 2013). It is still unclear
40 how metabolic pathways regulate the functions of T cell subsets specifically, but understanding
41 the underlining mechanisms would allow modulation of immunity in chronic disease and cancer
42 (Johnson et al., 2018).

43 Subsets of T cells that secrete IL-17 are the critical mediators in antimicrobial and pro-
44 inflammatory responses, as well as in pathogenesis in autoimmune or chronic inflammatory
45 diseases (Bettelli et al., 2006; Damasceno et al., 2020; Platt et al., 2020; Xu et al., 2020; Yasuda
46 et al., 2019). Th17 cells and a subset of Tregs are the major sources of IL-17 secretion, which are
47 also characterized by the expression of the transcription factor RORC and the chemokine receptor
48 CCR6 (Singh et al., 2008; Valmori et al., 2010; Wan et al., 2011). Several mouse studies have
49 revealed a role of glucose metabolism in Th17 differentiation, via mTORC1, Myc, and HIF1a
50 signaling (Kastirr et al., 2015; Perl, 2016; Sasaki et al., 2016; Shen & Shi, 2019; Shi et al., 2011).
51 Inhibition of glycolysis with 2-Deoxy-Glucose (2DG) or 3-bromopyruvate in mice for a short period
52 of time impaired Th17 differentiation, phenocopying the effect of HIF1a deficiency (Okano et al.,
53 2017; Shi et al., 2011). However, how glycolytic metabolism regulates the differentiation and
54 functions of human Th17 and IL-17 secreting Tregs remains unclear.

55 Another source of IL-17 producing T cells are mucosal associated invariant T (MAIT) cells (Coulter
56 et al., 2017; Willing et al., 2018). Human MAIT cells can be identified by a semi-invariant T cell
57 receptor(TCR) alpha chain(Va7.2) and CD161 expression (Chiba et al., 2018). MAIT cells can be
58 activated by a broad range of bacteria and yeasts, and can discriminate and fine-tune their
59 functional responses to complex human microbiota (Constantinides et al., 2019; Corbett et al.,
60 2020; Hinks & Zhang, 2020; Ioannidis et al., 2020; Tastan et al., 2018). Thus, IL17-secreting MAIT
61 cells play a role in infections and autoimmune diseases such as Tuberculosis and Multiple
62 Sclerosis (Coulter et al., 2017; Willing et al., 2018). While the frequency and distribution of MAIT
63 cells are currently under intense investigation, there is still much to understand in the metabolic
64 regulation of the function of the human MAIT cell subset.

65 In this study, we utilized 2DG, an analog and potent glycolysis inhibitor, to investigate the role of
66 glucose metabolism in the activation, differentiation, and effector function of human T cell subsets.
67 We showed that 2DG suppressed human T cell activation, cytokine production, and proliferation
68 upon early T cell receptor (TCR) activation. However, 2DG treated naive T cells showed a greater
69 reduced proliferative capacity and glycolytic function compared to memory T cells. Remarkably,
70 2DG treatment greatly enriched IL-17 producing CD4+ T cells in long term culture and during *in*
71 *vitro* differentiation of naive or naive regulatory T cells (TNreg) or from the MAIT cell subset.
72 Together, our findings suggest a differential impact of glycolysis in different subsets and
73 differentiation stages of human T cells.

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77 # Results

78 **Inhibitor of glucose uptake, 2DG, suppresses early activation of CD4+ T cells**

79 It has been reported that 2DG inhibits glucose metabolism (Xi et al., 2014), which may in turn
80 regulate T cell activation (Buck et al., 2015; Palmer et al., 2015). Therefore, we first examined the
81 effect of 2DG on T cell activation by assessing the expression of the IL-2 receptor alpha (CD25)
82 and the glucose transporter 1 (GLUT1), as both are upregulated upon T cell activation for IL-2
83 signaling and glucose uptake, respectively (Chapman et al., 2019). Human primary CD4+ T cells
84 were activated with anti-CD3/CD28 beads in media alone or in media supplemented with 0.3mM,
85 1mM, or 3mM 2DG. The expression of CD25 and GLUT1 in T cells was significantly reduced by
86 3mM 2DG compared to the control (**Figure 1A-B**), but lower concentrations of 2DG (0.3mM or
87 1mM) did not reach statistical significance (data not shown). Because 2DG suppresses glycolysis
88 (Xi et al., 2014), we also activated T cell subsets in the glucose-free media as a comparison. In
89 contrast to the effect seen with 2DG, T cells activated in glucose-free-media showed higher
90 GLUT1 expression and CD25 expression comparable to the control condition (**Figure 1A-B**). We
91 also observed a reduced expression of program cell death 1 (PD1) and Lymphocyte Activating 3
92 (LAG3) on 2DG-treated T cells, which are known to be induced upon TCR activation
93 (Lichtenegger et al., 2018) (**Figure 1C-D**). However, the expression of PD1 or LAG3 was not
94 significantly different between T cells activated in glucose-free or regular media (**Figure 1C-D**).

95 TCR-activation of T cells leads to a rapid production of cytokines such as TNF, IFNγ, and IL-2. As
96 such, to determine whether 2DG also suppresses cytokine production, we collected activated T
97 cell supernatants 2 days post CD3/CD28 activation and analyzed the cytokine levels using a
98 cytometric bead assay. We found that the levels of TNF and IFNγ secretion were significantly
99 reduced but there was no difference in IL-2 secretion in the presence of 2DG culture compared
100 to control media (**Figure 1E**). Together, these data suggest that 2DG suppresses early human T

101 cell activation and cytokine production.

102 **2DG has different effects on activation or effector functions of naive and memory T cells.**

103 It has been reported that naive and memory T cells may have different metabolic requirements
104 for activation and proliferation (Almeida et al., 2016; MacIver et al., 2013; van der Windt & Pearce,
105 2012). To determine whether 2DG modulates the activation of naive vs memory CD4+ T cells
106 differently, we sorted these subsets from human CD4+ T cells using CCR7+CD45RO- (naive)
107 and CCR7+/-CD45RO+ (memory) markers and activated with anti-CD3/CD28 beads in media
108 alone, in 3mM 2DG, or in glucose-free media. Both T cell subsets displayed a similar trend of
109 reduction in GLUT1 and CD25 expression on day 1 post activation **(Figure 2A)**; however, on day
110 4 post-activation, 2DG-treated memory T cells upregulated GLUT1 to levels comparable to control
111 cells whereas most naive T cells remained GLUT1 negative **(Figure 2B-C)**. Similar to unsorted
112 CD4+ T cells (**Figure 1A**), activation of naive or memory T cells in glucose-free media did not
113 significantly affect the expression of GLUT1 or CD25 (**Figure 2A-C**). Addition of IL-2 in these
114 activation experiments did not restore the 2DG-mediated down-regulation of GLUT1 or CD25
115 expression in either naive or memory T cells (**Supplemental Figure 1A-C**). Since we have
116 observed that 2DG suppresses T cell activation and glucose uptake, we hypothesized that 2DG
117 would also reduce T cell proliferative capacity. To address this, we labeled naive and memory T
118 cells with Celltrace violet (CTV) dye, and then activated with anti-CD3/CD28 coated beads.
119 Indeed, in 2DG-treated or in glucose-free media, naive or memory T cell subsets proliferated less
120 compared to control media (**Figure 2E**). Addition of IL-2 did not restore the low proliferation of
121 2DG-treated T cell subsets (S**upplemental Figure 1D**).

122 We next monitored the expansion of these activated T cells during 2-week culture in IL-2.
123 Expansion of 1 mM 2DG treated naive T cells were 30 fold after 14 days, compared to 150 fold
124 in the control, whereas memory T cells in the presence of 1mM 2DG expanded 85 fold compared

125 to 110 fold in the control media. We then asked whether 2DG has differential effects in the
126 glycolytic function of naive and memory subsets by using the Seahorse glucose stress test assay.
127 After T cell expansion in 2DG or control media for 14 days, 2DG was washed away and cells were
128 resuspended in Seahorse XF base media, and the metabolic functions were analyzed by the
129 Seahorse XFe96 Analyzer. The extracellular acidification rate (ECAR) was assessed after the
130 addition of glucose (gluc), oligomycin (oligo), and 2DG at indicated times and the glycolytic
131 capacity was determined. 2DG-treated memory T cells had comparable glycolytic function to the
132 control groups; however, 2DG-treated naive T cells showed significantly reduced glycolysis in
133 response to addition of glucose (**Figure 2F-G**).

134 **2DG enhances IL-17 producing CD4+ memory subsets.**

135 We next further explored which effector T cell subsets are modulated by suppressing glycolysis.
136 We first observed a significantly increased CD161+ and CCR6+ populations in 2DG-treated CD4+
137 T cells compared to control (**Figure 3A and 3B**). Since nearly all IL-17 secreting cells express
138 CCR6 and CCR6+ Th17 cells are reported enriched in the CD161+ subset (Acosta-Rodriguez et
139 al., 2007; Cosmi et al., 2008; Wan et al., 2011), we hypothesized that 2DG would preferentially
140 affect the function of IL-17 secreting T cell subsets. Indeed, after two weeks in culture with 2DG,
141 we observed that IL-17+ subsets were increased in both CCR6+ and CD161+ populations
142 compared to cells in control media, but interestingly, there was no difference in the low glucose
143 condition (**Figure 3C**). After 2DG was removed from the culture and cells reactivated with phorbol
144 myristate acetate (PMA) and calcium ionophore (ionomycin), we determined intracellular cytokine
145 secretion in the presence of brefeldin a (GolgiStop). We found that the frequency of cells that
146 expressed not only IL-17 but also IFNγ, IL-4, and IL-21 were significantly increased (**Figure 3C**
147 **and 3D**). However, no effect was seen in IL-2 and TNF production from CD4+ T cells

148 (**Supplemental Figure 3**). Taken together, these data demonstrate that 2DG-treatment can
149 modify the cytokine production of lineage-committed cells in long term *in vitro* cultures.

150 **2DG enriches IL-17+ cells within Th17 lineage-committed CCR6+ memory T cell subset.**

151 Since we observed a significant increase in IL-17 cytokine production from 2DG-treated T cells,
152 we hypothesized that 2DG could enhance the IL-17 production from Th17 cells compared with
153 other effector T cells. To test this hypothesis, we sorted CD4+ T cells into CCR6- and CCR6+
154 memory T cell subsets, activated them in the presence or absence of 2DG, and expanded for two
155 weeks in IL-2, as described above (**Figure 3A**). Consistent with our prior results, 2DG-treated
156 CCR6- and CCR6+ CD4+ T cells displayed higher CD161 expression than control cells (**Figure**
157 **4A**). IL-17 production from purified and expanded CCR6+ T cells were increased in the presence
158 of 2DG (**Figure 4B**). Notably, IFNγ+ and IL-21+ producing CCR6+ T cells were also increased by
159 2DG (**Figure 4B-E**). Interestingly, the percentage of IL-10+ and IL-17+ T cells was significantly
160 increased in 2DG-treated CCR6+ T cells (**Figure 4F and 4G)**. Therefore, we concluded that 2DG
161 increased CD161+ within both CCR6+ and CCR6- T cells, and also enhanced IL-17, IFNγ, IL-21,
162 and IL-10 production from the sorted CCR6+ memory T cell subset.

163 **2DG increases *in vitro* generation of IL-17-producing Tregs from human naive regulatory**
164 **T cell (TNreg) precursors**

165 A subset of Tregs that secretes IL-17 can preferentially arise from human naive precursors
166 (CD25+CD45RO-CCR7+ cells, termed TNreg) in the presence of polarizing-cytokines IL-1β, IL-
167 23, and TGF-β (Mercer et al., 2014; Valmori et al., 2010). To explore how 2DG regulates Th17
168 cell differentiation from TNregs, we used this cytokine polarization protocol (**Figure 5A**) to
169 differentiate and expand IL-17-secreting cells from highly purified naive or precursors naive
170 regulatory T cells (TNregs) in media alone or in the presence of 1 mM 2DG. The FOXP3+ cells

171 during polarization of TNregs with 2DG were statistically similar in frequency (**Figure 5B**).
172 However, 2DG significantly increased generation of IL-17+ Tregs from TNreg precursors (**Figure**
173 **5C**). Since HELIOS expression defines a phenotypically distinct population of Tregs (Thornton et
174 al., 2019), and *in vitro*-generated IL-17+ cells from TNreg precursors did not express HELIOS
175 (Mercer et al., 2014), we then asked whether 2DG could induce IL-17, IFNγ, and IL-21 production
176 from HELIOS+ or HELIOS- populations of naive and TNregs. We also compared the 2DG effects
177 between HELIOS+ and HELIOS- population of naive cells since HELIOS plays a role in naive T
178 cell differentiation (Ng et al., 2019). The frequency of IL-17+ T cells witihn HELIOS- naive T cells
179 was increased by 2DG in polarizing condition, but not within HELIOS+ subset (**Supplemental**
180 **Figure 4**). 2DG also significantly increased IL-17 and IFNγ populations from HELIOS- population
181 from TNregs in the polarization condition (**Figure 5D-F**). Notably, when comparing the intracellular
182 IL-21 from HELIOS+ and HELIOS- subset of Tregs, the frequency of IL-21+ cells was significantly
183 higher in 2DG-treated HELIOS- subset (**Figure 5G and 5H**). But there was no significant
184 difference in IL-21 cytokines secretion in HELIOS+ or HELIOS- Treg subsets in 2DG-treated naive
185 T cells compared to controls (**Figure 5H**). Intracellular IL-10 was also increased in HELIOS-
186 population of TNregs in polarizing cytokines, and was significantly increased in HELIOS+ and
187 HELIOS- population from naive T cells (**Figure 5J**). In addition, increased cytokine production of
188 IL-17A, IL-17F, IL-10, and IFNγ was also confirmed by corresponding levels of cytokines detected
189 in the culture supernatants (**Figure 5K**). Taken together, 2DG enhanced the generation of IL-17-
190 producing cells from both naive and TNregs, which was restricted to HELIOS- subsets. 2DG also
191 increased the production of IFNγ, IL-21, and IL-10 from TNregs in the polarizing condition.

192 **Mannose reverses or rescues 2DG effects on T cell activation and effector functions.**

193 2DG has previously been shown to inhibit *N*-linked glycosylation (Andresen et al., 2012; Xi et al.,
194 2014), which can be modulated through a branch of the glucose metabolism pathway called the

195 hexosamine biosynthetic pathway (HBP) (Akella et al., 2019). Further, mannose, which is a major
196 component of sugar moeity in glycoproteins, can reverse the apoptotic effect of 2DG on cancer
197 cells (Ahadova et al., 2015; Gu et al., 2017; Kurtoglu et al., 2007). Thus, we sought to determine
198 whether mannose can also reverse the 2DG effects on short term activation of T cells (**Figure 2**).
199 Accordingly, we activated CD4+ naive T cells with anti-CD3/CD28 beads in media alone, with
200 3mM 2DG or mannose alone, or with 3mM 2DG supplemented with 3mM mannose. While we did
201 not observe any difference in CD25 and GLUT1 expression on day 1 post activation
202 (**Supplemental Figure 5**), mannose reversed the 2DG-mediated downregulation of CD25 and
203 GLUT1 expression on day 4 (**Figure 6A and 6B**). Additional equimolar glucose did not reverse
204 the 2DG effects (**Figure 6B**). Equal molar of mannose significantly reverses the proliferative
205 capacity from 1 fold expansion with 2DG alone to 50 fold expansion with 2DG and mannose
206 comparing to 157 fold cell expansion in control media (**Figure 6C**). However, after long-term
207 culture, mannose did not rescue the impaired glycolytic function of 2DG-treated naive T cells
208 (**Figure 6D and 6E**).

209 Next, we asked whether mannose could reverse 2DG effects on IL-17 enrichment. After 14 days
210 cell expansion, we observed a reduction of CCR6+ and CD161+ population in a mannose-does
211 dependent manner (**Figure 6F and 6G**). In addition, the 2DG mediated-reduction of IL-17, IFNγ,
212 and IL-21 were also reversed by mannose (**Figure 6G**). Addition of equimolar glucose into 2DG
213 culture did not reverse the 2DG-mediated enrichment of IL-17 production (**Figure 6F and 6G**).
214 Together these findings suggest that the inhibitory effects of T cells activation and IL-17
215 production on 2DG-treated human T cells are mannose-dependent.

216

217 ## Discussion

218 In this study, we demonstrated that the glucose analog 2DG have remarkably different effects on
219 activation and differentiation of human T cell subsets. 2DG suppresses early human T cell
220 activation through the TCR, as assessed by cytokine production, and proliferation. However, in
221 long term cultures 2DG also profoundly increases the frequency of IL-17 production from lineage-
222 committed memory CCR6+CD4+ (Th17) T cells, MAIT cells and Tregs. In addition, inhibition of
223 glycolysis by 2DG in these memory subsets (Th17, MAIT and Tregs) does not affect their
224 expansion upon activation. On the other hand, naive CD4+ T cells are more sensitive to the
225 inhibition of glycolysis by 2DG and therefore have reduced their survival and function. Importantly,
226 we also demonstrated that the 2DG-mediated IL-17 enrichment could be reversed by addition of
227 equimolar mannose.

228 A major question derived from our findings is that how inhibition of glycolysis can significantly
229 increase the IL-17 production from already lineage-committed/differentiated memory Th17 cells?
230 Several mice studies have revealed a role of glucose metabolism in Th17 differentiation (Kastirr
231 et al., 2015; Perl, 2016; Sasaki et al., 2016; Shen & Shi, 2019; Shi et al., 2011) but showed
232 discrepancies on the effect of inhibiting glycolysis in Th17 differentiation and functions. For
233 example, in one study Tregs treated with 0.2mM 2DG for 5 days reduced IL-17 production *in vitro*
234 (Li et al., 2019) and in another study reduced production of IL-17 by T cells after 5 day-2DG
235 treatment was observed (Shi et al., 2011). However, another study reported an enhancement of
236 Th17 cell polarization and IL-17 production after treatment of 2mM 2DG (Brucklacher-Waldert et
237 al., 2017). In our study, we observed reduced IL-17 production by 2DG treated T cells for during
238 early activation period. However, frequency of IL-17 producing subset was greately increased
239 after long-term T cell culture (12-14 days), during reactivation in the absence of 2DG. Th17 can
240 be characterized as “pathogenic” and “non-pathogenic” Th17 cells (Wu et al., 2018). Pathogenic
241 Th17 cells express more effector molecules such as CXCL3, CCL4, CCL5, IL-3, and IL-22,
242 whereas non-pathogenic Th17 cells exhibit upregulation of immune suppressive molecules and

243 cytokines such as IL-10 (Gaublomme et al., 2015; Lee et al., 2012). We hypothesized that the
244 increased subset of IL-17-secreting Th17 cells belongs to the “non-pathogenic” subset, as we
245 also found an increase in IL-10 production from the same cells. Since IL-17+ Th17 cells are crucial
246 for mediating mucosal immunity against fungi and bacteria infection in human subjects (Brembilla
247 et al., 2018; McDonald, 2012), our findings may suggest that the metabolic changes affect the
248 function of Th17 against commensal microbes in the gastrointestinal tract which would lead to
249 mucosal damage and mucosal-relative diseases such as inflammatory bowel disease (IBD)
250 (Galvez, 2014). Since the functions of Th17 cells in the small intestine can be closely regulated
251 by gut microbiome (Evans-Marin et al., 2018; Garidou et al., 2015), our findings also suggest that
252 a dysregulated metabolites from dysbiotic ileum microbiota (Visconti et al., 2019) could impact
253 Th17 homeostasis, and were sufficient to induce metabolic disease such as Type 2 Diabetes
254 (T2D) (Garidou et al., 2015).

255 We found that 2DG also increased the frequency of IL-17+ Tregs and IL-17 cytokine secretion
256 from these cells in the presence of polarizing cytokines *in vitro* differentiation. These IL-17-
257 producing Tregs were found to be accumulated in the inflamed joints of patients with rheumatoid
258 arthritis and were functionally suppressive (Afzali et al., 2013; Jung et al., 2017). However, we did
259 not observe a significant difference in suppressive capacity of 2DG-treated Tregs in proliferation
260 assay (data not shown), which is consistent with a human Tregs study *in vitro* (Tanimine et al.,
261 2019). Inhibition of glycolysis may also contribute to mucosal diseases, as IL-17+Tregs may
262 contribute to the development of colon cancer (Knochelmann et al., 2018; Marshall et al., 2016),
263 and possibly to IBD (Galvez, 2014).

264 As noted, an increased level of IL-10 from memory T cells (such as Tregs in the presence of
265 polarizing cytokines, CCR6- and CCR6+ memory T cells) was also observed throughout the
266 experiments. IL-10 is an anti-inflammatory cytokines, plays a central role in limiting the regulation
267 of immune responses (Iyer & Cheng, 2012). Elevated IL-10 signaling can inhibit antigen

268 presenting cells maturation, and chemokine secretion of the host during chronic viral infection
269 (Granelli-Piperno et al., 2004) and autoimmune disease (Braat et al., 2003). It can be highly
270 produced by a subset of Tregs, called Type 1 regulatory cells (Tr1) (Zeng et al., 2015), which is
271 associated with autoimmune diseases such as IBD, multiple sclerosis(MS), and type 1 diabetes
272 mellitus when their frequency was found reduced (Jia et al., 2019). Thus our data may suggest a
273 potential role of glycolysis in the function of IL-10 secreting subsets and related diseases.

274 Another subset of cells, where found differential and specific effect of 2DG were MAIT cells, which
275 are characterized with expression of CD161 and a semi-invariant Va7.2+TCR, are activated by a
276 ligand from riboflavin metabolism called 5-ARU in the context of MR-1 molecule (Godfrey et al.,
277 2019). MAIT cells are also unique in that they can be CD8+CD4- or CD8-CD4- and can also
278 produce IL-17 and mostly migrate to mucosal regions, thus potentially play an important role in
279 mucosal homeostasis and microbiome regulation (Oh & Unutmaz, 2019). In our experiments, we
280 had found that 2DG-treated cells compared to control induced higher frequency of CD161+ cells,
281 which is also highly expressed on MAIT cells (CD161hiVa7.2+) (Cosmi et al., 2008; Kleinschek
282 et al., 2009). We also identified a distinct subset exhibiting medium-expression of CD161 which
283 is also enhanced by 2DG, but is neither Va7.2+ (MAIT) nor CCR6+ (Th17) cells. The identity of
284 this subset is not fully clear, but Klenerman et al reported a distinct functional subset in human T
285 cells which had mid-level CD161 expression, produced IL-17/IFNγ, and were found to be highly
286 enriched in chronic inflammation (Billerbeck et al., 2010). Future studies will be needed to better
287 characterize this subset and its functions, as these have also shown different glucose tolerance
288 than other effector cells.

289 Our findings that 2DG enhances IL-17 production also in MAIT cells, in addition to Th17 cells,
290 suggest a shared metabolic pathways that regulate the effector functions of these mucosa-
291 associated subsets. Interestingly IL-17+ MAIT cells are found specifically enriched from the

292 peripheral blood of multiple sclerosis (MS) patient, implicating a proinflammatory role in
293 autoimmune diseases (Willing et al., 2018). IL-17+ MAIT are also associated with inflamed
294 mucosal tissue, and are found activated and produce more IL-17 in IBD patients (Serriari et al.,
295 2014). In addition to the gut, CD8+ MAIT cells have also been shown to be resident in normal
296 skin and are thought to play a role in skin-associated inflammations, such as psoriasis and
297 dermatitis herpetimorfis (Willing et al., 2018) and tissue repair (Constantinides et al., 2019).
298 Therefore, it is tempting to speculate that 2DG or similar analogs of glucose could be used to
299 modulate pathogenic responses of these T cell subsets.

300 An increase in IL-21 production in 2DG-treated memory T cells was also shown significant in our
301 study. IL-21 has been documented to regulate the differentiation and function of several CD4+ T
302 cells subsets, including Th17 cells (Leonard & Wan, 2016; Nurieva et al., 2007; Zhou et al., 2007),
303 Th2 (Frohlich et al., 2007; Lajoie et al., 2014), and T follicular helper cells (TFH) (Vogelzang et
304 al., 2008). Thus, the enhanced IL-17 and IL-4 cytokine production in 2DG treated T cells may be
305 mediated partially by IL-21 signaling. IL-21 was also reported to induce IL-10 production in Th17
306 polarizing condition in mice (Spolski et al., 2009), which is consistent with our observation that
307 differentiation of IL-17 secreting subsets from TNreg with 2DG increase IL-21 and IL-10 secretion.
308 Furthermore, the functional significance of IL-21 in regulating effector functions of CD8+ T cells
309 is highlighted by its critical role in sustaining anti-viral CD8+T cells during chronic LCMV infections
310 (Cui et al., 2011; Elsaesser et al., 2009) and its potent effects to induce and expand cytotoxic
311 CD8+ T cells for cancer immunotherapy (Davis et al., 2015; Santegoets et al., 2013). An increase
312 production of IL-21 and IL-4 cytokines may suggest that 2DG enhance TFH cells, which have high
313 expression level of CXCR5 and produce high level of IL-21 (Crotty, 2014). This hypothesis is also
314 supported by that exogenous Ag-specific TFH cells do not require glycolysis (Choi et al., 2018).
315 TFH is critical for the formation and maintenance of germinal centers and provide help for B cells
316 generating antibody responses after immunizations (Bentebibel et al., 2013; Duan et al., 2014;

317 Spolski & Leonard, 2010). Therefore, regulating TFH cells with glucose metabolism would
318 potentially enhance development of new or improved vaccines (Crotty, 2014).

319 One potential mechanism of an increase frequency of IL-17-secreting T cell subsets in 2DG-
320 treated cells is that 2DG alters T cells metabolism from glucose to alternative energy sources or
321 metabolic pathways such as fatty acid oxidation or pentose phosphate pathway. Previous studies
322 showed an inhibition of glucose metabolism with a glucose transporter inhibitor (CG-5) promoted
323 fatty acid oxidation and the pentose phosphate pathway in CD4+ T cells in a similar manner as
324 with 2DG (Li et al., 2019). Fatty acid metabolism has also been suggested to be involved in Th17
325 inflammation, as blockade of carnitine palmitoyltransferase 1 (CPT1), an enzyme responsible for
326 catalyzing the breakdown of long-chain fatty acids, inhibits Th17-associated cytokine production
327 from type 2 diabetes (T2D) patients (Nicholas et al., 2019).

328 One of our important findings towards mechanism of action of 2DG in our system is that 2DG-
329 mediated increases in IL-17-secreting cells can be reversed by addition of equimolar mannose
330 (Berthe et al., 2018; Kurtoglu et al., 2007). This suggests that another possible mechanism of
331 2DG is inhibiting the initial step of glycolysis affects the downstream metabolic pathway of
332 mannose. Since mannose is a dominant monosaccharide in N-linked glycosylation (Imperiali &
333 O'Connor, 1999), our observation may further suggest that part of the 2DG effects on T cell
334 function are through modifying sugar moieties generated in central carbon metabolism (e.g. N-
335 linked glycosylation). N-linked glycosylation plays a role in modulating activation and cytokine
336 signaling (Baum & Cobb, 2017; Dean et al., 1979; Hauser et al., 2016), and thereby may affect
337 differentiation and function of T cell subsets. An evidence to support this notion was our finding
338 that mannose reversed 2DG-mediated down-regulation of CD25 expression upon activation.
339 Similarly, another inhibitor of N-linked glycosylation, glucosamine, attenuated CD25 surface
340 retention on T cells (Chien et al., 2015), and down-regulation of N-linked glycosylation promotes
341 Th17 differentiation (Chien et al., 2015). However, surprisingly, addition of equimolar glucose

342 could not reverse 2DG-mediated downregulation of CD25. We reason that 2DG efficiently inhibits
343 glycolysis, therefore 2DG-treated cells could not utilize additional glucose. This speculation could
344 be supported by seahorse glycolytic analysis showing that neither additional mannose nor
345 glucose could reverse 2DG-mediated glycolytic capacity. Overall, it is conceivable that 2DG may
346 have dual mechanism in enhancing the survival and effector functions of IL-17 secreting subsets.

347 Recently, 2DG has also been considered as a potential therapy during viral infections such as
348 COVID-19 (Yang et al., 2021). Indeed, 2DG was approved by the Indian Council of Medical
349 Research (OCMR) to treat COVID-19 patients in India (Sahu & Kumar, 2021). 2DG shortens the
350 time of COVID patients recovered from the infection and there was a higher proportion of patients
351 with improved symptoms who were free from supplemental oxygen dependence (Sahu & Kumar,
352 2021) . The underling mechanism of the effect of 2DG in these COVID patients is still unclear, but
353 it was suggested that inhibiting glycolysis with 2DG could affected viral life cycle (Passalacqua et
354 al., 2019) and an increased glucose level could enhance the suppressive function of monocytes
355 and therefore enhanced SARS-CoV-2 infection (Codo et al., 2020). Given our findings, we also
356 speculate that 2DG effects may have been through ameloriation of excessive or uncontrolled
357 immune response during COVID19, which is observed during later severe disease stages. 2DG
358 has also been tested to treat advanced cancer and proven safe in phase I studies (Raez et al.,
359 2013; Stein et al., 2010). Our data shows long-term culture of human primary T cells with 2DG
360 enriched IL-17 secreting T cells, which have been reported to exhibit pathogenic features (Basdeo
361 et al., 2015). However, a significant increase in IFNγ production by 2DG may enhance therapeutic
362 anti-tumor activity (Medrano et al., 2017). Taking together, our study provides insights for future
363 clinical trials and strategies for development of 2DG-related cancer therapies.

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367 ## Materials and Methods

368 **T cell purification, activation, and culture**

369 PBMCs from healthy individuals (New York Blood Center, New York, NY) were isolated using
370 Ficoll-Paque plus (GE Healthcare). CD4+ T cells were isolated using Dynal CD4+ isolation kits
371 (Invitrogen) and were 99% pure. Purified CD4+ cells were sorted in some experiments by flow
372 cytometry (FACSAria; BD Biosciences) based on CD45RO, CCR7, CD25, and chemokine
373 receptors expression into: 1) naive T cells (CD45RO-CCR7+CD25-), 2) memory T cells
374 (CD45RO+CD25-), 3) naive regulatory T cells (Tregs; CD45RO-CD25+), 4) Th17 cells
375 (CD45RO+CCR6+). Sorted subsets were 98% pure. All purified cells were kept at 37°C and 5%
376 CO_2 in complete RPMI 1640 media (RPMI 1640 supplemented with 10% FBS; Atlanta Biologicals,
377 Lawrenceville, GA), 8% GlutaMAX (Life Technologies), 8% sodium pyruvate, 8% MEM vitamins,
378 8% MEM nonessential amino acid, and 1% penicillin/streptomycin (all from Corning Cellgro). To
379 activate cells for expansion *in vitro*, anti-CD3/CD28 Dynabeads (Invitrogen) were used at a bead:
380 cell ratio of 1:2 and the activated cells were cultured in complete RPMI 1640 medium (Thermo
381 Fisher Scientific) or complete 1640 RMPI no glucose medium supplemented with IL-2 (10 ng/ml).
382 For MAIT experiments, MAIT cells were activated by adding riboflavin metabolite 5ARU (Toronto
383 Research, Ontario, Canada) into PBMC culture.

384

385 **Flow cytometry staining and analysis**

386 Cells were stained with fluorochrome-conjugated Abs in FACS buffer(PBS+2% FCS and 0.1%
387 sodium azide) for 30 min at 4°C (or room temperature for CCR6 staining). Abs used in surface
388 staining are IL-2R (CD25), PD1, CD161, Va7.2, CCR6 (from BioLegend), LAG3, CD3, and CD8

389 (from Invitrogen). For intracellular cytokine staining, cells were stimulated for 4 h at 37°C with
390 PMA (10ng/ml for PBMC and CD4+ T cells for 40 ng/ml) and ionomycin (500 ng/ml) (both from
391 Sigma-Aldrich) together with GolgiStop (BD Biosciences). Cells were then stained with fixable
392 viability dye (eBioscience) in PBS and surface marker Abs in PBS or FACS for 30 minutes, then
393 fixed and permeabilized using eBioscience fixation/permeabilization buffers for 30 min at 4°C
394 according to the manufacturer's instructions, before staining for intracellular cytokines for 30 min
395 at 4°C. Abs used for intracellular cytokines are IFNγ, TNF, IL-17A, IL-10, and IL-21(BioLegend).
396 Ab used for Regulatory T cells transcription factor intracellular staining are Foxp3 and Helios Abs
397 (BioLegend). Flow cytometry analyses were performed using an LSRFortessa X-20 flow
398 cytometer (BD Biosciences) and SP6800 spectral cell analyzer (Sony Biotechnology).

399 **Seahorse metabolism assay**

400 Seahorse assays were performed according to the manufacturer's instructions to analyze
401 glycolysis metabolism using the Seahorse XF Glycolysis Stress Test Kit (Agilent Technologies).
402 Briefly, 14 day post activation, naive and memory T cells expanded in indicated conditions were
403 washed and resuspended in glucose-free media (Gibco). 300k cells per well were spun down
404 onto plates coated with Cell-Tak (Fisher scienctific). Four replicates were set up for each
405 condition. Glucose, oligomycin, and 2DG were serially injected to measure metabolic function.
406 Plates were analyzed using an XF^e 24 Extracellular Flux Analyzer (Agilent Technologies).
407 Glycolysis was calculated as average post-glucose ECAR values minus average basal ECAR
408 values.
409

410 ***In vitro* cytokine polarization assay**

411 Sorted TN and TNreg were activated with anti-CD3/CD28 beads and cultured in complete RPMI
412 media containing IL-2 10ng/ml (Chiron), together with IL-1β (10ng/ml), TGF-β (10ng/ml), and IL-
413 23 (100ng/ml) (R&D Systems). Cells were expanded for 2 weeks in media replenished for 2DG

414 and IL-2.

415

416 **Statistical analysis**

417 Data recorded by flow cytometry were analyzed using FlowJo (Tree Star, Ashland, OR). Statistical
418 analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA).
419 Error bars represent SEM. Results were compared using two-tailed t tests. Bonferroni corrections
420 were applied for multiple comparisons. For all experiments, significance was defined as $*p < 0.05$,
421 $**p < 0.01$, $***p<0.001$

422

423 Conflict of Interest Statement

424 The authors declare that the research was conducted in the absence of any commercial or
425 financial relationships that could be construed as a potential conflict of interest.

426

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435 ## Figures Legends:

436 **Figure 1. 2DG suppresses early activation of CD4+ human T cells.** T cell subsets were
437 activated with anti-CD3/CD28 beads in media alone, 3mM 2DG or in glucose-free media. (A)
438 Surface expression of CD25 and GLUT1 on resting and 24h-activated CD4+ T cells with indicated
439 conditions. (B) Fold difference in GLUT1 expression and mean florescent intensity (MFI) of CD25
440 on 24h-activated CD4+ T cells in 3mM 2DG or in glucose-free media compared to control. (C)
441 Flow cytometry plot of LAG3 and PD1 expression on resting and activated CD4+ T cell in media
442 alone, 3mM 2DG, or in glucose-free media for 24h. (D) Statistical analysis of fold difference in
443 PD1, LAG3, and double positive expression of CD4+ T cells activated for 24 h in 3mM 2DG and
444 in glucose-free media. (E) CD4+ T cells were activated with anti-CD3/CD28 beads. Cell
445 supernatant were collected from the indicated conditions after 2 days post beads activation. The
446 levels of TNF, IFNg, and IL-2 cytokines were determined by FlowCytomix Multiplex bead assay.
447 Data represent three independent experiments. **$p < 0.01$, ***$p < 0.001$

448

449 **Figure 2. 2DG has differential effects on activation of naive and memory T cell subsets.** (A)
450 Naive and memory CD4+ T cells were activated with anti-CD3/CD28 beads in media alone, 3mM
451 2DG, or in glucose-free media. Media was not supplemented with IL-2. Representative flow
452 cytometry plot of GLUT1 and CD25 surface expression on resting, and day 1(A)- or day 4(B)- post
453 activated Naive (top) and memory T cells (bottom) in indicated conditions were shown. (C) Fold
454 difference in frequency of GLUT1 expression or mean fluorescence intensity of CD25 expression
455 on naive or memory T cells on day 1 (black)- and day 4 (grey)-post activation in indicated
456 conditions. (D) Naive and memory CD4+ T cells were labeled with CellTrace violet (CTV) dye
457 followed by anti-CD3/CD28 beads activation in indicated conditions. Representative histogram
458 plot of CTV-labeled naive (up) and memory (bottom) CD4+ T cells in 3mM 2DG (red) or in glucose-

459 free media (blue) after day 4 and day 6 post TCR activation. (E) Representative fold expansion
460 of naive and memory CD4+ T cells in 1 or 3mM 2DG or in glucose-free media after 2 week T cells
461 expansion. (F and G) Naive and memory CD4+ T cells were activated and expanded in media
462 alone (control, black) or 1mM 2DG (red) for 14 days. Metabolic functions were analyzed using
463 Seahorse glucose stress test assay. The extracellular acidification rate (ECAR) was assessed
464 after the addition of glucose (gluc), oligomycin (oligo), and 2DG at indicated times and the
465 glycolytic capacity was determined. Each set of data is representative of three donors. *$p < 0.05$,
466 **$p < 0.01$, ***$p<0.001$
467
468 **Figure 3. 2DG enhances IL-17 production in primary CD4+ T cells.** Purified CD4+ T cells
469 were activated with aCD3/CD28 beads in media alone, 3mM 2DG, and in media with 0.5mM
470 glucose. (A) The representative flow cytometry plot of CCR6 and CD161 expression on day14
471 post- activated T cells were shown. (B) Statistical analysis of fold difference in frequency of
472 CCR6+ and CD161+ T cells in indicated conditions. (C) After 14 days' cell culture, T cells activated
473 and expanded in indicated condition were re-stimulated with PMA/ionomycin for 4h, followed by
474 intracellular staining as described in the method section. The percentage of IL-17 production from
475 CD161+ and CCR6+ T cells were shown. (D) Statistical analysis of fold difference in frequency of
476 IL17+, IFNg, IL-4, and IL-21+ T cells from indicated conditions was shown. Each set of data is
477 representative of three donors. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$
478

479 **Figure 4: 2DG enriches the frequency of IL-17 from sorted CCR6+ memory T cells.** (A) sorted
480 CCR6+ and CCR6- memory cells were activated with aCD3/CD28 beads in media alone or with
481 3mM 2DG for 14 days. The frequency of CD161+ cells in day-14 post activated sorted CCR6+
482 and CCR6- subsets in 3mM 2DG compared to control were shown. (B) 14 days post activated T
483 cells were re-stimulated with PMA and ionomycin for 4h, followed by surface and intracellular

484 staining. The frequency of IFNg and IL-17 was determined from sorted CCR6- and CCR6+ T cells.
485 (C) Statistic analysis of the frequency of IL-17+, IFNg +, and IL17+ IFNg+ T cells from CCR6- and
486 CCR6+ T cells as in Figure 4B. The representative plot (D) and the statistical analysis (E) of the
487 frequency of IL-21, IL-10, and IL-17 was determined from sorted CCR6- and CCR6+ T cells after
488 14 days expansion and re-activation with PMA and ionomycin. The representative plot (F) and
489 the statistical analysis (G) of the frequency of IL-10 and IL-17 was determined from sorted CCR6-
490 and CCR6+ T cells after 14 days expansion as in Figure 4D and E. Each set of data is the
491 representative of three donors. *$p < 0.05$, ***$p < 0.001$

492 **Figure 5. 2DG enhances *in vitro* generation of IL-17-producing cells from TNreg cells.** (A)
493 Polarization protocol with Naive or TNreg cells. (B) Representative FOXP3/HELIOS expression
494 of TNreg in two-week polarization cultures with or without 1mM 2DG. (C) Fold difference of IL-17
495 production from 1mM 2DG treated TNreg cells with polarizing cytokines (P.C.) or without (-)
496 compared with control after 14-day expansion. (D) Representative flow cytometry plots of
497 intracellular IFNγ and IL-10 secretion within HELIOS+ and HELIOS- gated populations of TNreg
498 after 14-day expansion. (E) Statistical analysis of IL-17 production of indicated subsets from 1mM
499 treated naive T cell (left)/TNreg(right) with or without polarizing cytokines. (F) Statistical analysis
500 of IFNγ from 1mM treated TNreg in polarizing cytokines (G) Representative flow cytometry plots
501 of intracellular IL-21 and IL-17 secretion within HELIOS+ and HELIOS- gated populations of
502 TNreg after 14-day expansion. (H) Statistical analysis of IL-21 production of indicated subsets
503 from 1mM treated naive T cell (left)/TNreg(right) with or without polarizing cytokines. (I)
504 Representative flow cytometry plots of intracellular IL-10 and IL-17 secretion within HELIOS+ and
505 HELIOS- gated populations of TNreg after 14-day expansion. (J) Statistical analysis of fold
506 difference of IL-10 production of indicated subsets from 1mM treated naïve T cell
507 (left)/TNreg(right) with or without polarizing cytokines. (K) Day-14 expanded TNreg with or without
508 polarization condition were re-stimulated with anti-CD3/CD28 beads. Cell supernatant were

509 collected from the indicated conditions after 2 days post beads activation. IL-17A, IL-17F, IFNγ,
510 and IL-10 productions from supernatant were determined by FlowCytomix Multiplex bead assay.
511 Data represent three independent experiments. *p < 0.05, **p < 0.01, ***p<0.01

512 **Figure 6. Mannose reverses or rescues 2DG effects on T cell subsets.** (A) Naive CD4+ T
513 cells were activated with aCD3/CD28 beads in indicated conditions. Representative flow
514 cytometry plots of GLUT1 and CD25 surface expression. (B) Statistical analysis of fold difference
515 in GLUT1 and Mean Florescent Intensity (MFI) of CD25 on activated naive T cells as in Figure
516 6A. (C) Representative fold expansion of naive CD4+ T cells in indicated conditions after 2-week
517 T cells expansion. (D and E) Naive T cells were activated and expanded in the indicated
518 conditions for 14 days. 2DG was washed away and the metabolic functions were analyzed using
519 Seahorse glucose stress test assay. The extracellular acidification rate (ECAR) was assessed
520 after the addition of glucose (gluc), oligomycin (oligo), and 2DG at indicated times and the
521 glycolytic capacity was determined. (F) CD4+ T cells were activated with aCD3/CD28 beads in
522 media alone, 3mM 2DG alone, 3mM 2DG plus 1mM or 3mM mannose. 14-days post activated T
523 cells were re-stimulated with PMA and ionomycin for 4h, followed by surface and intracellular
524 staining. Representative flow cytometry plots and statistical analysis of T cells expressed CCR6,
525 CD161, IL-21, and IL-17. Data represent three independent experiments. *p < 0.05, **p < 0.01,
526 ***p<0.01

527 ## Supplemental Figure Legends

528

529 **Supplemental Figure 1. Addition of IL-2 did not restore 2DG effects on naive and memory**
530 **T cell subsets.** (A) Naive and memory CD4+ T cells were activated with aCD3/CD28 beads in
531 media alone, 3mM 2DG, or in glucose-free media. Media was supplemented with IL-2.
532 Representative flow cytometry plots of GLUT1 and CD25 surface expression on resting, and day

533 1-(A) or day 4- post(B) activated Naïve (top) and memory T cells (bottom) in indicated conditions
534 were shown. (C) Fold difference in frequency of GLUT1 expression or mean fluorescence
535 intensity of CD25 expression on naive or memory T cells on day 1- and day 4-post activation in
536 media without IL-2. (D) Naive and memory CD4+ T cells were labeled with CTV dye followed by
537 aCD3/CD28 beads activation in media alone (control), 3mM 2DG, or in glucose-free media.
538 Representative histogram plot of CTV-labeled naïve (up) and memory (bottom) CD4+ T cells in
539 3mM 2DG (Red) or in glucose-free media(blue) after day 4 and day 6 post beads activation. Data
540 represent three independent experiments. ***p<0.01
541
542 **Supplemental Figure 2. GLUT1 expression was down-regulated on naive T cells but not**
543 **memory T cells after long-term culture.** Naive and memory CD4+ T cells were activated with
544 aCD3/CD28 beads in media alone (control in grey), or 1mM 2DG (in black). Media was not
545 supplemented with IL-2. Fold difference in frequency of GLUT1 expression on naïve or memory
546 T cells on day 14-post activation was shown. Data represent three independent experiments.
547 ***p<0.01
548
549 **Supplemental Figure 3. 2DG did not change IL-2 and TNF production by CD4+ T cells.**
550 Purified CD4+ T cells were activated with aCD3/CD28 beads in media alone, with various doses
551 of 2DG, and in glucose-free media. After 14 days' cell culture, T cells activated and culture in
552 indicated condition were re-stimulated with PMA/ionomycin for 4h, followed by intracellular
553 staining as described in the method section. The percentage of IL-2 and TNF production from
554 CD4+ T cells were shown. Data represent three independent experiments.
555
556 **Supplemental Figure 4. 2DG enhances *in vitro* generation of IL-17-producing cells from**
557 **naive T cells.** Fold difference of IL-17 production from 1mM 2DG treated naive T cells with
558 polarizing cytokines (P.C.) or without (-) compared with control after 14-day expansion.

559

560 **Supplemental Figure 5. Mannose did not reverse GLUT1 expression 2DG-treated naive T**
561 **cells.** Naive CD4+ T cells were activated with aCD3/CD28 beads in media alone, 3mM 2DG
562 alone, 3mM 2DG plus 1mM or 3mM mannose. Representative flow cytometry plots of GLUT1 and
563 CD25 surface expression on naive T cells activated for 24 h in indicated conditions were shown.
564 Data represent three independent experiments.

565

566

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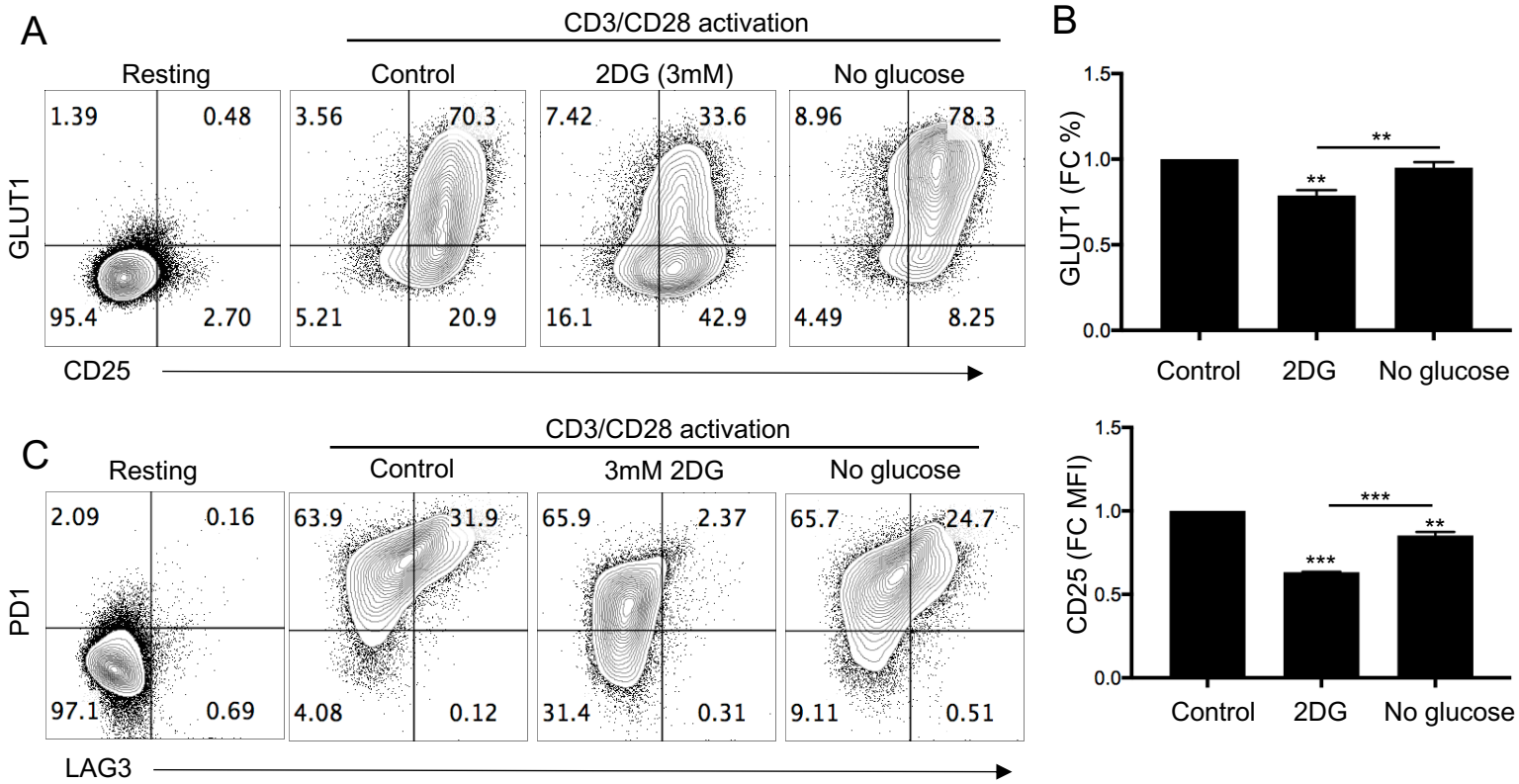
946

Figure 1


A
CD3/CD28 activation
Resting
Control
2DG (3mM)
No glucose
GLUT1
CD25
1.39
0.48
95.4
2.70
3.56
70.3
5.21
20.9
7.42
33.6
16.1
42.9
8.96
78.3
4.49
8.25
B
GLUT1 (FC %)
Control
2DG
No glucose
C
CD3/CD28 activation
Resting
Control
3mM 2DG
No glucose
PD1
LAG3
2.09
0.16
97.1
0.69
63.9
31.9
4.08
0.12
65.9
2.37
31.4
0.31
65.7
24.7
9.11
0.51
CD25 (FC MFI)
Control
2DG
No glucose

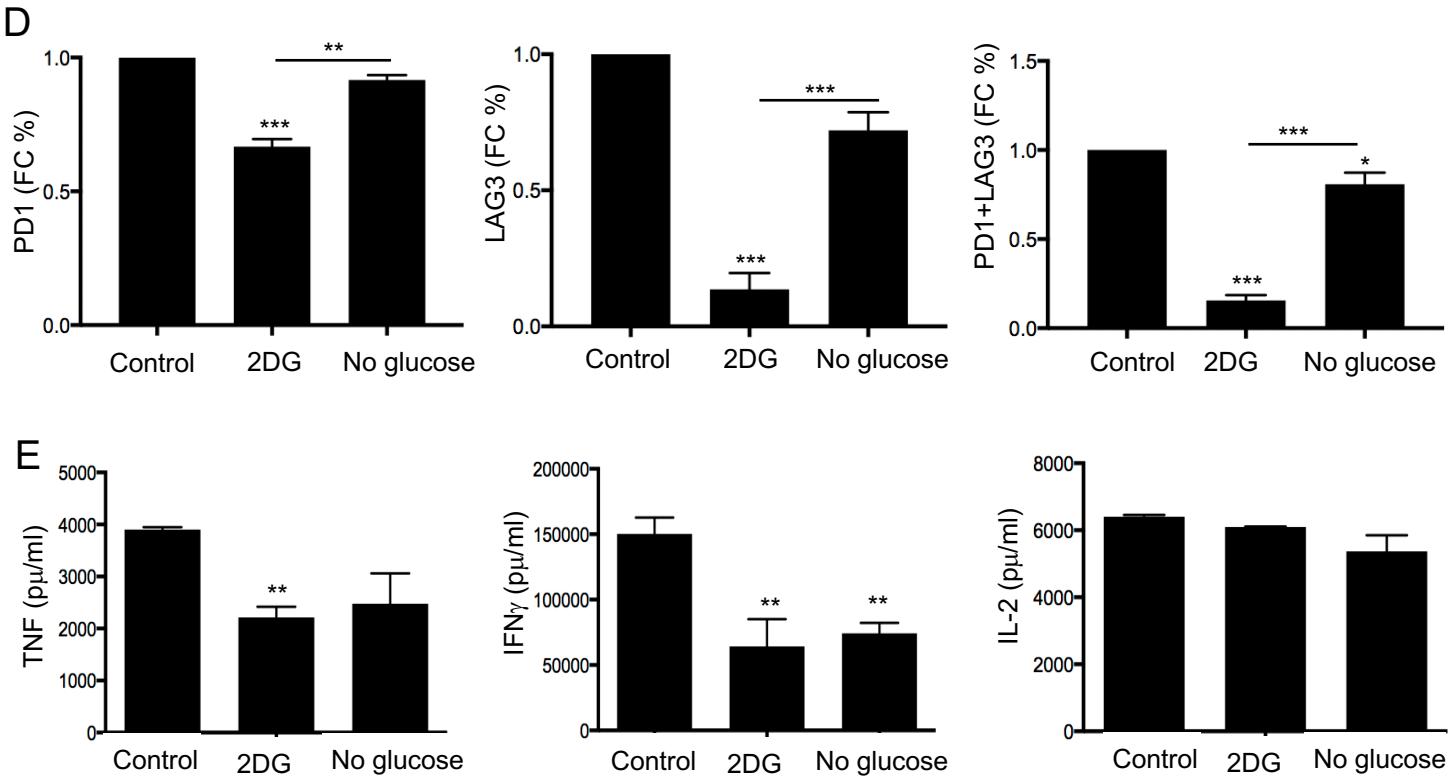

D
PD1 (FC %)
Control
2DG
No glucose
LAG3 (FC %)
PD1+LAG3 (FC %)
E
TNF (pμ/ml)
IFNγ (pμ/ml)
IL-2 (pμ/ml)

Figure 2

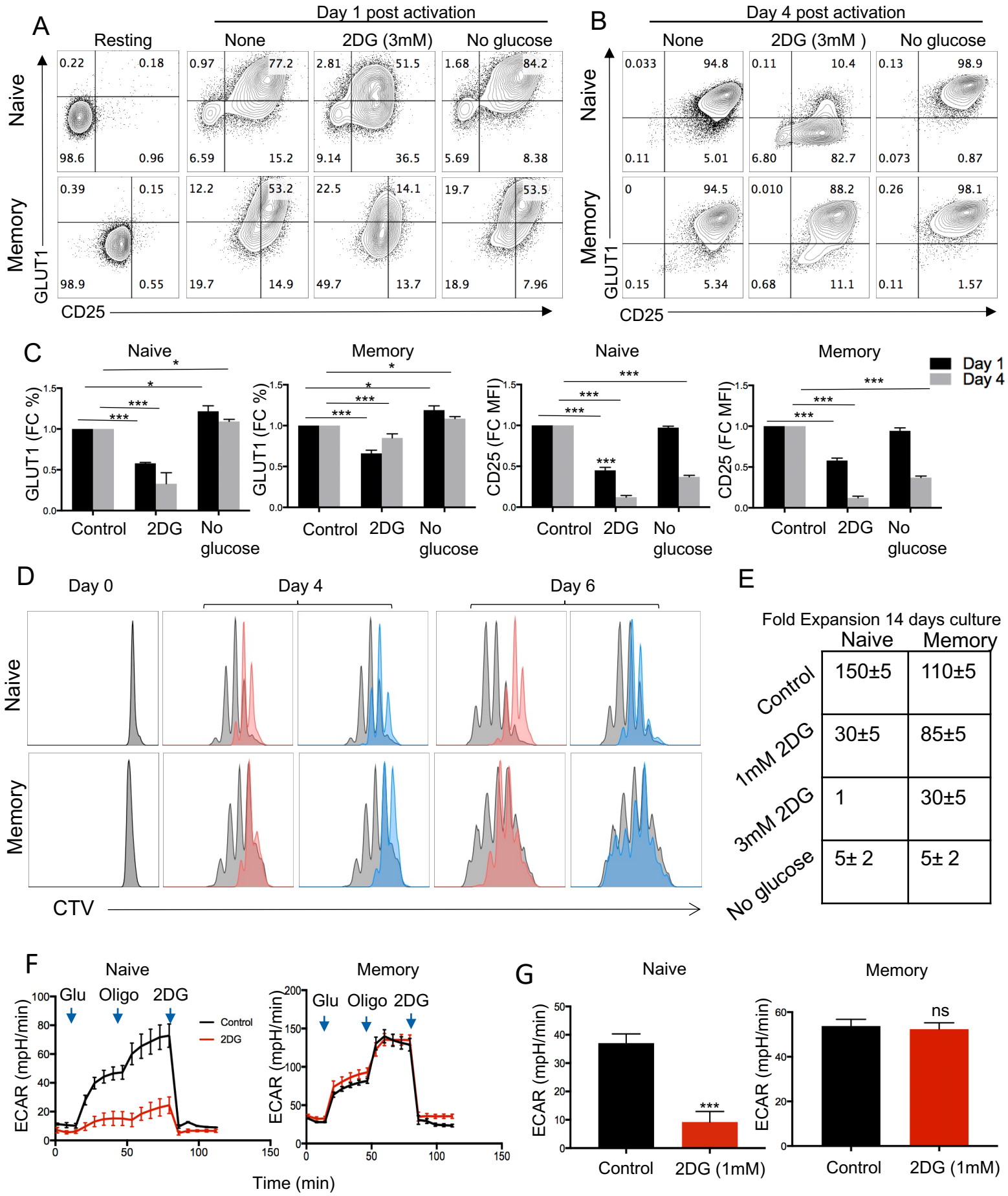

A
Day 1 post activation
Resting
None
2DG (3mM)
No glucose
Naive
Memory
GLUT1
CD25
0.22
0.18
98.6
0.96
0.97
77.2
6.59
15.2
2.81
51.5
9.14
36.5
1.68
84.2
5.69
8.38
0.39
0.15
98.9
0.55
12.2
53.2
19.7
14.9
22.5
14.1
49.7
13.7
19.7
53.5
18.9
7.96
B
Day 4 post activation
None
2DG (3mM)
No glucose
0.033
94.8
0.11
5.01
0.11
10.4
6.80
82.7
0.13
98.9
0.073
0.87
0
94.5
0.15
5.34
0.010
88.2
0.68
11.1
0.26
98.1
0.11
1.57
C
Naive
Memory
GLUT1 (FC %)
CD25 (FC MFI)
Control
2DG
No glucose
Day 1
Day 4
D
Day 0
Day 4
Day 6
CTV
E
Fold Expansion 14 days culture
Naive
Memory
Control
1mM 2DG
3mM 2DG
No glucose
150±5
110±5
30±5
85±5
1
30±5
5± 2
5± 2
F
Glu
Oligo
2DG
Control
2DG
ECAR (mpH/min)
Time (min)
G
ns

Figure 3

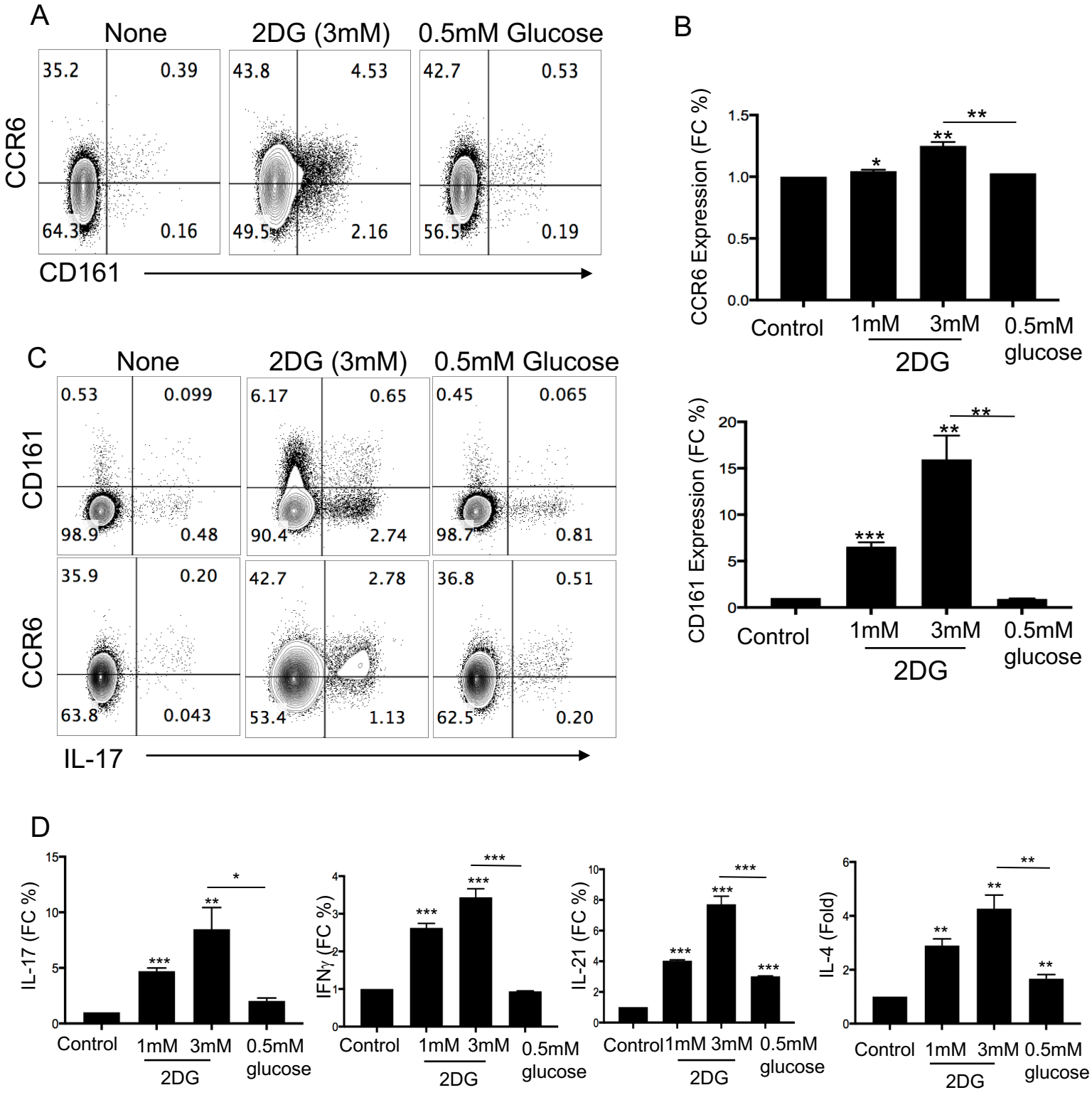

A
None
2DG (3mM)
0.5mM Glucose
CCR6
CD161
35.2
0.39
64.3
0.16
43.8
4.53
49.5
2.16
42.7
0.53
56.5
0.19
B
CCR6 Expression (FC %)
Control
1mM
3mM
0.5mM glucose
2DG
CD161 Expression (FC %)
C
CD161
CCR6
IL-17
0.53
0.099
98.9
0.48
6.17
0.65
90.4
2.74
0.45
0.065
98.7
0.81
35.9
0.20
63.8
0.043
42.7
2.78
53.4
1.13
36.8
0.51
62.5
0.20
D
IL-17 (FC %)
IFNγ (FC %)
IL-21 (FC %)
IL-4 (Fold)

Figure 4

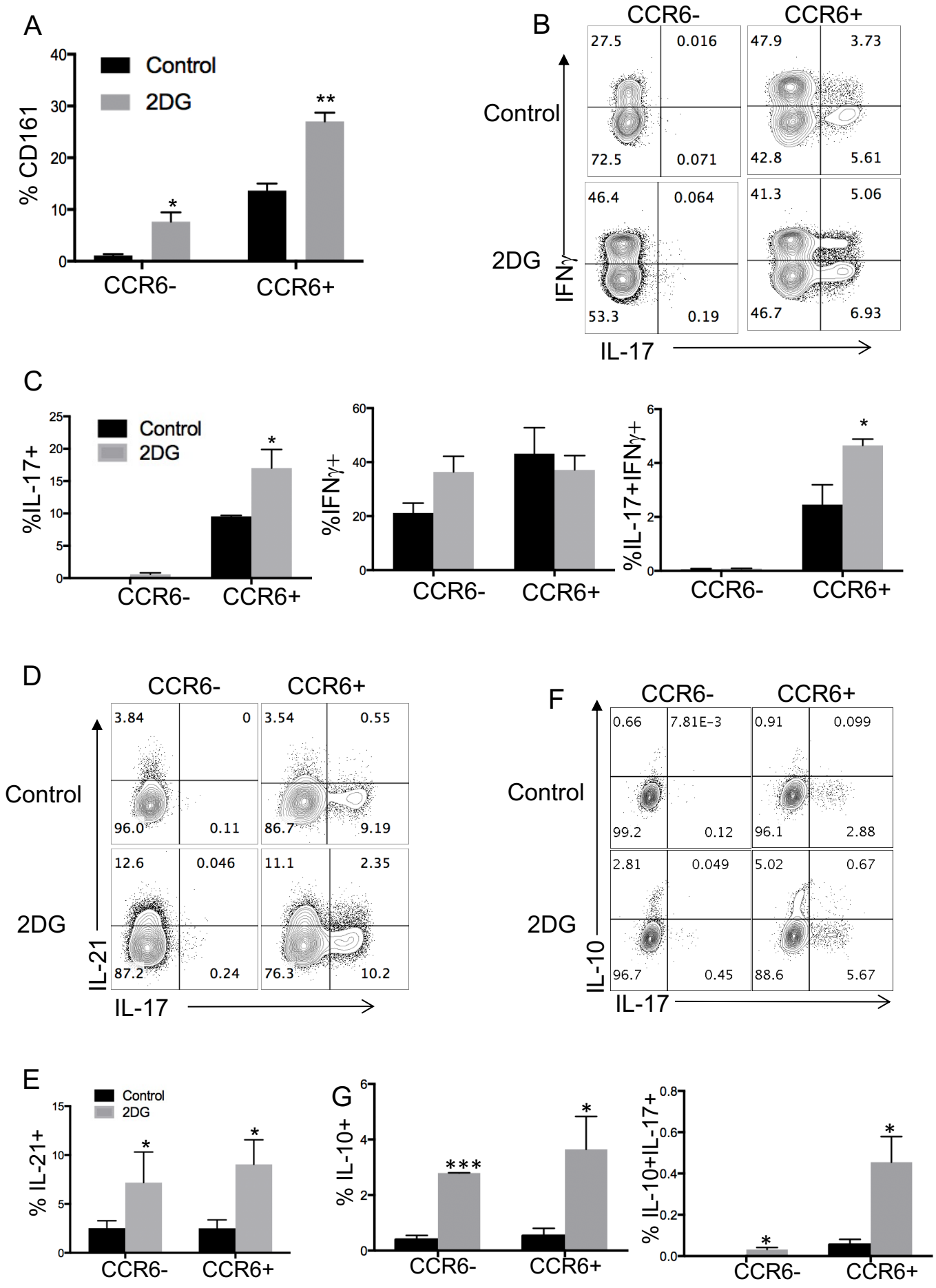

A
Control
2DG
% CD161
CCR6-
CCR6+
B
CCR6-
CCR6+
Control
2DG
IFNγ
IL-17
27.5
0.016
72.5
0.071
47.9
3.73
42.8
5.61
46.4
0.064
53.3
0.19
41.3
5.06
46.7
6.93
C
%IL-17+
%IFNγ+
%IL-17+IFNγ+
D
3.84
0
96.0
0.11
3.54
0.55
86.7
9.19
12.6
0.046
87.2
0.24
11.1
2.35
76.3
10.2
IL-21
F
0.66
7.81E-3
99.2
0.12
0.91
0.099
96.1
2.88
2.81
0.049
96.7
0.45
5.02
0.67
88.6
5.67
IL-10
E
% IL-21+
G
% IL-10+
% IL-10+IL-17+

Figure 5

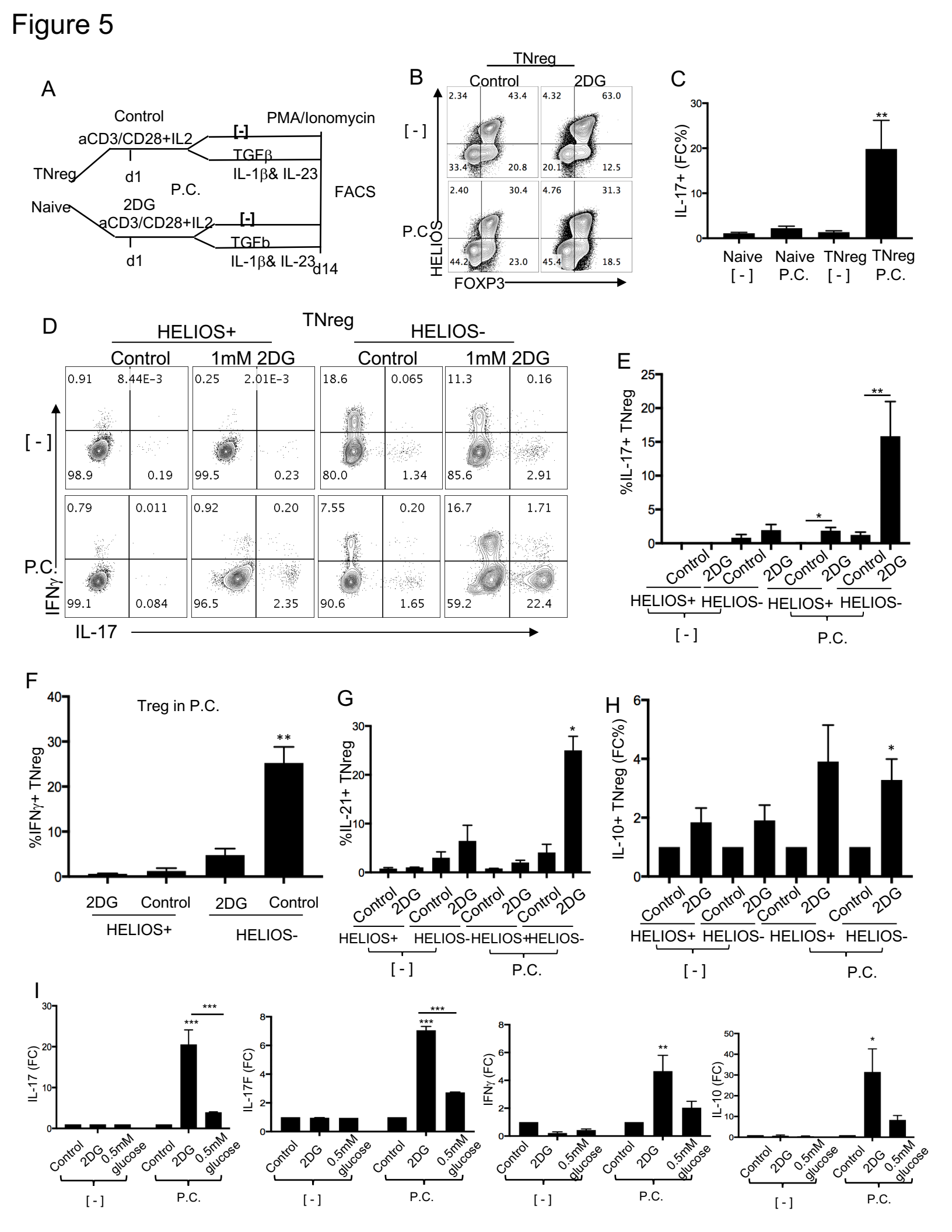
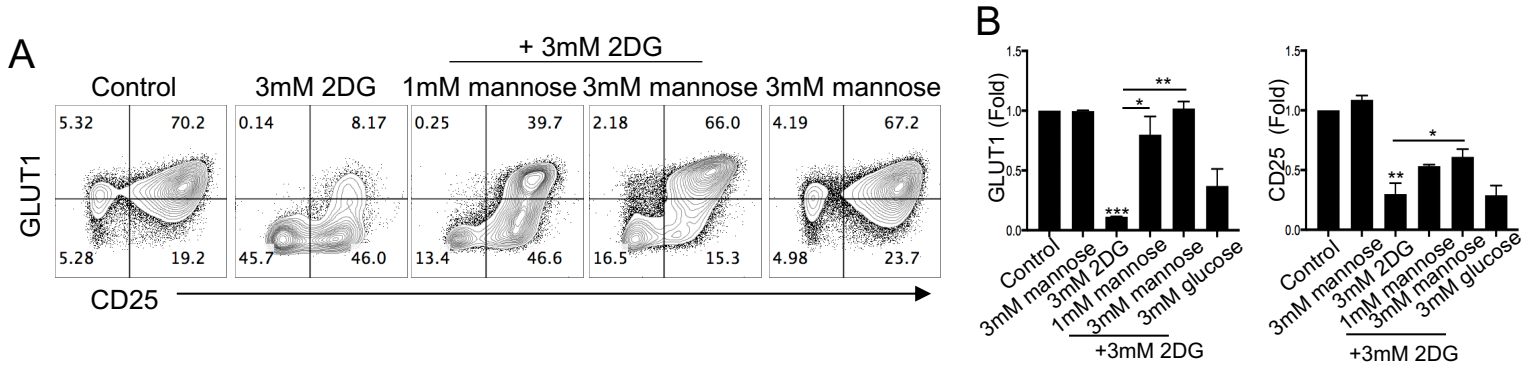
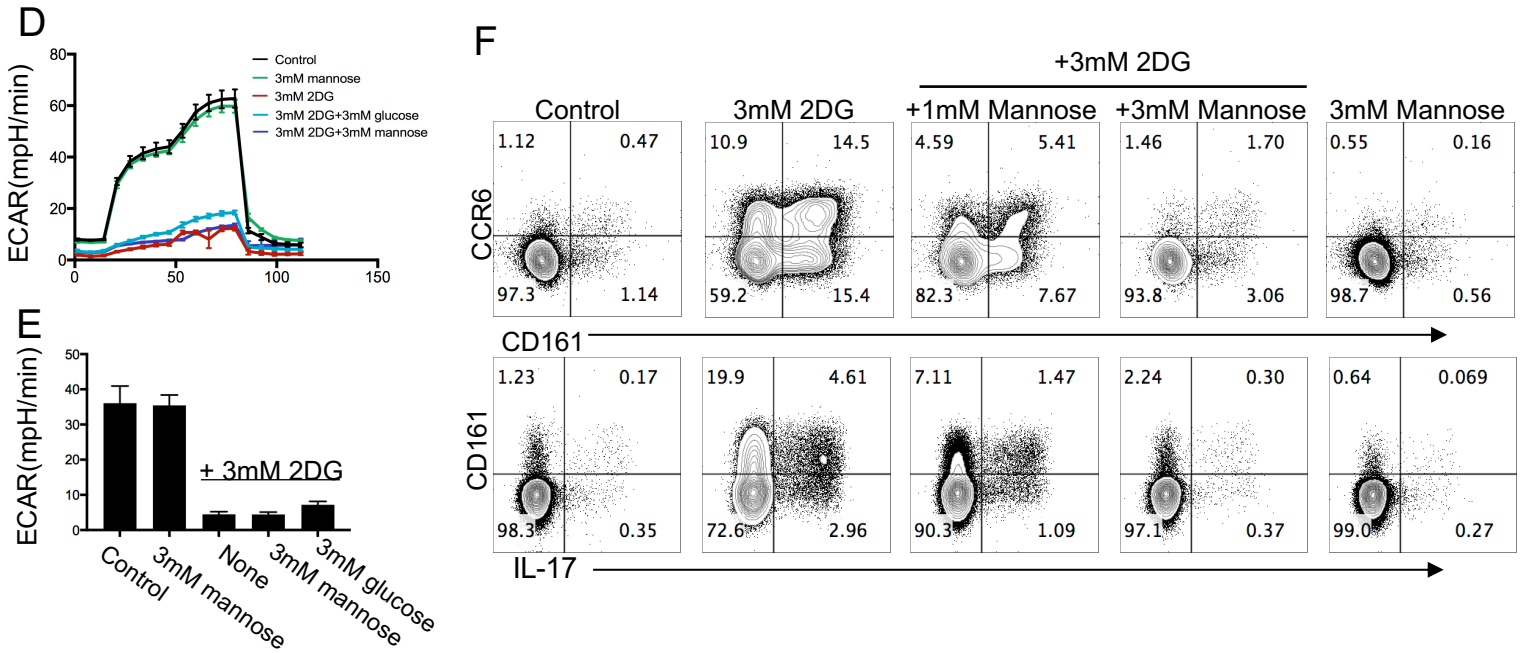

A
Control
aCD3/CD28+IL2
PMA/Ionomycin
[-]
TGFβ
IL-1β& IL-23
TNreg
d1
P.C.
FACS
Naive
2DG
aCD3/CD28+IL2
[-]
TGFb
IL-1β& IL-23
d1
d14
B
TNreg
Control
2DG
[-]
P.C.
HELIOS
FOXP3
2.34
43.4
33.4
20.8
4.32
63.0
20.1
12.5
2.40
30.4
44.2
23.0
4.76
31.3
45.4
18.5
C
IL-17+ (FC%)
Naive [-]
Naive P.C.
TNreg [-]
TNreg P.C.
D
TNreg
HELIOS+
HELIOS-
Control
1mM 2DG
Control
1mM 2DG
[-]
P.C.
IFNγ
IL-17
0.91
8.44E-3
98.9
0.19
0.25
2.01E-3
99.5
0.23
18.6
0.065
80.0
1.34
11.3
0.16
85.6
2.91
0.79
0.011
99.1
0.084
0.92
0.20
96.5
2.35
7.55
0.20
90.6
1.65
16.7
1.71
59.2
22.4
E
%IL-17+ TNreg
Control
2DG
HELIOS+
HELIOS-
[-]
P.C.
F
Treg in P.C.
%IFNγ+ TNreg
2DG
Control
HELIOS+
HELIOS-
G
%IL-21+ TNreg
Control
2DG
HELIOS+
HELIOS-
[-]
P.C.
H
IL-10+ TNreg (FC%)
Control
2DG
HELIOS+
HELIOS-
[-]
P.C.
I
IL-17 (FC)
IL-17F (FC)
IFNγ (FC)
IL-10 (FC)
Control
2DG
0.5mM glucose
[-]
P.C.

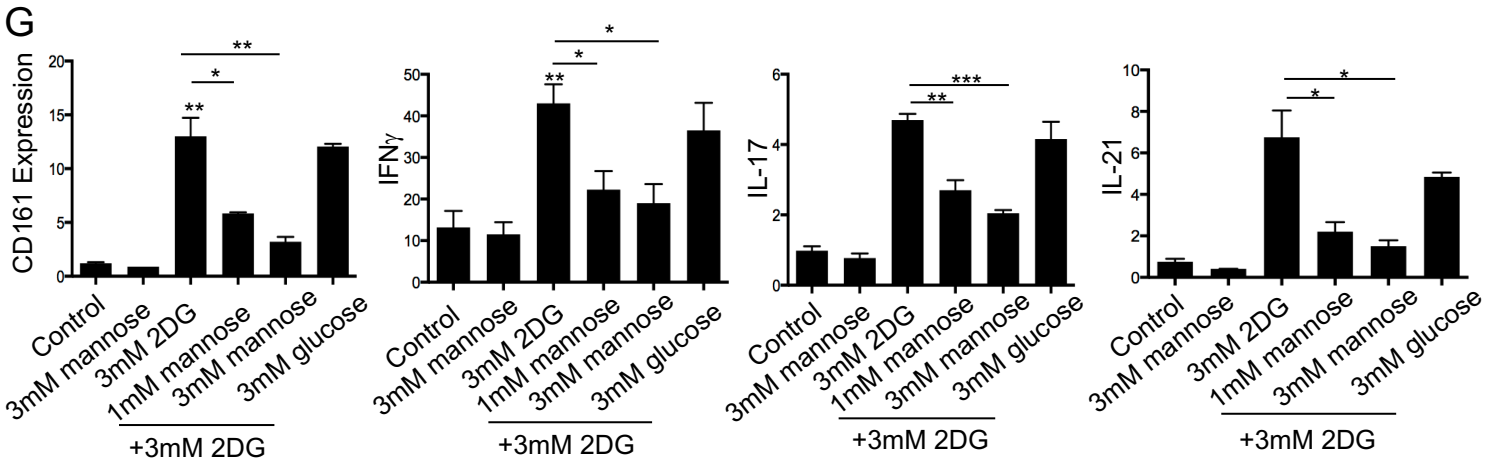
Figure 6



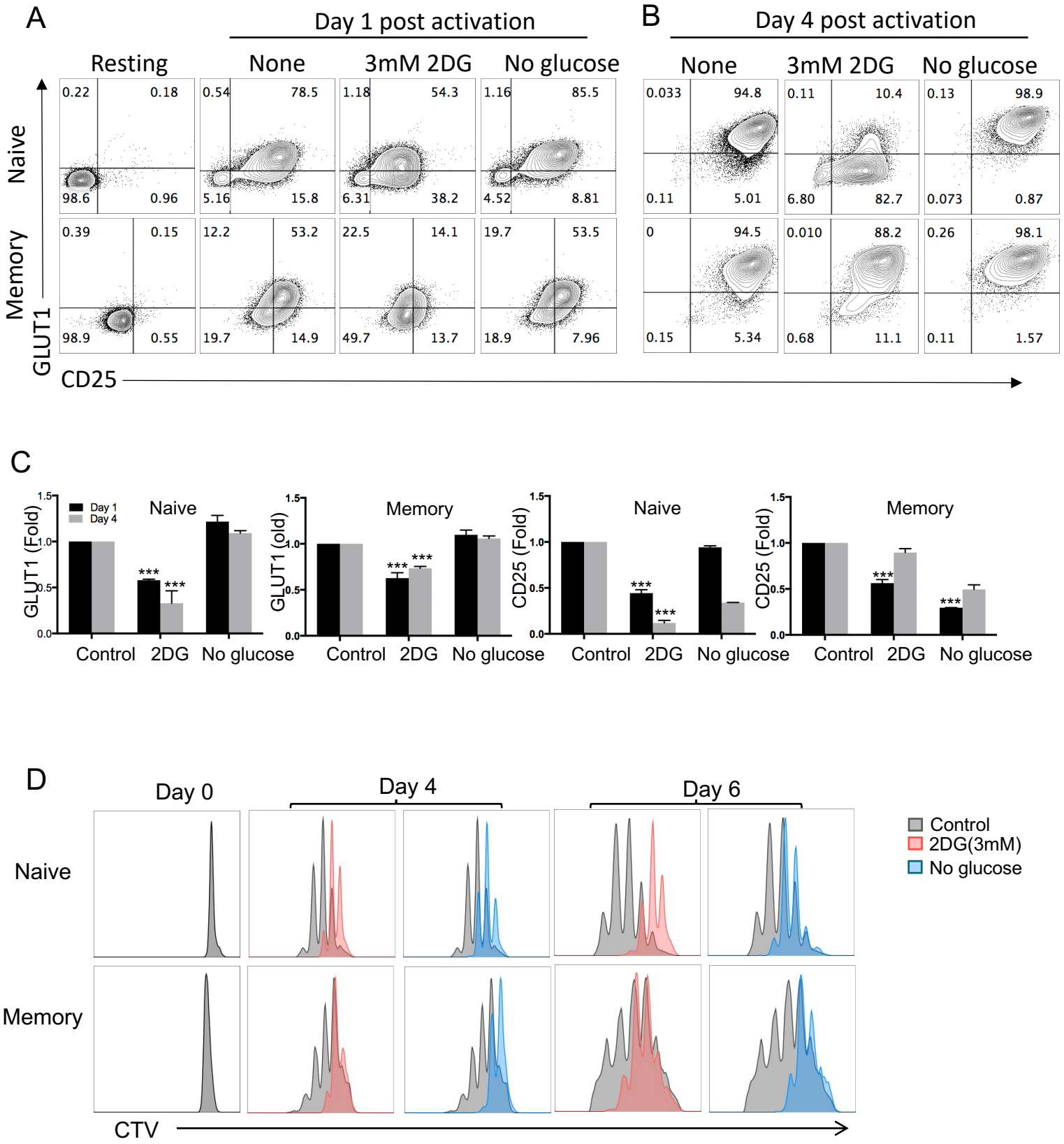
C Naive T Cell Expansion after 2 weeks culture

		+ 3mM 2DG			
Control	3mM mannose	None	1mM mannose	3mM mannose	3mM glucose
157±5	100±5	1	30±5	50±5	16±5

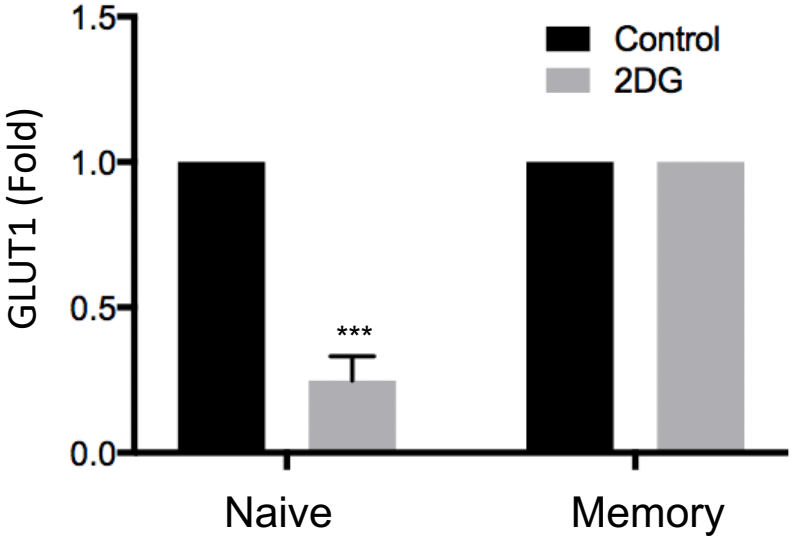




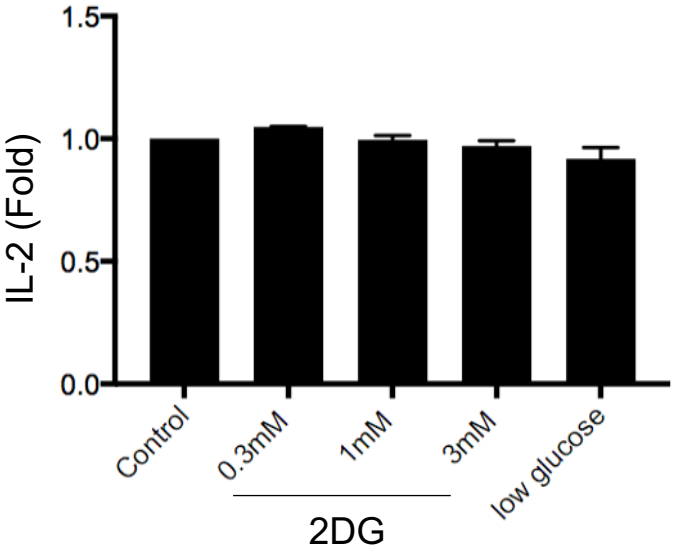
Supplemental Figure 1

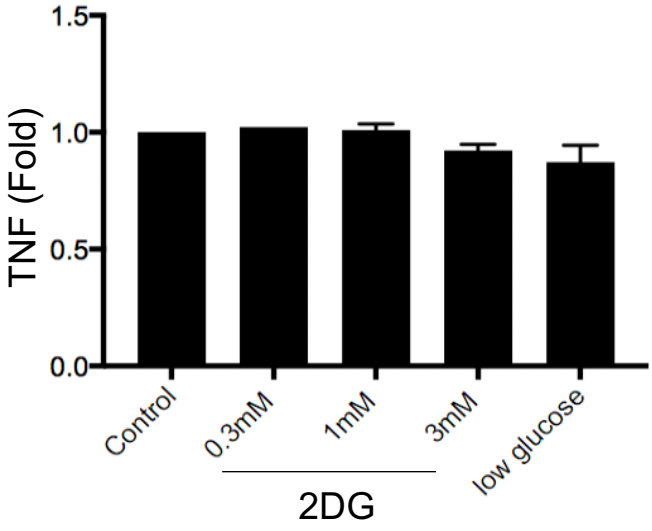


Supplemental Figure 2

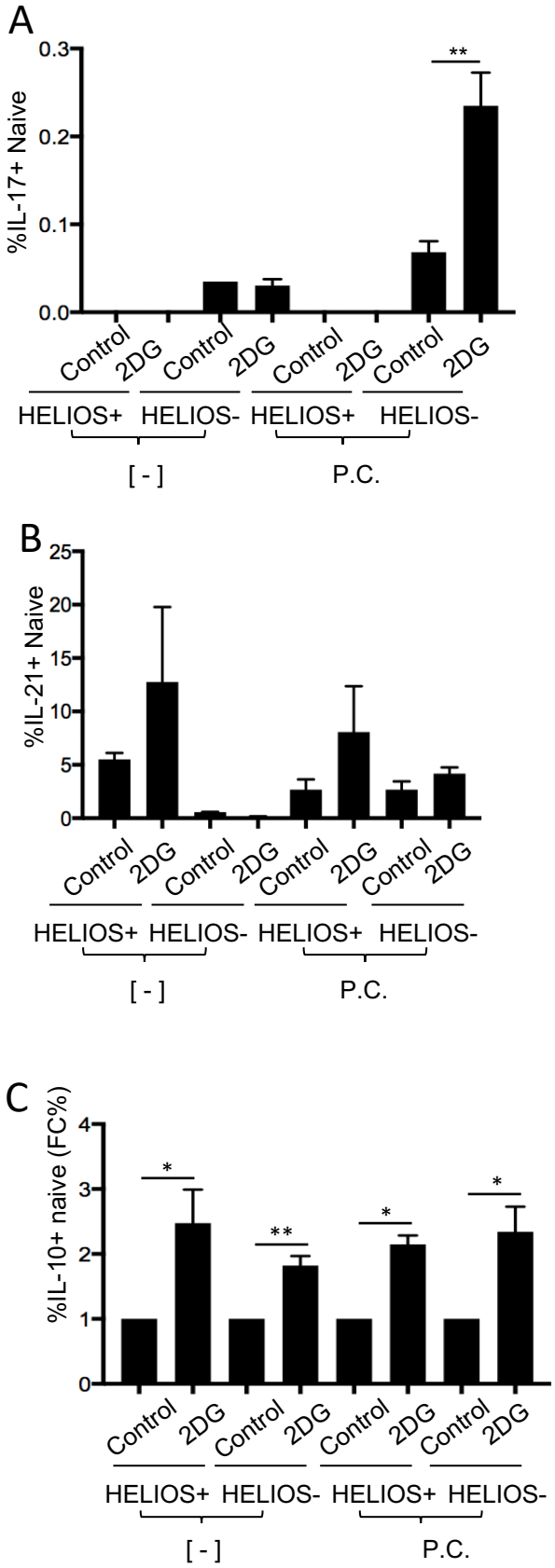
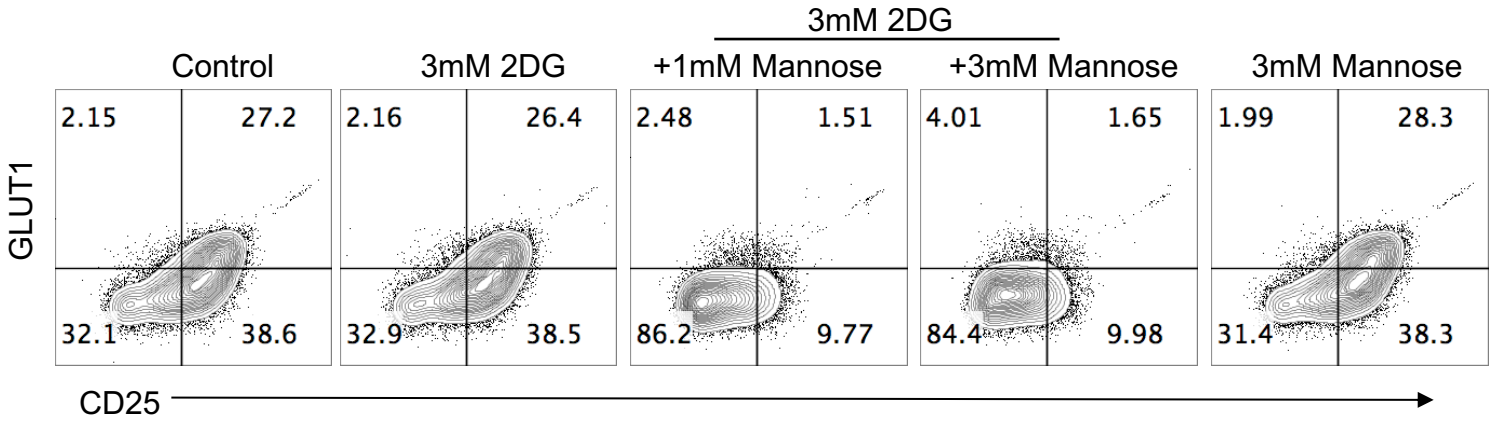


Supplemental Figure 3





Supplemental Figure 4



Supplemental Figure 5