Hypoxia is a dominant remodeler of the CD8⁺ T cell surface proteome relative to activation and regulatory T cell-mediated suppression

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ABSTRACT

Immunosuppressive factors in the tumor microenvironment (TME) impair T cell function and limit the anti-tumor immune response. T cell surface receptors that influence interactions and function in the TME are already proven targets for cancer immunotherapy. However, surface proteome remodeling of primary human T cells in response to suppressive forces in the TME has never been characterized systematically. Using a reductionist cell culture approach with primary human T cells and SILAC-based quantitative cell surface capture glycoproteomics, we examined how two immunosuppressive TME factors, regulatory T cells (Tregs) and hypoxia, globally affect the activated CD8⁺ surface proteome (surfaceome). Surprisingly, the CD8⁺/Treg co-culture only modestly affected the CD8⁺ surfaceome, but did reverse several activation-induced surfaceomic changes. In contrast, hypoxia dramatically altered the CD8⁺ surfaceome in a manner consistent with both metabolic reprogramming and induction of an immunosuppressed state. The CD4⁺ T cell surfaceome similarly responded to hypoxia, revealing a novel hypoxia-induced surface receptor program. Our findings are consistent with the premise that hypoxic environments create a metabolic challenge for T cell activation, which may underlie the difficulty encountered in treating solid tumors with immunotherapies. Together, the data presented here provide insight into how suppressive TME factors remodel the T cell surfaceome and represent a valuable resource to inform future therapeutic efforts to enhance T cell function in the TME.

56 INTRODUCTION

57 Cytotoxic CD8⁺ T cells promote tumor cell killing, a function that is substantially modulated in the
58 tumor microenvironment (TME)¹. The TME is a complex mixture of tumor, somatic, and immune cells that
59 create a unique environment in and around the tumor. Tumor-infiltrating CD8⁺ T cells encounter many cell
60 types in the TME, including immunosuppressive regulatory T cells (Tregs).^{2,3} Tregs express immune
61 suppressors such as CTLA-4⁴ and produce immunosuppressive adenosine⁵ and cytokines such as TGFβ,
62 interleukin (IL)-10⁶, and IL-35⁷. Furthermore, Tregs and CD8⁺ cells compete for IL-2 for proliferation;
63 high expression of the high affinity IL-2 receptor on Tregs allows them to out-compete CD8⁺ T cells for
64 available IL-2.⁸ Tregs can also directly kill CD8⁺ T cells via the perforin pathway.⁹ Consistent with these
65 many immunosuppressive activities, increased Treg tumor infiltration is associated with poor prognosis in a
66 number of cancers, including non-small cell lung, hepatocellular, renal cell, breast, cervical, ovarian, and
67 gastric cancers, as well as melanoma.^{3,10,11}

68 Another hallmark of the TME is hypoxia due to poor and variable vascularization within the
69 tumor.^{1,12} Hypoxia is common in the core of tumors and induces dramatic transcriptional changes.^{1,12-17}
70 Tumor-associated hypoxia strongly influences the function of numerous immune cells from both the
71 myeloid¹⁸ and lymphoid lineages¹⁹⁻²¹, with both stimulatory and inhibitory effects reported²². Hypoxia
72 induces expression of CD39 and CD73 that catalyze the production of immunosuppressive adenosine from
73 ATP.²³ Hypoxia also induces the Warburg effect, which leads to tumor acidification, decreased CD8⁺ T cell
74 proliferation, and reduced cytotoxic activity.²⁴ Hypoxia additionally promotes recruitment of Tregs to the
75 tumor²⁴, and CD8⁺ cells have been observed to be excluded from areas of tumor hypoxia²⁵. Recently,
76 hypoxia was also linked to T cell exhaustion²⁶. Inhospitable, hypoxic regions in solid tumors may also limit
77 the function of CAR-T cells²⁷, which could contribute to the limited success of targeting solid tumors with
78 CAR-T cells.

79 The cell surface proteome, or surfaceome, mediates T cell interactions with the external
80 environment, and the effect of external environmental factors on the T cell surfaceome has not yet been

81 studied globally. Not only does the surfaceome help T cells sense and respond to the environmental
82 conditions of the TME, but membrane proteins are useful surface markers and key regulators of the anti-
83 tumor function of CD8⁺ cells. For example, proteins such as PD1 play crucial roles in the suppression of
84 CD8⁺ cells.²⁸ Consequently, many current immunotherapies target and modulate T cells through blockade
85 or engagement of surface proteins (e.g. anti-PD1 or anti-CTLA-4 therapy, bispecific T cell engagers).²⁹
86 Therefore, profiling how the CD8⁺ surfaceome changes in response to TME factors, such as Treg-mediated
87 suppression or hypoxia, should expand our understanding of the basic biological response to these
88 modulators.

89 We have taken a reductionist cell culture approach to begin to understand how Tregs and hypoxia
90 modulate the cell surface proteome of primary CD8⁺ T cells. We first identified global and bi-directional
91 changes in the CD8⁺ T cell surfaceome following classic activation with agonistic antibodies to CD3 and
92 CD28 using quantitative cell surface capture mass spectrometry methods^{30,31}. We discovered that co-
93 culturing with Tregs, and especially hypoxic culture, significantly alter the activated CD8⁺ surfaceome in a
94 manner consistent with reduced CD8⁺ activation. Although our *in vitro* model conditions are much less
95 complex than what would be found in an *in vivo* TME, this approach allowed us to control and separately
96 assess the impact of T cell activation, Treg co-culture, and hypoxia on the CD8⁺ T cell surfaceome.
97 Collectively, these findings help illuminate how surfaceomic remodeling contributes to suppression of
98 CD8⁺ T cells in the presence of Tregs and hypoxia, and provides a resource to identify potential markers
99 for selective therapeutic targeting of suppressed CD8⁺ T cells in the TME or the design of new cellular
100 therapies to overcome TME-mediated suppression.

101

102 RESULTS

103 Activation dramatically alters the CD8⁺ T cell surfaceome in a bi-directional fashion

104 Our strategy to study how the CD8⁺ cell surfaceome responds to activation, and how this response
105 is altered by Treg suppression or hypoxia, is shown in Figure 1. Primary CD8⁺ cells were isolated from

106 healthy donors and expanded 10- to 100-fold with anti-CD3 and anti-CD28 stimulation in the presence of
 107 IL-2. Cells were grown in medium containing light or heavy isotope-labeled lysine and arginine to
 108 quantitatively compare stimulation conditions using SILAC (stable isotopic labeling with amino acids in
 109 cell culture) coupled with a glycoprotein cell surface capture technique and LC-MS/MS^{30–32} (Figure 1A).
 110 This led to quantitative and uniform labeling as assessed by isotope distribution on four abundant and
 111 constitutively-expressed proteins (Supplemental Figure 1). We first compared the activation-induced
 112 changes in the CD8⁺ surfaceome before and after activation with anti-CD3/anti-CD28 for three days, and
 113 then examined how the program was altered by the addition of primary Tregs or hypoxic culture (Figure
 114 1B).

115 Surfaceomic analysis of unstimulated and anti-CD3/anti-CD28-stimulated CD8⁺ T cells from four
 116 donors identified a total of 669 surface proteins (Figure 2A, Supplemental Table 1). Although there was
 117 donor-to-donor variation (Supplemental Figure 2), assessment of a compiled dataset including fold-change
 118 data from the four donors revealed about 16% of these proteins (106/669) consistently showed significant
 119 ($P<0.05$) 1.5-fold up- or downregulation. These significantly-altered proteins showed strong correlation
 120 between most donors (Supplemental Figure 2). We observed changes in classic markers of T-cell
 121 activation, including upregulation of two classic T cell activation markers (CD69³³ and the transferrin
 122 receptor [TFRC]³⁴), and downregulation of the IL-7 receptor (internalized upon activation) and VIPR1³⁵
 123 (Figure 2B).

124 Large-scale network analysis of the significantly-altered proteins demonstrates the roughly
 125 symmetrical, bi-directional response of the T cell surfaceome to activation, with 66 proteins upregulated
 126 and 40 proteins downregulated (Figure 2C). In addition to CD69 and TFRC, numerous well-established T
 127 cell activation markers were also upregulated, including CD63³³, CD83³³, CD97³⁶, and CD109³⁷.
 128 Importantly, multiple solute carrier (SLC) transporters were also upregulated on activated CD8⁺ T cells,
 129 including the amino acid transporters SLC1A5 and SLC7A5 that have been previously implicated in
 130 supporting T cell activation.³⁸ Of note, comparison of our proteomics data with RNAseq data from

131 activated versus resting CD8⁺ cells in the DICE database revealed a loosely positive correlation ($R=0.25$,
132 $P<0.0001$, Supplemental Figure 3A).³⁹ It is well known that protein and RNA levels show only mild
133 correlations because of differences in stability and regulation. Assessment of only proteins that were
134 significantly changed in our proteomics data revealed a stronger correlation ($R=0.53$, $P<0.0001$,
135 Supplemental Figure 3B). However, several proteins that demonstrated significant change in our data
136 showed minimal or divergent change in the RNAseq data (e.g. CD70, 2.09 vs. -0.41; SLC5A6, 1.62 vs.
137 0.46; IL7R, -1.80 vs. 0.73 [$\log_2(\text{enrichment ratio})$ for proteomics vs. RNAseq, respectively]). These
138 discrepancies could be due to differential activation conditions, but nonetheless underscore the importance
139 of protein-level profiling to capture surface protein remodeling in immune cells.

140 More globally, pathway analysis of up- and downregulated cell surface proteins revealed the most
141 significant enrichment for proteins implicated in immune function, with a slight trend towards upregulation
142 of these proteins (Figure 2D, proteins annotated for GO.0002376: immune system process are indicated
143 with green borders in Figure 2C). Collectively, these surfaceomic data identify classic (e.g. CD69) and
144 some newly-recognized (e.g. integrin α_X [ITGAX], SLC39A14, BST2) markers for immune activation of
145 primary CD8⁺ T cells.

146

147 **Co-culture with Tregs modulates the activated CD8⁺ surfaceome**

148 We next analyzed the effect that primary Tregs have upon the surfaceome of activated primary
149 CD8⁺ T cells in a 1:1 ratio co-culture after three days (Figure 3A, Supplemental Table 2). Relative to
150 activation alone, Treg co-culture had a mild global impact on the surfaceome of activated CD8⁺ cells, with
151 significant ($P<0.05$) up- or downregulation (± 1.5 -fold change) of only 34 out of 675 proteins detected
152 (Figure 3A). Changes in these proteins were again largely consistent between donors (Supplemental Figure
153 4). Of note, upregulation of TFRC and the pro-inflammatory cytokine receptor IL18R1 on the cell surface
154 was blunted by the addition of Tregs (Figure 3B). In the presence of Tregs, IL7R showed variable
155 upregulation between donors, opposite of the trend seen with cell activation in monoculture (Figures 2B

and 3B). Similarly, L-selectin (SELL), a protein which is typically downregulated with T cell activation⁴⁰, was slightly upregulated on CD8⁺ T cells, again consistent with a suppressive effect of Tregs (Figure 3B). Due to the smaller number of significantly changed proteins in this dataset, network analysis was not as striking as in the activation dataset (Figure 3C). However, in contrast to the monoculture CD8⁺ T cell activation dataset, which demonstrated an upward trend in proteins implicated in immune processes, there is a downward trend in immune-annotated proteins when CD8⁺ cells are activated in the presence of Tregs, consistent with an immunosuppressive effect (Figure 3D). Furthermore, many proteins that were upregulated on activated CD8⁺ T-cells in monoculture (Figure 2) were downregulated upon activation in the presence of Tregs (11 of 23 downregulated proteins, Figure 3C). Among these proteins are several SLCs, including SLC1A5 and SLC7A1. Collectively, these data suggest that the presence of Tregs reverses at least part, but not all, of the activation-induced surfaceomic response in CD8⁺ T cells.

Hypoxia triggers large-scale surfaceomic changes in activated CD8⁺ T cells consistent with immunosuppression and anaerobic reprogramming

We next wanted to test how hypoxia affects the surfaceome of activated CD8⁺ T cells. Interestingly, over three days in culture, activated CD8⁺ T cells proliferated only ~1.3-fold faster in normoxia (20% O₂) compared to hypoxia (1% O₂), and there were no substantial differences in cell viabilities (Supplemental Figure 5). However, surface proteomics of CD8⁺ T cells activated in normoxic or hypoxic conditions revealed substantial remodeling of the surface proteome. Of a total of 1064 proteins identified, 196 were significantly ($P < 0.05$) up- or down-regulated (± 1.5 -fold change, Figure 4A, Supplemental Table 3) in hypoxia relative to normoxia. The fold changes observed for these significantly-altered proteins showed higher correlation among donors (Supplemental Figure 6) than seen in the activation (Supplemental Figure 2) and Treg co-culture (Supplemental Figure 4) datasets. The upregulation of the hypoxia-induced glucose transporter SLC2A3 (GLUT3)⁴¹ we observed is consistent with a shift towards glycolysis. We also

180 observed downregulation of activin receptor type-1 (ACVR1), which is sequestered in endosomes under
 181 hypoxic conditions⁴² (Figure 4B).

182 Network visualization of proteins significantly altered by hypoxia revealed multiple clusters of up-
 183 and down-regulated proteins (Figure 4C). Interestingly, gene ontology analysis did not identify significant
 184 enrichment for proteins involved in response to hypoxia (GO.0001666) or cellular response to hypoxia
 185 (GO.0071456). However, four of these significantly-altered proteins are found in the “Hallmark Hypoxia”
 186 gene set (SDC4, SLC6A6, B3GALT6, and SLC2A3).⁴³ We did observe marked upregulation of proteins
 187 involved in protein glycosylation and glycoprotein metabolic processes (Figure 4C and D). Tumor hypoxia
 188 is well established to cause glycan remodeling of tumor cell surface proteins⁴⁴, and our data suggest the
 189 same may be true for hypoxic T cells. Among the 132 hypoxia-upregulated proteins were numerous
 190 proteins involved in the unfolded protein response (e.g. ERMP1⁴⁵, FKBP11⁴⁶, MBTPS2⁴⁷) and regulation
 191 of autophagy (ATG9A⁴⁸, ERGIC1, SEC22B⁴⁹). In contrast, proteins implicated in immune function were
 192 strongly represented in the pool of 64 hypoxia-suppressed proteins (Figure 4C and D). In addition to the
 193 late activation marker IL2RA (CD25), co-stimulatory receptors, such as CD80 and CD86, as well as
 194 receptors for interleukins-3, -12, and -18 were significantly downregulated in hypoxic conditions (Figure
 195 4B and C). Several SLCs implicated in T cell function, including SLC1A5³⁸, and the pro-proliferative
 196 cytokines TNFSF4 (OX40 ligand) and TNFSF8 (CD30 ligand) were also downregulated. Of note, hypoxia
 197 led to downregulation of some immunosuppressive proteins, including CD70⁵⁰ and TIGIT⁵¹, but consistent
 198 upregulation of the checkpoint molecule VISTA (C10orf54)⁵² (Figure 4B and C). To validate these
 199 observations, we performed flow cytometry for IL18R1 and CD70 on CD8⁺ cells from four different
 200 donors activated in normoxia and hypoxia. Both proteins were upregulated on normoxic activated cells
 201 relative to resting cells, although the degree of upregulation was variable (Supplemental Figure 7). Hypoxia
 202 resulted in blunted cell surface induction of both proteins (Supplemental Figure 7), consistent with our
 203 proteomics results. Collectively, these data reveal that hypoxia induces dramatic remodeling of the

204 activated CD8⁺ T cell surfaceome and markedly regulates numerous proteins important for T cell
205 activation.

206

207 **CD8⁺ and CD4⁺ T cells demonstrate similar hypoxia-induced surface remodeling**

208 Given the dramatic effect of hypoxic culture on the surface proteome of activated CD8⁺ T cells, we
209 next determined how hypoxia modulates surface protein expression on another cell subset important for the
210 anti-tumor immune response: CD4⁺CD25⁻ conventional T cells. Analysis of CD4⁺CD25⁻ T cells from the
211 same donors as in Figure 4 again revealed dramatic surfaceomic remodeling when these cells were
212 activated in hypoxic conditions (Figure 5A, Supplemental Table 4). Of the 1144 proteins identified, 992
213 were also identified in our CD8⁺ hypoxia dataset. The fold-change ratios of these commonly-identified
214 proteins showed significant correlation ($R=0.82$, $P<0.0001$) between CD8⁺ and CD4⁺CD25⁻ cells (Figure
215 5B). Interestingly, more proteins were significantly up- (306) or downregulated (204) in the CD4⁺CD25⁻
216 dataset, and the magnitude of these changes was larger than those observed for the CD8⁺ cells. However,
217 there was a large overlap in the sets of significantly-altered proteins (Figure 5C and D). Consequently,
218 functional enrichment for these overlapping proteins was similar to that observed when analyzing the CD8⁺
219 response alone, with significant downregulation of proteins involved in immune-related processes (e.g.
220 CD70 and IL18R1) and upregulation of proteins involved in solute transport (e.g. SLC2A3, SLC16A3) and
221 protein glycosylation. Taken together, these data show there is substantial similarity in the surfaceomic
222 response of CD8⁺ and CD4⁺ T cells to hypoxia.

223

224 **Analysis of all CD8⁺ datasets reveals a conserved response to immunosuppressive stimuli**

225 We next cross-referenced the observed surfaceomic changes associated with Treg co-culture and
226 hypoxia. Interestingly, gene set enrichment analysis comparing the expression level of all proteins
227 identified in our datasets revealed significant enrichment for a number of biological processes, namely
228 organic and amino acid transmembrane transport (Figure 6A). Consistent with this observation, analysis of

229 proteins significantly upregulated with CD8⁺ activation but blunted due to hypoxia or Tregs identified three
 230 solute carriers (Figure 6B). Of these, SLC1A5 and SLC7A1 have been previously implicated in supporting
 231 T cell function following activation. Furthermore, the pro-inflammatory cytokine receptor IL18R1 and
 232 potentially inhibitory CD70⁵³ that were upregulated with CD8⁺ activation exhibited blunted surface
 233 induction with hypoxia and Tregs. Strikingly, when examining proteins downregulated upon CD8⁺
 234 activation in standard conditions but upregulated with hypoxia and Tregs, there are no commonly regulated
 235 proteins (Figure 6C). This suggests that protein upregulation seen during activation in hypoxic conditions
 236 may be primarily a general response to hypoxia. Together, the downregulation of a small but common set
 237 of surface proteins by both Treg co-culture and hypoxia may represent a conserved response of the T cell
 238 surface proteome to these two immunosuppressive stimuli.

239

240 **DISCUSSION**

241 A major obstacle encountered by CD8⁺ T cells when mounting an anti-tumor immune response is
 242 the immunosuppressive TME. Although Tregs and hypoxia are known to be immunosuppressive, we have
 243 little global understanding of how these affect the CD8⁺ T cell surface proteome. Our studies illuminate the
 244 bi-directional surfaceomic changes on CD8⁺ T cells associated with cell activation and with
 245 immunosuppression following co-culture with Tregs or hypoxic culture. The presence of Tregs partly
 246 reversed activation-induced changes in CD8⁺ T cells, consistent with suppressed cell activation. Perhaps
 247 surprisingly, the effect of hypoxia was much larger. Hypoxia triggered large-scale surfaceomic remodeling
 248 consistent with a general cellular response to the new metabolic demands of a low oxygen environment.
 249 Most importantly, cross-referencing of the effects of both Treg co-culture and hypoxia on the surface
 250 proteome of activated CD8⁺ T cells exposed a small, but intriguing list of common changes in the
 251 expression of proteins primarily involved in nutrient transport. This finding suggests that induced
 252 downregulation of surface proteins important to support CD8⁺ activation may be one mechanism of
 253 immunosuppression in the TME.

Global cell-surface capture proteomics not only provided a means of visualizing upregulation of known activation markers to validate our experimental system, but also generated an unbiased list of additional proteins demonstrating activation-associated changes. Importantly, our approach identified significant cell surface upregulation of numerous well-established activation markers (e.g. CD69, TFRC, CRTAM) that support T cell function. We also observed upregulation of TNFRSF18 (GITR), a molecule vital for CD8⁺ anti-tumor function and agonism of which synergizes with anti-PD1 checkpoint therapy.⁵⁴ The exhaustion marker LAG3 was upregulated, as was ENTPD1 (CD39), which has been reported to increase following T cell activation⁵⁵. Upregulation of CD39 is especially interesting because it facilitates conversion of extracellular ATP to ADP, which is the first step to generating immunosuppressive adenosine. Although this could represent a negative feedback loop to dampen T cell activation, we observed that CD73 (NT5E), which converts ADP to adenosine, was downregulated following activation. This is consistent with the recent observation that following activation human T cells strongly upregulate CD39, whereas CD73 remains near baseline levels.⁵⁵

Another class of proteins that demonstrated divergent trends are cell adhesion molecules. Many were significantly upregulated upon activation, including CD84⁵⁶, ALCAM, which stabilizes the immunological synapse⁵⁷, and integrin α X, which is associated with improved migratory potential⁵⁸. However, NRCAM, ICAM1, and ITGA4, the latter of which supports T cell migration⁵⁹, were downregulated. The polarity of this response following activation may be representative of the complex interactions an activated T cell must make with its environment to not only infiltrate an immunologically-active zone, but also interact with target cells. Lastly, we observed significant upregulation of a number of solute transporters involved in transporting amino acids, vitamins, and other nutrients. Of these, several (SLC29A1, SLC2A1, SLC7A1) have previously been implicated in supporting T cell function, and are crucial to help T cells respond to the metabolic demands following activation.^{60,61} However, the choline transporter SLC44A2 and potassium-chloride cotransporter SLC12A7 were downregulated. Collectively, these data not only validate our approach and provide a broad picture of the surfaceomic remodeling

279 following activation, but also serve as a benchmark for assessing the effects of hypoxia and Tregs on the
280 activated CD8⁺ surfaceome.

281 Activation of CD8⁺ T cells in the presence of Tregs did not induce dramatic surfaceomic shifts
282 relative to the hypoxia or activation datasets. However, co-culture showed several of the classic markers
283 up-regulated in activation, TFRC, SLC1A5, and SLC7A1³⁸, are downregulated upon addition of Tregs. Of
284 note, the exhaustion marker HAVRC2 (TIM-3) was also downregulated on CD8⁺ T cells activated in the
285 presence of Tregs. This may be a consequence of diminished CD8⁺ activation in the presence of Tregs. As
286 our experiments were limited to three days of co-culture, the trends we observe likely represent changes
287 that occur relatively early in an environment containing both CD8⁺ cells and Tregs. Longer-term culture
288 conditions may demonstrate even more dramatic changes associated with prolonged Treg-mediated
289 suppression of CD8⁺ cells.

290 Multiple studies have specifically examined the direct effect of hypoxia on CD8⁺ T cells, either by
291 modulating the activity of the canonical hypoxia-associated transcription factor hypoxia-inducible factor-1 α
292 (HIF-1 α) or using hypoxic cell culture. Doedens et al. showed that knockdown of the negative HIF
293 regulator VHL enhanced the cytotoxic signature of CD8⁺ cells and led to sustained effector function.⁶²
294 Similarly, Gropper et al. showed that CD8⁺ T cells cultured in 1% O₂ exhibited enhanced cytolytic
295 activity.⁶³ However, these cells proliferated only half as quickly as normoxic cells during the culture
296 period, which may counteract the enhanced effector function of these cells. Taken with the results of other
297 studies examining CD8⁺ function in hypoxia, low oxygen tension appears to exert a net immunosuppressive
298 effect on the antitumor response.²² Our data add to these focused functional studies and revealed that
299 hypoxia led to the most substantial surfaceomic changes of all conditions tested with respect to the number
300 of proteins demonstrating significant change. Additionally, the response of CD4⁺ T cells to hypoxia was
301 tightly correlated with that of CD8⁺ cells, suggesting hypoxia has similar impacts on both cell types.

302 Analysis of the altered surface proteins in hypoxic culture (e.g. GLUT3 upregulation), reveals
303 changes consistent with a metabolic change toward glycolysis. One protein that showed robust upregulation

in both CD8⁺ and CD4⁺CD25⁻ cells was SLC16A3, a hypoxia-induced lactate transporter that helps export lactate produced from glycolysis which has previously been implicated in supporting tumor growth.⁶⁴ Interestingly, the related SLC16A1 was recently shown to help intratumoral Tregs metabolically cope with high lactate levels in areas of tumor hypoxia.⁶⁵ The role of SLC16A3 upregulation on hypoxic CD8⁺ and CD4⁺CD25⁻ cells remains unclear and further functional follow up is needed, but previous studies indicate inhibition of this transporter can enhance effector function.⁶⁶ Intratumoral hypoxia is also known to dramatically alter the glycoproteome of tumor cells, changes that in turn regulate cell migration and metastasis. Our data suggest the same could be true for T cells, which may have similar consequences for T cell migration within the tumor and could represent another feature that could be harnessed to selectively target hypoxic T cells. Further studies are needed to profile the hypoxic T cell glycoproteome.

Intriguingly, proteins involved in hypoxia-induced autophagy (ATG9A⁴⁸, ERGIC1, SEC22B⁴⁹) and the unfolded protein response (ERMP1⁴⁵, FKBP11⁴⁶, MBTPS2⁴⁷) were also upregulated. These two pathways are associated with a survival response under hypoxic conditions.⁶⁷ However, activation of the unfolded protein response pathway is usually observed only at very low levels of oxygen (<0.1%)⁶⁷, and is often associated with cell death. Although we did not observe rampant cell death in our experiments (performed at 1% oxygen), even lower oxygen levels within dividing T cell clusters or the compounded metabolic demands of hypoxic conditions and activation may have triggered these pathways. Interestingly, T cell activation has also been associated with induction of the endoplasmic reticulum stress response and autophagy.^{68,69} Collectively, these observations suggest that hypoxia places additional stress on the activation-associated metabolic shift, ER stress, and autophagic responses that are typically induced in T cells following activation. This added stress may impair T cell function and the ability for T cells to cope with sustained stimulation in the hypoxic TME.

Finally, one global observation from our hypoxic CD8⁺ data is that although a large number of proteins show significant change, the magnitude of these changes is mild, with only 28/196 (14%) of significantly-altered proteins showing a change of greater $\pm$ two-fold change. This may suggest that the T

cell response to hypoxia results in a distributed biology, where many small changes in protein abundance collectively lead to a suppressed state. This complicates functional follow-up of proteins identified in this study, but nonetheless this will be a focus of future efforts to provide greater resolution of the surface protein changes that lead to hypoxia-induced T cell suppression.

Taken together, our data provide the opportunity to search for common surfaceomic changes in response to both Treg co-culture and hypoxia. Gene set enrichment analysis comparing the three datasets revealed very strong enrichment for transmembrane amino acid transport in the CD8⁺ monoculture activation dataset. These transporters are vital for fueling macromolecule production needed for both proliferation and effector function, with both the glutamate transporter SLC1A5 and amino acid transporter SLC7A1 previously implicated in supporting T cell activation. Interestingly, SLC5A6 is a sodium-dependent multivitamin transporter that has not been previously implicated in T cell function. Together, these three transporters may represent a common set of proteins whose expression is modulated by immunosuppressive factors. Downregulation of these transporters by the suppressive forces tested here suggests these factors blunt the T cell activation response by limiting the increased metabolism required to sustain proliferation and effector function (Figure 7). This premise is consistent with a recent study finding that hypoxia-induced metabolic stress promotes T cell exhaustion.²⁶ It remains to be seen if these solute transporters are also downregulated on infiltrating T cells *in vivo*, but our findings begin to illuminate how TME-associated immunosuppression of CD8⁺ cells may result from effectively starving T cells as they attempt to mount an anti-tumor response.

In conclusion, our cell surface proteomics data revealed upregulation of well-established activation markers following CD8⁺ stimulation, but also revealed many additional upregulated proteins that may augment T cell effector function and help T cells cope with the increased metabolic demands associated with rapid proliferation. Both immunosuppressive factors tested caused significant changes to the CD8⁺ surfaceome consistent with diminished T cell function. Interestingly, however, these factors converged on mediating downregulation of transporters crucial for linking the T cell activation response and the

354 metabolic shift that follows stimulation, uncovering a potential metabolic mechanism for
 355 immunosuppression in the TME. Future work will aim to validate and screen these proteins for potential
 356 strategies to support the function of T cells in hypoxic environments. Such strategies could either block the
 357 function of inhibitory proteins upregulated in the TME, or could mediate selective delivery of beneficial
 358 cytokines via antibody-cytokine fusions (Figure 7B). Furthermore, our results provide a starting point to
 359 identify proteins that could be modulated in adoptive T cellular therapies (e.g. CAR-T) to enhance function
 360 of these cells in the solid TME (Figure 7B). Collectively, our findings provide important insight into the
 361 plasticity of the T cell surfaceome and lay the foundation for future efforts to not only further characterize,
 362 but also potentially therapeutically engage, T cell surface proteins within the TME.

363

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378

379 **AUTHOR CONTRIBUTIONS**

380 J.R.B. and A.M.W. designed the research, performed experiments, and analyzed data. E.S. and J.C.
381 performed experiments. L.K. provided valuable technical assistance and training. A.A., A.M., and J.A.W.
382 conceived and supervised the research. J.R.B. and J.A.W. co-wrote the manuscript, and all authors
383 provided edits and approved the final version.

385 **DISCLOSURES**

386 A.A. is a co-founder of Tango Therapeutics, Azkarra Therapeutics, Ovibio Corporation; a consultant for
387 SPARC, Bluestar, ProLynx, Earli, Cura, GenVivo and GSK; a member of the SAB of Genentech,
388 GLAdiator, Circle and Cambridge Science Corporation; receives grant/research support from SPARC and
389 AstraZeneca; holds patents on the use of PARP inhibitors held jointly with AstraZeneca which he has
390 benefitted financially (and may do so in the future). A.M. is cofounder, member of the Boards of Directors
391 and member of Scientific Advisory Boards of Spotlight Therapeutics and Arsenal Biosciences. A.M. has
392 served as an advisor to Juno Therapeutics, was a member of the scientific advisory board at PACT Pharma
393 and was an advisor to Trizell. A.M. has received honoraria from Merck and Vertex, a consulting fee from
394 AlphaSights, and is an investor in and informal advisor to Offline Ventures. A.M. owns stock in Arsenal
395 Biosciences, Spotlight Therapeutics and PACT Pharma. The Marson lab has received research support
396 from Juno Therapeutics, Epinomics, Sanofi, GlaxoSmithKline, Gilead and Anthem. J.A.W. is co-founder
397 of Soteria Therapeutics, is on the SAB of Jnana Therapeutics, Inception Therapeutics, IgGenix Inc, Red
398 Tree Capital, Spotlight Therapeutics, Inzen Therapeutics, and receives research support from Bristol-
399 Myers-Squibb, TRex Bio and Merck, Inc.

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404 **METHODS**

405 **Cell isolation**

406 Primary human T cells were isolated from leukoreduction chamber residuals following Trima Apheresis
 407 (Blood Centers of the Pacific, San Francisco, CA) using established protocols⁷⁰. Briefly, peripheral blood
 408 mononuclear cells (PBMCs) were isolated using Ficoll separation in SepMate tubes (STEMCELL
 409 Technologies, Vancouver, Canada) in accordance with the manufacturer's instructions. CD8⁺ T cells were
 410 isolated from PBMCs using either the EasySep™ Human CD8⁺ T cell Isolation Kit or the RosetteSep™
 411 Human CD8⁺ T Cell Enrichment Cocktail (STEMCELL), following the manufacturer's protocol.
 412 CD4⁺CD25⁻ conventional T cells and CD4⁺CD127^{low}CD25⁺ Tregs were isolated from PBMCs with the
 413 EasySep™ Human CD4⁺CD127^{low}CD25⁺ Regulatory T Cell Isolation Kit (STEMCELL). Isolated cell
 414 populations were analyzed for purity by flow cytometry on a Beckman Coulter CytoFlex flow cytometer
 415 using a panel of antibodies (anti-CD3 [UCHT1], anti-CD4 [OKT4], anti-CD8a [RPA-T8], anti-CD25 [M-
 416 A251], anti-CD45RA [HI100], and anti-CD127 [A019D5], all from BioLegend, San Diego, CA). Pilot
 417 experiments comparing FACS sorting and magnetic bead separation showed similar results with respect to
 418 cell purity, so magnetic bead separation was used.

419

420 **Cell culture/SILAC labeling**

421 Following isolation, cells were adjusted to 1e6 cells/mL in RPMI 1640 Medium for SILAC (ThermoFisher)
 422 supplemented with 1000 U/mL Penicillin/Streptomycin (Gemini Bio-Products), 10 mM HEPES (UCSF
 423 Cell Culture Facility), 100 μM non-essential amino acids (Lonza), 1 mM sodium pyruvate (VWR), 55 μM
 424 2-mercaptoethanol (Gibco), 10 mM N-acetyl-cysteine (Sigma), and 10% dialyzed FBS (Gemini). Media
 425 was also supplemented with either light L-[12C6,14N2] lysine/L-[12C6,14N4] arginine (Sigma) or heavy
 426 L-[13C6,15N2] lysine/L-[13C6,15N4] arginine (Cambridge Isotope Laboratories). CD8⁺ and CD4⁺ T
 427 effectors were stimulated by addition to tissue culture flasks coated with anti-CD3 (produced in-house,
 428 clone OKT3) and anti-CD28 ([D2Z4E], Cell Signaling) antibodies or with anti-CD3/anti-CD28 Dynabeads

(Thermo) at a 1:1 bead:cell ratio in the presence of 50 U/mL recombinant human IL-2 (Thermo). Following two days of culture, the stimuli were removed and cells allowed to expand in heavy or light SILAC media with 50 U/mL IL-2 for 12 additional days. Tregs were stimulated using anti-CD3/anti-CD28 Dynabeads (Thermo) at a 1:1 bead:cell ratio and maintained in 300 U/mL IL-2. Following two days of culture, the stimuli were removed and cells allowed to expand in heavy or light SILAC media with 300 U/mL IL-2 for 9 days and then restimulated with more beads at 1:1 bead:cell ratio. At day 14, Tregs were counted and put into co-culture with CD8 T cells at a 1:1 Treg: CD8⁺ T cell ratio.

Baseline CD8⁺ activation

CD8⁺ T cells were stimulated using anti-CD3/anti-CD28 Dynabeads (Thermo) at a 1:1 bead:cell ratio and maintained in 50 U/mL IL-2. Following two days of culture, the stimuli were removed and cells allowed to expand in heavy or light SILAC media with 50 U/mL IL-2 for 12 additional days, at which point CD8⁺ T cells were counted and were re-stimulated 1:1 with anti-CD3/anti-CD28 Dynabeads for three days. Isotopically-labeled activated and resting cells were then combined 1:1 for downstream processing.

Co-culture

On day 14, isotopically-labeled CD8⁺ T cells were put into co-culture with Tregs at a 1:1 Treg:CD8⁺ T cell ratio. In addition, at the time of initiation of co-culture, anti-CD3/anti-CD28 Dynabeads were added at a 1:1 bead:CD8⁺ T cell ratio. At the time of co-culture, the CD8⁺ T cells grown in heavy SILAC media were co-cultured with Tregs, while CD8⁺ T cells were kept in monoculture and grown in light SILAC media. At the end of three days of co-culture, CD8⁺ T cells were isolated from co-culture using STEMCELL CD8⁺ enrichment kits and then combined in equal numbers with the CD8 T cells grown in mono-culture in the light SILAC media. This was done in the opposite combination of light/heavy SILAC media as well to ensure there was no bias in the experiment in assigning light or heavy SILAC media to those T cells grown in co- or mono-culture.

454 **Hypoxic T cell activation**

455 To model activation in hypoxic conditions, expanded, SILAC-labeled CD8⁺ or CD4⁺ cells were collected,
456 resuspended in fresh SILAC media, and stimulated using anti-CD3/anti-CD28 Dynabeads at a 1:10
457 bead:cell ratio. Cells were then either cultured at 37°C, 5% CO₂ in normoxic (20% O₂) or hypoxic (1% O₂)
458 conditions for three days. Hypoxic culture was performed in a Coy Laboratory Products hypoxic cabinet
459 using a nitrogen/5% CO₂ balance blend. Cells were then separated from the Dynabeads and heavy and light
460 cells mixed at a 1:1 ratio in both forward and reverse SILAC mode before surface protein capture. For flow
461 cytometry of IL-18R1 and CD70, cells were similarly isolated and activated but were cultured in
462 STEMCELL Immunocult-XF T cell expansion media. Cells were stained with GHOST viability dye from
463 Tonbo Biosciences and flow cytometry was performed using anti-IL18R1 [H44] and anti-CD70 [113-16]
464 antibodies, both from Biolegend. Flow cytometry data was analyzed using FlowJo (v10.7.1).

466 **Cell surface capture**

467 Cell surface glycoproteins were captured as previously described.³² Briefly, immediately after combining
468 isotopically-labeled cells, the cells were washed in PBS, pH 6.5 and glycoproteins oxidized with 1.6 mM
469 NaIO₄ (Sigma) in PBS, pH 6.5 for 20 minutes at 4°C. Oxidized vicinal diols were subsequently biotinylated
470 with 1 mM biocytin hydrazide (Biotium) in the presence of 10 mM aniline (Sigma) in PBS, pH 6.5 for 90
471 minutes at 4°C. Cells were then flash frozen and stored at -80°C before further preparation. To isolate
472 glycoproteins for mass spectrometry, cell pellets were lysed with commercial RIPA buffer (VWR)
473 supplemented with 1X Protease Inhibitor Cocktail (Sigma) and 1 mM EDTA (Sigma) for 30 minutes at
474 4°C. Cells were further disrupted with probe sonication and biotinylated glycoproteins pulled down with
475 NeutrAvidin coated agarose beads (Thermo) for one hour at 4°C. Beads were transferred to Poly-Prep
476 chromatography columns (Bio-Rad, Hercules, CA) and sequentially washed with RIPA (PBS pH 7.4 with
477 0.5% sodium deoxycholate [Thermo], 0.1% sodium dodecylsulfate [Fisher Scientific, Waltham, MA], 1%
478 Nonidet P-40 substitute [VWR]), high salt PBS (PBS pH 7.4, 2 M NaCl [Sigma]), and denaturing urea

479 buffer (50 mM ammonium bicarbonate, 2 M Urea). After washing, beads were collected and glycoproteins
 480 reduced with 5 mM TCEP (Calbiochem) for 30 minutes at 37°C and alkylated with 11 mM iodoacetamide
 481 (Sigma) for 30 minutes at room temperature. Beads were washed with urea buffer and trypsinized on-bead
 482 overnight at room temperature with 20 µg trypsin (Promega). The next day, the tryptic fraction was
 483 collected using Pierce Spin Columns before the beads were again transferred to PolyPrep columns and
 484 washed with RIPA, high salt buffer, and urea buffer before a final wash with 50 mM ammonium
 485 bicarbonate. Beads were transferred to a fresh tube and glycopeptides liberated with 5000 U/mL PNGaseF
 486 for 3 hours at 37°C. This PNGaseF fraction was collected as above. Both tryptic and PNGaseF fractions
 487 were then desalted with SOLA HRP SPE columns (Thermo) following standard protocols, dried, and
 488 dissolved in 0.1% formic acid, 2% acetonitrile prior to LC-MS/MS analysis.

489

490 **Mass spectrometry**

491 Mass spectrometry was performed as previously described³², with some slight adjustments. All peptides
 492 were separated using an UltiMate 3000 UHPLC system (Thermo) with pre-packed 0.75mm x 150mm
 493 Acclaim Pepmap C18 reversed phase columns (2µm pore size, Thermo) and analyzed on a Q Exactive Plus
 494 (Thermo Fisher Scientific) mass spectrometer. For tryptic fractions, 1 µg of resuspended peptides was
 495 injected and separated using a linear gradient of 3-35% solvent B (solvent A: 0.1% formic acid, solvent B:
 496 80% acetonitrile, 0.1% formic acid) over 230 mins at 300 µL/min. Due to the low peptide yield of the
 497 PNGase fraction, the entire fraction was injected and subsequently separated using the same gradient over
 498 170 mins. Data-dependent acquisition was performed using a top 20 method (dynamic exclusion 35
 499 seconds; selection of peptides with a charge of 2, 3, or 4). Full spectra with a resolution of 140,000 (at 200
 500 m/z) were gathered in MS1 using an AGC target of 3e6, maximum injection time of 120 ms, and scan
 501 range of 400 - 1800 m/z. Centroided data from MS2 scans were collected at a resolution of 17,500 (at 200
 502 m/z) with an AGC target of 5e4 and maximum injection time of 60 milliseconds. The normalized collision
 503 energy was set at 27 and an isolation window of 1.5 m/z with an isolation offset of 0.5 m/z was used.

504 **Data analysis/Statistics**

505 SILAC proteomics data were analyzed as previously described³². Briefly, each individual dataset was
 506 searched for peptides using ProteinProspector v5.13.2 against the human proteome (Swiss-prot database,
 507 August 3, 2017 release). Enzyme specificity was set to trypsin with up to two missed cleavages. Cysteine
 508 carbamidomethyl was set as the only fixed modification; methionine oxidation, N-terminal glutamate to
 509 pyroglutamate, and lysine/arginine SILAC labels were set as variable modifications. Asparagine
 510 deamidation was also listed as a variable modification for the PNGaseF fractions. During the search, the
 511 peptide mass tolerance was 6 ppm, fragment ion mass tolerance was 0.4 Da, and peptide identification was
 512 filtered by peptide score of 0.0005 in ProteinProspector, resulting in a false discovery rate (FDR) of <1%
 513 calculated using the number of decoy peptides in the SwissProt database. Skyline (UWashington)⁷¹
 514 software was used to perform quantitative analysis of SILAC ratios using an MS1 filtering function against
 515 a curated list of extracellular proteins generated via searches for “membrane” but not “mitochondrial” or
 516 “nuclear” using UniProt subcellular localization annotations, as previously described.³² For datasets
 517 collected in forward and reverse SILAC mode, spectral libraries of experiments were analyzed
 518 simultaneously to allow MS1 peaks without an explicit peptide ID to be quantified using an aligned peptide
 519 retention time. The Skyline report was subsequently exported for ratiometric analysis using a previously
 520 reported custom R script³². Briefly, low quality identifications (isotope dot product <0.8) were removed.
 521 For the tryptic fraction, only proteins with two or more peptides were included in downstream analysis,
 522 whereas for the PNGase fraction, only peptides with N to D deamidation were included. Ratios derived
 523 from both fractions were then combined, centered on a mean of zero, and presented as median log₂
 524 enrichment values. Significance was determined using a Mann-Whitney test of peptide ratios for all
 525 peptides for a given protein. Keratin 2, vimentin, and prothrombin showed dramatic enrichment in some
 526 light-labeled SILAC samples, suggesting these were contaminants and were therefore removed from
 527 downstream analysis. Heatmaps comparing expression levels between donors were generated using
 528 heatmapper.ca and other graphs were generated using GraphPad Prism (v8).

RNAseq data for naïve and activated CD8⁺ T cells was downloaded from the Database of Immune Cell eQTLs, Expression, and Epigenomics (DICE)³⁹. Expression data was gathered for all overlapping proteins found in the CD8⁺ activation surfaceomics dataset, and an average expression level was calculated from all available donors in the DICE database. The expression ratio between activated and naïve was then calculated and compared with enrichments observed in the surfaceomics data. STRING analysis was performed using the online STRING Database (v11.0) and visualized using Cytoscape (v3.7.2). Gene-set enrichment analysis was performed using GSEA (v4.0.1) and the compiled data for all detected proteins within each dataset. The biological process annotated gene set (c5.bp.v7.0.entrez.gmt) used was obtained from the MSigDB collection. The CD8⁺ monoculture activation dataset was compared with both the Treg co-culture and hypoxia datasets to find pathways commonly regulated by the two immunosuppressive stimuli tested.

Data availability

The raw proteomics data, peaklists, ProteinProspector results, and Skyline quantification results have been deposited to the ProteomeXchange Consortium via the PRIDE⁷² partner repository with the dataset identifier PXD024789. For the purposes of peer review, the data can be accessed using reviewer credentials (login: reviewer_pxd024789@ebi.ac.uk; password: xz6yqlTq). Full outputs from SILAC analysis can be found in Supplemental Tables 1-4. Flow cytometry data and all other data presented is available upon reasonable request.

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724 FIGURES

Figure 1. Overall strategy for cell surface glycoproteomic characterization of primary CD8⁺ T cells under various conditions.

725

726 **Figure 1. Overall strategy for cell surface glycoproteomic characterization of primary CD8⁺ T cells**

727 **under various conditions.** (A) Schematic depicting expansion and SILAC labeling workflow. Primary

728 human CD8⁺ T cells were isolated and expanded using anti-CD3/anti-CD28 stimulation in media

729 supplemented with IL-2 and either heavy or light arginine and lysine. After expansion, cells were

730 stimulated in varying conditions before surface protein enrichment and protein identification with LC-

731 MS/MS. (B) Strategy for assessing the effect of immunosuppressive stimuli on the activated CD8⁺ T-cell

732 surfaceome. First, the surfaceomic changes associated with CD8⁺ activation in monoculture under

733 normoxic conditions were analyzed. These changes then served as a baseline for later experiments

734 examining the surfaceomic consequences of activating CD8⁺ T cells in co-culture with primary Tregs or in

735 hypoxic culture.

Figure 2. Surface proteomics reveals both well-established and novel activation-induced changes in surface protein levels.

737 **Figure 2. Surface proteomics reveals both well-established and novel activation-induced changes in**
738 **surface protein levels.** (A) Volcano plot of surface protein changes following stimulation of CD8⁺ T cells
739 with anti-CD3/anti-CD28 beads. Data represent compiled results from N=4 donors. Proteins with a ± 1.5 -
740 fold change and $P < 0.05$ were included in downstream analysis. Proteins significantly down- (blue) or
741 upregulated (red) are indicated. (B) $\log_2(\text{Enrichment Ratio})$ of indicated proteins. Each dot represents data
742 from an individual donor. Line represents the mean and error bars are standard deviation. (C) STRING
743 analysis of all significantly-altered proteins in (A). Network is overlaid with a color gradient representing
744 $\log_2(\text{Enrichment Ratio})$ for each individual protein. Proteins with a gene ontology (GO) biological process
745 annotation of “immune system process” are indicated with green borders. (D) Significantly-altered proteins
746 were subjected to GO biological process pathway analysis using the STRING database. The number of
747 proteins identified, the direction of regulation, and analysis FDR for each process are indicated.

Figure 3. Treg co-culture causes significant changes in the surfaceome of activated CD8⁺ T cells consistent with immunosuppression.

749 **Figure 3. Treg co-culture causes significant changes in the surfaceome of activated CD8⁺ T cells**
750 **consistent with immunosuppression.** (A) CD8⁺ T cells were stimulated with anti-CD3/anti-CD28 beads
751 either in the absence or presence of Tregs. Following culture, CD8⁺ cells were isolated for cell surface
752 proteomics. Volcano plot shows compiled results from N=4 donors. Proteins with a ± 1.5 -fold change and
753 $P < 0.05$ were included in downstream analysis. Proteins significantly down- (blue) or upregulated (red) are
754 indicated. (B) Log₂(Enrichment Ratio) of indicated proteins. Each dot represents data from an individual
755 donor. Line represents mean and error bars are standard deviation. (C) STRING analysis of all
756 significantly-altered proteins in (A). Network is overlaid with a color gradient representing
757 log₂(Enrichment Ratio) for each individual protein. Proteins with a gene ontology (GO) biological process
758 annotation of “immune system process” are indicated with green borders. (D) Significantly-altered proteins
759 were subjected to GO biological process pathway analysis using the STRING database. The number of
760 proteins identified, the direction of regulation, and analysis FDR for each process are indicated.

Figure 4. Hypoxia induces surfaceomic changes representative of both immunosuppression and a general response to hypoxia.

Figure 4. Hypoxia induces surfaceomic changes representative of both immunosuppression and a general response to hypoxia. (A) CD8⁺ T cells were stimulated with anti-CD3/anti-CD28 beads either normoxic (20% O₂) or hypoxic (1% O₂) for three days. Volcano plot shows compiled results from N=3 donors. Proteins with a ± 1.5 -fold change and $P < 0.05$ were included in downstream analysis. Proteins significantly down- (blue) or upregulated (red) are indicated. (B) Log₂(Enrichment Ratio) of indicated proteins. Each dot represents data from an individual donor. Line represents mean and error bars are standard deviation. (C) STRING analysis of all significantly-altered proteins in (A). Network is overlaid with a color gradient representing log₂(Enrichment Ratio) for each individual protein. Proteins with a gene ontology (GO) biological process annotation of “immune system process” are indicated with green borders and proteins with an annotation of “protein glycosylation” with purple borders. Proteins annotated for both processes are indicated with an orange border. (D) Significantly-altered proteins were subjected to GO biological process pathway analysis using the STRING database. The number of proteins identified, the direction of regulation, and analysis FDR for each process are indicated.

Figure 5. CD8⁺ and CD4⁺ surface proteomes respond similarly to hypoxia.

Figure 5. CD8⁺ and CD4⁺ surface proteomes respond similarly to hypoxia. (A) CD4⁺CD25⁻ T cells were stimulated with anti-CD3/anti-CD28 beads either normoxic (20% O₂) or hypoxic (1% O₂) for three days. Volcano plot shows compiled results from N=3 donors. Proteins with a $\pm$ 1.5-fold change and $P < 0.05$ were included in downstream analysis. Proteins significantly down- (blue) or upregulated (red) are indicated. (B) Spearman correlation comparing the log₂(Enrichment Ratio [ER]) for both CD8⁺ and CD4⁺ T

781 cells activated under hypoxic conditions. Venn diagrams showing proteins commonly down- (C) or
 782 upregulated (D) in hypoxia on both CD8⁺ and CD4⁺ cells. Below each Venn diagram is are results from a
 783 GO biological process pathway analysis for commonly regulated proteins using the STRING database. The
 784 number of proteins identified and analysis FDR for each process are indicated.

Figure 6. Analysis of all datasets reveals commonalities in the effects of Tregs and hypoxia on the activated CD8⁺ T cell surfaceome.

786 **Figure 6. Analysis of all datasets reveals commonalities in the effects of Tregs and hypoxia on the**
787 **activated CD8⁺ T cell surfaceome.** (A) Gene set enrichment analysis (GSEA) output of all commonly-
788 detected proteins in all three datasets. The five biological processes with FDR q-values <0.05 are shown, as
789 are the normalized enrichment scores (NES) for each process. A heat map of proteins associated with
790 GO_AMINO_ACID_TRANSMEMBRANE_TRANSPORT is also shown with the three datasets indicated.
791 Red and blue indicate high and low enrichment, respectively. (B) Venn diagram showing intersection of
792 proteins that were upregulated upon CD8⁺ activation, but downregulated by Treg co-culture or hypoxia.
793 The five proteins at the intersection of all three datasets are indicated. (C) Venn diagram showing
794 intersection of proteins downregulated upon CD8⁺ activation, but upregulated by Treg co-culture or
795 hypoxia.
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Figure 7. Metabolic model of CD8⁺ immunosuppression and potential therapeutic strategies.

Figure 7. Metabolic model of CD8⁺ immunosuppression and potential therapeutic strategies. (A)

Tregs and hypoxia suppress expression of nutrient transporters on CD8⁺ T cells, limiting T cell activation and the anti-tumor response. (B) Proteins identified in our studies could be utilized for targeted antibody-based therapies to enhance CD8⁺ function in the TME. Hypoxia-induced antigen (HIA) used as an example. Antibody-cytokine fusion targeting an HIA could be used to deliver pro-proliferative cytokines to CD8⁺ T cells (left). Alternatively, TME-induced antigens that inhibit T cell function could be blocked with mono- or bi-specific antibodies. Lastly, adoptive cellular therapies could be reprogrammed to express higher levels of SLCs to help cope with the metabolic stresses found within the TME, which may enhance the efficacy of these therapies against solid tumors.