

1 Identification of human CD4⁺ T cell populations 2 with distinct antitumor activity

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28 Abstract:

29
30 How naturally arising human CD4⁺ T helper subsets impact tumor immunity is unknown. We
31 reported that human CD4⁺CD26^{high} T cells elicit potent immunity against solid tumor
32 malignancies. As CD26^{high} T cells secrete type-17 cytokines and have been categorized as Th17
33 cells, we posited these helper populations would possess similar molecular properties. Herein,
34 we reveal that CD26^{high} T cells are epigenetically and transcriptionally distinct from Th17 cells.
35 Of clinical significance, CD26^{high} T cells engineered with a chimeric antigen receptor (CAR)
36 ablated large human tumors to a greater extent than enriched Th17, Th1, or Th2 cells. Moreover,
37 CD26^{high} T cells mediated curative responses in mice, even when redirected with a suboptimal
38 CAR and without the aid of CD8⁺ CAR T cells. CD26^{high} T cells co-secreted effector cytokines
39 at heightened levels and robustly persisted. Collectively, our work reveals the potential of human
40 CD4⁺ T cell populations to improve durability of solid tumor therapies.

43 **Introduction**

44 We previously reported that CD26 distinguishes three human CD4⁺ T cell subsets with varying
45 degrees of responsiveness to human tumors: one with regulatory properties (CD26^{neg}), one with a
46 naive phenotype (CD26^{int}), and one with a durable stem memory profile (CD26^{high}) (1).
47 Adoptively transferred tumor-specific CD26^{high} T cells persisted and regressed difficult-to-treat
48 malignancies superior to CD26^{neg} T cells and surprisingly, slightly better than naive CD26^{int} T
49 cells. CD26^{high} T cells secreted Th17 cytokines, including IL-17A, TNF α and IL-22. These
50 previous findings reveal that CD26^{high} T cells are promising for immunotherapies.

51 The therapeutic potency of Th17 cells over Th1 or Th2 cells has been reported by many
52 groups using mouse model systems (2-5). Surprisingly, the impact of tumor-specific *human*
53 Th17 cells have not been fully explored in the context of adoptive T cell transfer (ACT) therapy
54 for cancer. Given the sizeable expression of master transcription factor ROR γ t and production of
55 IL-17 by human CD26^{high} T cells (1), we postulated that these cells would eradicate tumors to the
56 same extent as classic Th17 cells (i.e. CCR4⁺CCR6⁺CD4⁺) when redirected with a chimeric
57 antigen receptor (CAR) and infused into hosts bearing human tumors (6). Moreover, the
58 therapeutic potential of naturally arising human CD4 subsets—sorted from the peripheral blood
59 via classic surface markers—engineered with a CAR has yet to be elucidated.

60 We report herein that CD26^{high} T cells are molecularly and functionally distinct from Th17
61 cells. CD26^{high} T cells are robustly therapeutic compared to Th17, as demonstrated by their
62 capacity to persist and eradicate large human tumors in mice. Additional investigation uncovered
63 that CD26^{high} T cells were more effective than Th1 or Th2 cells as well. We found that the
64 molecular and epigenetic properties of CD26^{high} T cells are distinct from Th17 cells, which might
65 support their persistence and sustained responses to large tumors.

66 **Results**

67 **CD26^{high} T cells possess a dynamic cytokine profile**

68 We reported that CD4⁺ T cells expressing high CD26 levels (termed CD26^{high} T cells) secrete IL-
69 17A and elicit potent tumor immunity when redirected with a CAR compared to sorted CD26^{int}
70 or CD26^{low} T cells (1). While CD26^{high} T cells are categorized as Th17 cells, the functional
71 profile of sorted human CD26^{high} T cells compared to classic Th17 cells as well as other known
72 helper subsets has never been tested. Given the abundance of IL-17 produced by CD26^{high} T
73 cells, we suspected that they would possess a similar cytokine profile as classic Th17 cells. To
74 first address this question, we measured the level and type of cytokines produced by various
75 CD4⁺ subsets, which were sorted from the peripheral blood of healthy individuals via
76 extracellular markers (Figure 1A) (6). This sort yielded Th1 (CXCR3⁺CCR4⁻CCR6⁻), Th2
77 (CXCR3⁻CCR4⁺CCR6⁻), Th17 (CCR4⁺CCR6⁺CXCR3^{+/-}) and CD26^{high} T cells with high purity
78 (>90%). As expected, Th1 cells expressed CXCR3, Th2 cells expressed CCR4, and Th17 cells
79 expressed CCR4 and CCR6. CD26^{high} cells expressed high CXCR3 and CCR6 but nominal
80 CCR4 on their surface (Figure 1B).

81 CD26^{high} T cells were not restricted to a Th17-like functional profile (Figure 1C). Instead,
82 CD26^{high} T cells secreted more IL-17A (58 vs. 15%), IL-22 (27 vs. 4%) and IFN- γ (73 vs. 25%)
83 than Th17 cells. CD26^{high} T cells produced nearly as much IFN- γ (73 vs. 88%) as Th1 cells but
84 far less IL-4 (3 vs 29%) than Th2 cells. We consistently observed this functional pattern in
85 CD26^{high} T cells from several healthy individuals (Figure 1C-E). On a per-cell basis, CD26^{high} T
86 cells concomitantly secreted 4 (35%) to 5 (7%) cytokines, a dynamic process not manifested in
87 other subsets (Figure 1F). Collectively, our data suggest that CD26^{high} T cells have a distinct
88 functional profile from classic Th17 cells.

89

90 **CD26^{high} T cells display a unique chromatin landscape**

91 Given the functional profile of CD26^{high} T cells, we hypothesized that the epigenetic landscape
92 of these cells at resting state would be different than Th17 cells. To test this idea, we sorted
93 naïve, Th1, Th2, Th17 and CD26^{high} T cells from the blood of 5 different healthy donors and
94 profiled their chromatin accessibility with Assay for Transposase-Accessible Chromatin with
95 high-throughput sequencing (ATAC-seq). CD26^{high} T cells contained peaks displaying enhancer
96 accessible regions near various transcription factors (TF) known to direct Th1 (such as *Tbx21*
97 and *EOMES*) and Th17 (*RORC*) cell lineage development while displaying suppressor regions
98 near TF genes known to regulate Th2 development, such as *GATA3* (Figure 2A-B). While *Tbx21*
99 and *EOMES* were more accessible in both Th1 and CD26^{high} T cells, they were repressed in
100 naïve, Th2 and Th17 cells (Figure 2B). Moreover, a core of other accessible regions in Th1-
101 related TFs, such as *MGA*, *STAT2*, *STAT1* and *STAT5A*, were pronounced in Th1 and CD26^{high} T
102 cells (Figure 2A). As expected, accessible regions surrounding *GATA3* were enhanced in Th2
103 cells and interestingly in Th17 cells. Other enhancer accessible regions surrounding Th2-like
104 TFs, such as *GATA1*, *GATA2*, *GATA4*, *GATA5*, *GATA6*, *PAX4*, *YY1*, *PITX2* and *GFII* were
105 distinguished in Th2 and Th17 cells (Figure 2A). Similar to Th17 cells, chromatin accessible
106 regions near the *RORA*, *RORB*, and *STAT3* loci were enhanced in CD26^{high} T cells but
107 suppressed in naïve, Th1 and Th2 cells (Figure 2A-B). Th1 cells more closely aligned with the
108 epigenetic landscape of naïve cells, as they both expressed accessible chromatin regions
109 neighboring TFs in the stem and development pathways, including *TCF1*, *LEF1*, *CTCF*, *DNMT1*
110 and *ZFP161* (Figure 2A). Yet, certain accessible regions in naïve cells were also heightened in
111 both CD26^{high} and Th1 subsets, including *STAT1*, *STAT2*, *IRF1*, *IRF2*, *IRF3*, *IRF5*, *IRF7*, *IRF8*
112 and *ZNF683* (Figure 2A).

113 Despite overlap with Th1 and Th17 cells, CD26^{high} T cells possessed a unique set of
114 differentially accessible elements relative to other subsets. Open accessible regions in the
115 CCAAT/enhancer-binding protein family (C/EBP), which function as TFs in processes including
116 cell differentiation, motility and metabolism, were among the most unique and differentially
117 expressed in CD26^{high} T cells (Figure 2A-B). Along with *CEBPs*, *ELK3*, important for cell
118 migration and invasion, and *RUNX*, which promotes memory cell formation, were enhanced in
119 CD26^{high} T cells. Principal component analysis of the genome-wide open chromatin landscape of
120 these 25 samples showed that CD26^{high} T cells cluster separately from naïve, Th1, Th2 and Th17
121 cells (Figure 2C). We verified the distinct characteristics of CD26^{high} versus Th17 cells using
122 gene array (Figure S1A-B). Further, as helper subsets have been reported to express a particular
123 TCR β repertoire (7), we defined the frequency and likelihood of TCR β clonotype overlap
124 between various sorted subsets and found nominal overlap between CD26^{high} cells and other
125 helper subsets (Figure 2D & Figure S1C). Collectively, we conclude that the epigenetic
126 landscape and TCR repertoire of CD26^{high} cells differs substantially from that of classic CD4⁺
127 subsets.

128

129 **Single cell sequencing reveals that CD26^{high} T cells are molecularly unique from Th17 cells**

130 Single-cell transcriptome analysis also supported that CD26^{high} T cells are distinguished from
131 Th17 cells based on differential clustering from that of bulk CD4⁺ and Th17 cells (Figure 3A).
132 Interestingly, a cluster of Treg-like cells was present within the sorted Th17 population (Figure
133 3B), as demonstrated by heightened *FOXP3*, *IL2RA*, and *TIGIT* and reduced *IL7R* transcript, but
134 was not found within CD26^{high} T cells. A small cluster of Th1-like cells was identified within the
135 sorted bulk CD4⁺ population, as indicated by elevated *TBX21*, *GZMH*, *PRF1*, *CCL5* and *CXCR3*

136 but nominal transcripts associated with Th17 or Treg cells, such as *CCR6*, *CCR4*, *RORC* and
137 *FOXP3*. Transcripts describing naïve-like cells including *SELL*, *CCR7*, *CD27*, and *LEF1* were
138 expressed at slightly higher levels in bulk CD4⁺ cells than other populations. In concurrence with
139 their chromatin accessibility, *CEBPD* transcripts were elevated in CD26^{high} T cells compared to
140 bulk CD4⁺ or Th17 cells, potentially indicating a bioenergetic profile resistant to oxidative stress
141 (8). Taken together, these data suggest that CD26^{high} T cells are unique from Th17 cells, yet their
142 relative clinical potential in cancer immunotherapy remained unknown.

143

144 **CD26^{high} T cells lyse tumor target cells *in vitro***

145 Given the pronounced capacity of CD26^{high} T cells to co-secrete multiple cytokines, we tested if
146 they would be more effective at lysing human tumors than Th1, Th2 or Th17 cells *in vitro*. To
147 address this, we engineered these helper populations to express chimeric antigen receptor that
148 recognizes mesothelin (meso-CAR) and co-cultured them with mesothelin-positive K562 tumor
149 cells (Figure S2A). As anticipated, CD26^{high} T cells lysed tumor targets at a lower effector to
150 target (E:T) ratio compared to all other subsets when co-cultured overnight (Figure S2B). In this
151 assay, Th1, Th17 and bulk CD4⁺ T cells similarly lysed targets at equal E:T ratios, whereas a
152 greater number of Th2 cells were needed to lyse targets. Finally, after co-culture with target
153 cells, CD26^{high} T cells produced as much IFN- γ and IL-17 as Th1 and Th17 cells, respectively
154 (Figure S2C). Thus, CD26^{high} T cells are highly polyfunctional and mount robust responses
155 against tumors *in vitro*.

156

157 **CD26^{high} T cells demonstrate enhanced tumor immunity compared to other helper subsets**

158 Next, we set out to test the relative antitumor activity of these CD4⁺ T helper populations *in vivo*.
159 As in Figure S2A, we engineered sorted Th1, Th2, Th17, CD26^{high}, and bulk CD4⁺ T cells to
160 express mesothelioma specific-CAR and infused them into NSG mice bearing a large established
161 tumor. Note that we used a 1st generation meso-CAR, reported by our colleagues to be less
162 therapeutic than 2nd generation meso-CARs (9), as we surmised this approach would generate a
163 treatment window to address whether CD26^{high} T cells lyse tumor to a greater extent than other
164 subsets. CD8⁺ T cells (10 day-expanded) were also redirected with this 1st-generation CAR and
165 co-infused with these various CAR-CD4⁺ subsets (Figure 4A). CD26^{high} T cells eradicated
166 tumors while Th17 cells only regressed tumors short term (Figure 4B-C). Th17 cells were more
167 effective than Th1 or bulk CD4⁺ T cells at transiently clearing tumors, while Th2 cells were the
168 least effective (Figure 4B-C). Ultimately, mice treated with CD26^{high} T cells survived
169 significantly longer (Figure 4D), which was associated with higher CD4⁺ and CD8⁺ CAR T cell
170 persistence compared to other helper subsets (Figure 4E). Moreover, co-transferred
171 CD4⁺CD26^{high} cells improved the function of CD8⁺ CAR-engineered T cells, as both persistence
172 of CD8⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺/IL-2⁺/TNF- α ⁺ CAR T were heightened in the spleen (Figure
173 S3A-C). These findings suggested that CD4⁺CD26^{high} CAR T cells persisted and promoted the
174 function of co-transferred CD8⁺ CAR T cells.

175 We sought to uncover if CD8⁺ T cells partnered with CD26^{high} T cells to mediate the long-
176 term survival in mice administered this therapy. Given the polyfunctionality of CD26^{high} cells *in*
177 *vitro*, we posited that CD26^{high} CAR T cells may not require CD8⁺ CAR T cells for productive
178 immunity. To address this question, we transferred CD4⁺CD26^{high} CAR T cells with or without
179 CD8⁺ CAR T cells into NSG mice bearing M108 tumors (Figure S4A-B). Indeed, CD4⁺CD26^{high}

180 CAR T cells did not require the presence of CD8⁺ CAR T cells to regress tumors, and CD8⁺
181 CAR T cells alone were not therapeutic long term (Figure S4B).

182 Finally, we questioned whether the CAR signaling in CD4⁺CD26^{high} cells was critical to
183 improve persistence of CD8⁺ CAR T cells in the tumor, or whether their presence alone (i.e.
184 redirected with a non-signaling CAR) could support CD8⁺ CAR T cells. To address this
185 question, CD8⁺ and CD26^{high} T cells were redirected with either a full-length signaling meso-
186 CAR- ζ or a truncated TCR- ζ domain without signaling capability ($\Delta\zeta$) but could still recognize
187 mesothelin and analyzed their presence in tumors. We found that meso- ζ -CD26^{high} cells, either
188 co-infused with meso- $\Delta\zeta$ -CD8⁺ or with meso- ζ -CD8⁺ T cells, promoted CD45⁺ immune
189 infiltration in M108 tumors 84 days post adoptive transfer (Figure S4C). Conversely, CAR T
190 cells did not persist if transferred with meso- $\Delta\zeta$ -CD26^{high} cells. Collectively, our work reveals
191 that meso-CAR CD4⁺CD26^{high} cells are cytotoxic *in vitro* and *in vivo*, regress tumors in the
192 absence of CD8⁺ T cells and require tumor-reactive CD3 ζ signaling to persist.

193 Discussion

194 CAR T cells are therapeutic in many patients with hematological malignancies but have been
195 less effective thus far against solid tumors, owing in part to the oppressive tumor
196 microenvironment and poor persistence. Many efforts for overcoming these obstacles include
197 modulating T cell trafficking, targeting, cytokine delivery, co-stimulation, and improving cell
198 persistence among other strategies reviewed previously (10). CD4⁺ T cells help cytotoxic CD8⁺
199 T cells and when CAR engineered, have the ability to improve longevity of responses against
200 hematological malignancies (11, 12). Here we reveal that naturally arising CD4⁺ T cell subsets in
201 the peripheral blood differentially impact efficacy of CAR T cell therapy. For the first time, we
202 demonstrate that CD4⁺CD26^{high} T cells redirected with CAR possess enhanced functional and

203 antitumor activity versus classic subsets (Th1, Th2, Th17) or unselected CD4⁺ T cells, as
204 summarized visually in Figure 5.

205 CD26^{high} T cells derived from the peripheral blood of healthy individuals were
206 polyfunctional, co-secreting elevated IL-17A, IFN- γ and IL-22 while classic Th1, Th2 or Th17
207 cells lacked this dynamic functional profile. Moreover, CD26^{high} T cells have unique epigenetic
208 and molecular properties versus Th17 cells. As well, their TCR β repertoire does not overlap
209 profoundly with other helper subsets. Of clinical significance, CD4⁺CD26^{high} T cells persisted
210 long-term and ablated mesothelioma in mice when *ex vivo* engineered with CAR unlike bulk
211 CD4⁺, Th1, Th2, or Th17 cells. These cells could improve persistence of co-transferred CD8⁺
212 CAR T cells yet did not require CD8⁺ T cells for tumor regression. Notably, sorting Th17 cells
213 by CCR4⁺CCR6⁺ yielded an IL-17⁺ population also containing FoxP3⁺IL-2R α ^{high} Tregs, which
214 was not present when sorting CD26^{high} T cells. This work could yield future insight into new
215 methods of sorting T cells to improve CAR therapies by generating more functional T cells.

216 CD26 has many properties that could impact T cell immunity and our work supports this
217 concept (13). CD26 regulates distinct T cell functions, including: a) enzymatic cleavage of
218 chemokines that regulate migration (14); b) induction of CD86 on APC via CD26/Caveolin-1 co-
219 stimulation, in turn activating T cells (15, 16); c) conversion of adenosine (in tumors) to non-
220 suppressive inosine via docking adenosine deaminase (17, 18) and d) binding extracellular
221 matrix proteins (19), which may help CD26^{high} T cells infiltrate and remain in tumor. CD26
222 expressing cells further have high levels of chemokine receptors on their cell surface including
223 CCR2 which promote their recruitment and migration capability and are associated with rapid
224 functional recall responses (1, 20). CD26⁺ T cells have been associated with exacerbating
225 various autoimmune manifestations, including rheumatoid arthritis (RA) (21), multiple sclerosis

226 (MS) (22), graft versus host disease (GVHD) (23, 24) and diabetes (25). Conversely, levels of
227 CD26 enzymatic activity and the number of CD26⁺ T cells decrease in the blood of melanoma
228 patients as their disease progresses (26). This clinical data might suggest that CD26 itself plays a
229 role in augmenting T cell-mediated tumor immunity. Indeed, the CD26 molecule possesses many
230 functions that could be attributed to enhanced antitumor responses, but which one(s), if any, have
231 not yet been elucidated.

232 It remains possible that none of the many CD26 properties are responsible for regulating
233 the remarkable antitumor activity of these cells. Rather, high CD26 expression may mark
234 lymphocytes with durable persistence. CD26^{high} T cells may have a competitive advantage in the
235 tumor compared to other lymphocyte populations due to their function or perhaps resistance to
236 oxidative stress within the tumor microenvironment suggested by open chromatin and
237 heightened transcription of *CEBPD*. Study of the importance of *CEBPD* to CD26^{high} T cell
238 immunity is underway in our lab. Finally, while our work shows that enriching T cell subsets can
239 improve sub-optimal CAR constructs lacking costimulation, it will be important to clinically
240 elucidate the impact of costimulatory domains on persistence and durability of CD4⁺ T cell
241 populations. Future investigation of the unique CD26^{high} T cell signature discovered herein will
242 reveal the importance of these characteristics to T cell function and provide novel approaches to
243 enhance tumor immunity.

244 There are many implications from our findings given the significant antitumor responses
245 mediated by CD26^{high} T cells in a mouse model of large established human mesothelioma. The
246 epigenetic and molecular landscape of these helper subsets will permit investigators to address
247 novel questions regarding their function in the immune system. Future work to translate, target
248 and redirect these cells to eradicate tumors or target cells inducing autoimmunity in the clinic

249 could provide new treatment options for a vast array of diseases. Clinical trials are now
250 underway based on our findings to evaluate the potential of CD4⁺CD26^{high} T cells in patients.

251

252 **Methods**

253 **Study Design**

254 *Sample Size:* As these experiments were exploratory, there was no estimation to base the
255 effective sample size; therefore, we based our animal studies using sample sizes ≥ 5 . *Rules for*
256 *Stopping Data Collection:* experimental endpoints were designated prior to study execution.
257 Tumor control studies were conducted over ~ 70 days. *Data Inclusion/exclusion:* for experiments
258 reported herein, animals were only excluded if tumors were very small or not measurable, a rule
259 established prospectively prior to any therapy initiation. *Outliers:* Outliers were reported.
260 *Endpoints:* Tumor endpoint was reached when tumor area exceeded 400mm². Remaining mice
261 were euthanized and spleens were harvested when more than half of the mice in a group reached
262 tumor endpoint. *Randomization:* Prior to therapy, mice were randomized based on tumor size.
263 *Blinding:* Tumors were measured using L x W measurements via calipers by personnel blinded
264 to treatment group.

265

266 **Statistical Analysis**

267 Tumor area results were transformed using the natural logarithm for data analysis. Mixed effects
268 linear regression models with a random component to account for the correlation of the repeated
269 measure within a mouse were used to estimate tumor area over time. In circumstances where
270 linearity assumptions were not met, polynomial regression models were used (27). Linear
271 combinations of the resulting model coefficients were used to construct estimates for the slope
272 differences with 95% confidence intervals where applicable. For polynomial models, estimates

273 were constructed for the differences in area between groups on the last day where at least one
274 mouse was alive in all groups. Experiments with multiple groups were analyzed using one-way
275 analysis of variance (ANOVA) with post comparison of all pair wise groups using Tukey's range
276 test. Experiments comparing two groups were analyzed using a Student's t test. The center
277 values are the mean and error bars are calculated as the SEM. TCR β sequencing analysis was
278 based on the log-linear model and the 'relative risks' calculated with a 95% confidence interval.

279

280 **Subset Isolation**

281 De-identified, normal human donor peripheral blood cells were purchased as a buffy coat
282 (Plasma Consultants) or leukapheresis (Research Blood Components). PBL were enriched using
283 Lymphocyte Separation Media (Mediatech). CD4⁺ T cells were negatively isolated using
284 magnetic bead separation (Dynabeads, Invitrogen) and plated in culture medium with a low
285 concentration of rhIL-2 (20 IU/ml; NIH repository) overnight. For *in vivo* studies, CD8⁺ T cells
286 were positively isolated prior to the enrichment of CD4⁺ T cells. The following morning CD4⁺ T
287 cells were stained using PE-CD26 (C5A5b), AlexaFluor647-CXCR3 (G025H7), PECy7-CCR6
288 (G034E3, Biolegend), FITC-CCR4 (205410, R&D Systems) and APCCy7-CD4 (OKT4, BD
289 Pharmingen). Cells were sorted based on the following gating strategies: bulk CD4: CD4⁺; Th1:
290 CD4⁺CCR6⁻CCR4⁻CXCR3⁺; Th2: CD4⁺CCR6⁻CCR4⁺CXCR3⁻; Th17: CD4⁺CCR6⁺CCR4⁺;
291 CD26: CD4⁺CD26^{high}. Cells were sorted on a BD FACSAria Iiu Cell Sorter or on a Beckman
292 MoFlo Astrios High Speed Cell Sorter.

293

294 **T cell culture**

295 T cell subsets were expanded in RPMI 1640 culture medium supplemented with non-essential
296 amino acids, L-glutamine, sodium pyruvate, HEPES, Pen/Strep, β -mercaptoethanol and FBS.
297 Cells were cultured at either a 1:1 or 1:10 bead to T cell ratio. Magnetic beads (Dynabeads, Life
298 Technologies) coated with antibodies to CD3 (OKT3) and/or ICOS (ISA-3, eBioscience) were
299 produced in the lab according to manufacturers' protocols. One hundred IU/ml rhIL-2 (NIH
300 repository) was added on day 2 and media was replaced as needed.

301

302 **T cell transduction**

303 To generate mesothelin-specific T cells, α CD3/ICOS-activated, sorted CD4⁺ and bulk CD8⁺ T
304 cells were transduced with a chimeric anti-mesothelin single-chain variable fragment (scFv)
305 fusion protein containing the T cell receptor ζ (TCR ζ) signaling domain (1st-gen-Meso-CAR) or
306 a truncated CD3 ζ non-signaling domain ($\Delta\zeta$) that was generated as described previously (9).
307 CAR expression was determined using a flow cytometry antibody specific for the murine F(ab')₂
308 fragment (Jackson ImmunoResearch, 115-606-006).

309

310 **Flow cytometry**

311 For intracellular staining data, cells were stimulated with PMA/Ionomycin. After one hour,
312 Monensin (Biolegend) was added and incubated for another 3 hours. Following surface staining,
313 intracellular staining with antibodies was performed according to the manufacturer's protocol
314 using Fix and Perm buffers (Biolegend). Data were acquired on a BD FACSVerser or LSRII X-20
315 (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

316

317 **MicroArray**

318 RNA was isolated from sorted CD4⁺ T cells using the Qiagen RNeasy Mini kit and frozen. RNA
319 was submitted to Phalanx Biotech Group for processing on their OneArray platform (San Diego,
320 CA). *Heatmap and PCA clustering*: Graphing was performed in R (version 3.1.2) using gplots
321 (version 2.16.0). Log₂ values for CD4⁺ cells were averaged and used as baseline for the genes of
322 interest. For each individual sample the fold change relative to baseline was calculated and the
323 median value for the triplicates was calculated and used for generating figures.

324

325 **ATAC sequencing**

326 Sorted CD4⁺ T cells were cryopreserved in CryoStor and sent for analysis. Naïve cells were
327 sorted based on expression of CCR7 and CD45RA. ATAC-seq was performed by Epinomics
328 according to the protocol described by Buenrostro et al. (28). Fifty thousand sorted T cells were
329 frozen using Cryostor CS10 freeze media (BioLife Solutions) and shipped on dry ice for
330 processing and analysis to Epinomics (Menlo Park, CA).

331

332 **T Cell Receptor β sequencing**

333 Sorted T cells were centrifuged and washed in PBS, and genomic DNA was extracted using
334 Wizard Genomic DNA purification kit (Promega). The quantity and purity of genomic DNA was
335 assessed through spectrophotometric analysis using NanoDrop (ThermoScientific).
336 Amplification of TCR genes was done within the lab using the ImmunoSEQ hsTCR β kit
337 (Adaptive Biotechnologies Corp., Seattle, WA) according to the manual. Survey sequencing of
338 TCR β was performed by the Hollings Cancer Center Genomics Core using the Illumina MiSeq
339 platform.

340

341 **Single cell RNA sequencing**

342 Sorted Th17, CD26^{high} T cells or bulk CD4⁺ T cells were cryopreserved and sent for analysis to
343 David H. Murdock Medical Research Institutes (DHMRI) Genomics core. Genomic DNA was
344 analyzed using the Chromium Controller instrument (10X Genomics, Pleasanton, CA) which
345 utilizes molecular barcoding to generate single cell transcriptome data (29). Sequencing of the
346 prepared samples was performed with a HiSeq2500 platform (Illumina). Data were analyzed
347 with Long Ranger and visualized with Loupe (10X Genomics).

348

349 ***In vitro* cytotoxicity assay**

350 Sorted CD4⁺ T cell subsets were activated with α CD3/ICOS beads and engineered to be
351 mesothelin-specific using a lentiviral CAR. Following a 10-day expansion, equal numbers of
352 transduced T cells were co-cultured overnight with target cells. For the CAR, meso-expressing
353 K562 cells (pre-stained with Cell Trace Violet, Molecular Probes) serially diluted in the presence
354 of CD107A (Pharmingen). K562 lysis was determined by 7-AAD (Pharmingen) uptake. K562-
355 meso cells were tested for mycoplasma (MycoAlert, Lonza) and mesothelin (R&D Systems,
356 FAB32652) expression during expansion.

357

358 **Mice and tumor line**

359 NOD SCID gamma chain knockout mice (NSG, The Jackson Laboratory) were bred at the
360 University of Pennsylvania or at the Medical University of South Carolina. NSG mice were
361 given ad libitum access to autoclaved food and acidified water. M108 xenograft tumors (gift
362 C.H. June), described previously (9), were tested for mycoplasma during expansion (Lonza).

363

364 **Ethics Approval:** Human peripheral blood was not collected specifically for the purposes of this
365 research and all samples were distributed to the lab in a deidentified manner. Therefore, this
366 portion of our research was not subject to IRB oversight. All animal studies were approved by
367 the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South
368 Carolina.

369
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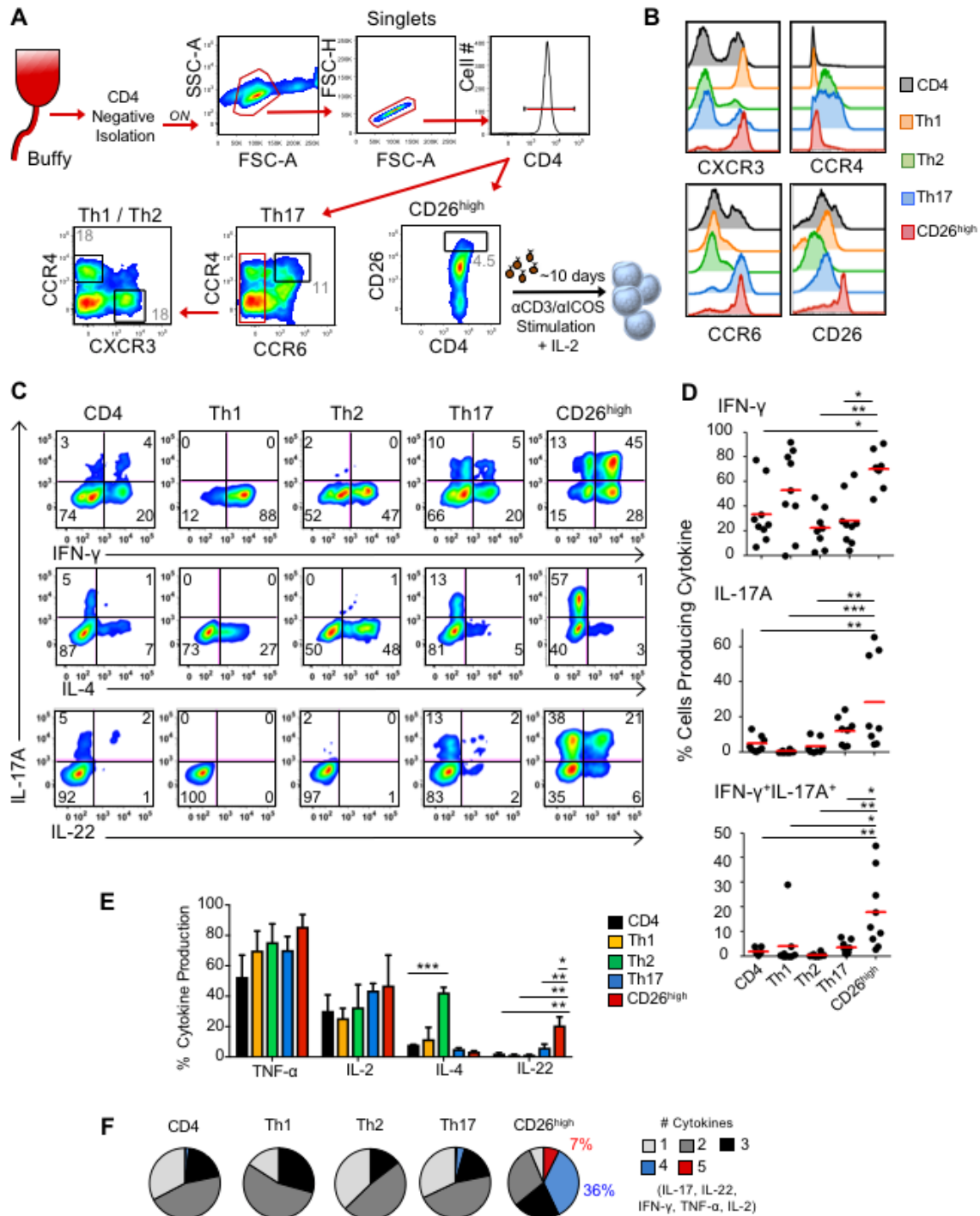
496 **Author contributions:** M.H.N. designed and executed experiments, analyzed the data, created
497 the figures, and wrote and edited the manuscript; H.M.K performed experiments, analyzed data,
498 created figures, wrote and edited the manuscript; S.R.B, J.S.B., L.W.H., K.M., and M.M.W
499 performed experiments; K.E.A., P.G., and M.J.Z analyzed the data, S.M., M.P.R., M.I.N., H.E.B
500 designed experiments and edited the manuscript, and C.M.P. directed the project, designed
501 experiments, and edited the manuscript. All authors critically read and approved the manuscript.

502 **Competing interests:** C.M.P has a patent for the expansion of Th17 cells using ICOSL-
503 expressing aAPCs. M.H.N, S.R.B and C.M.P have a patent for the use of CD26^{high} T cells for the
504 use in adoptive T cell transfer therapy. All other authors have no disclosures.

505

506 **Data and materials availability:** For original data, please contact paulos@musc.edu.
507 Microarray data can be found at GEO accession number GSE106726.

508 **Figures and Tables**



509

510 **Figure 1. CD4⁺CD26^{high} T cells possess a dynamic cytokine profile. A) CD4⁺ subset sorting**

511 **scheme. CD4⁺ lymphocytes were negatively isolated using magnetic beads from normal donor**

512 PBL. Th17 cells were sorted from CCR6⁺CCR4⁺ gate. Th1 and Th2 cells are both CCR6⁻ and
513 subsequently sorted via CXCR3 or CCR4, respectively. CD26^{high} cells were sorted independently
514 based on CD26 expression. **B)** Chemokine receptor profile post sort. **C)** CD4⁺ T cell subsets
515 were stimulated with α CD3/ICOS beads at a ratio of 1 bead:10 T cells and expanded in IL-2
516 (100IU/ml). Ten days following activation, the 5 different cell subsets were examined for their
517 intracellular cytokine production. Dot plot representation of IL-17, IFN- γ , IL-4, and IL-22
518 expression by flow cytometry. **D)** Graphical representation of at least 8 normal donors from
519 independent experiments demonstrating IFN- γ and IL-17 single and double producing cells by
520 flow cytometry. **E)** Graphical representation of 10 normal donors demonstrating cytokine-
521 producing cells by flow cytometry. 2-3 replicates each. Compared to CD26^{high} *, $P < 0.05$; **,
522 $P < 0.01$; ***, $P < 0.001$; ANOVA, Tukey post-hoc comparisons. **F)** Cells were gated on cytokine-
523 producing cells to quantify cells that produced between one and five cytokines simultaneously.
524 Cytokines of interest were IL-17, IFN- γ , IL-2, IL-22 and TNF- α . Representative of 5
525 experiments.

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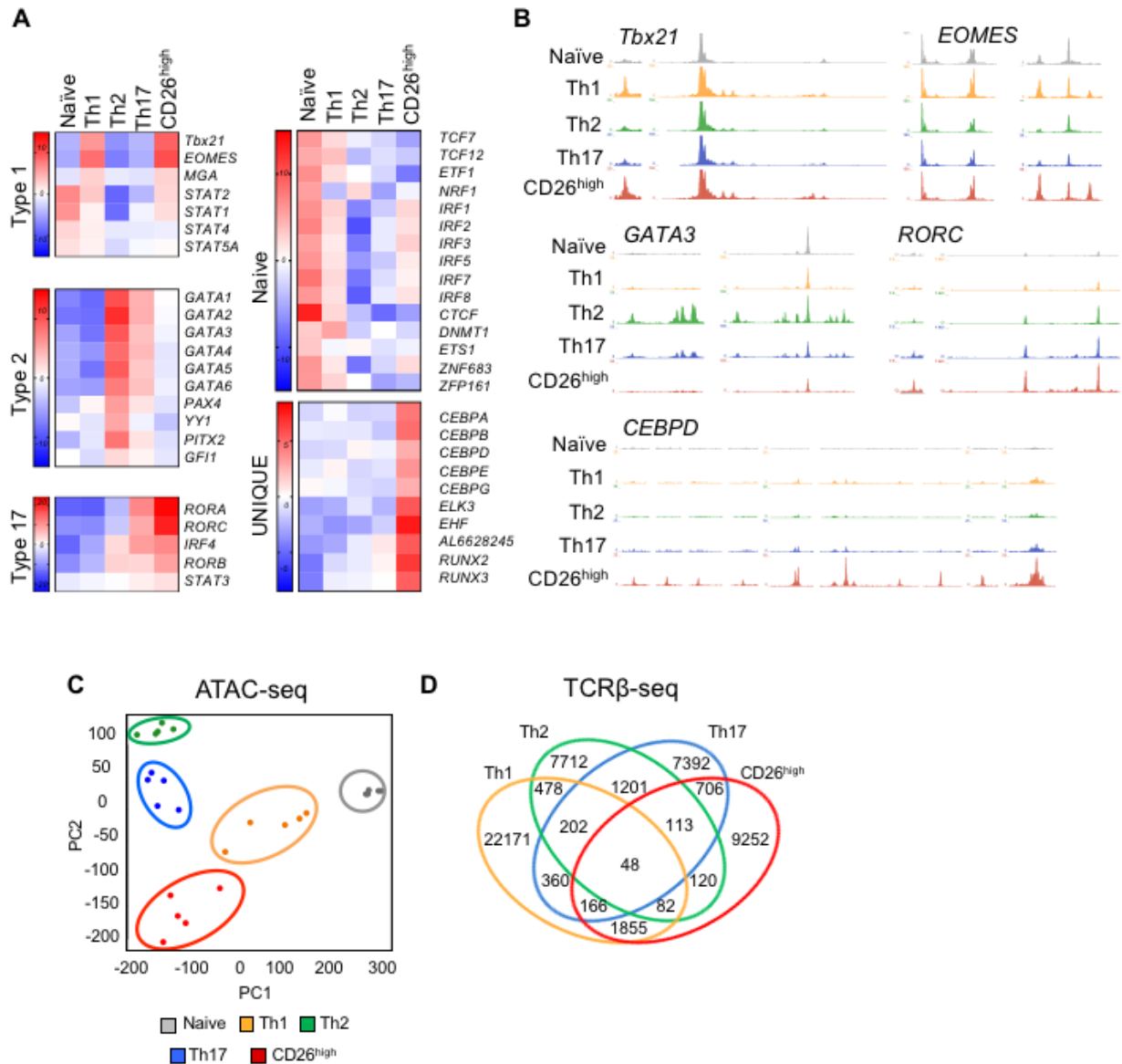
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535 **Figure 2. The epigenetic and molecular signature of CD26^{high} T cells are unique.** A) Assay

536 for Transposase-Accessible Chromatin sequencing (ATAC-seq) analysis describing chromatin

537 accessibility in FACS sorted CD4⁺ subsets (Naive, Th1, Th2, Th17, CD26^{high}) organized by

538 transcription factor networks known to describe Th1, Th2, Th17 and naive subsets. Accessible

539 transcription regions unique to CD26^{high} T cells are also shown. Compiled from 5 healthy

540 donors. B) UCSC genome browser tracks for sorted CD4⁺ subsets around classical T helper

541 transcription factors from ATAC-seq analysis. **C)** ATAC-seq principal component analysis of
542 sorted T cell subsets analyzed at resting state. n=5 donors. **D)** TCR β sequencing of CD26^{high},
543 Th17, and Th1 cells sorted from peripheral blood of healthy donors demonstrates unique or
544 shared clonotypes. Venn diagram illustrates percentage of unique or shared TCR β sequences.
545 The relative frequencies (standardized to sum to 1.0): CD26^{high} only = 0.237, Th1 only = 0.487,
546 Th17 only = 0.196, CD26^{high} & Th1 = 0.041, CD26^{high} & Th17 = 0.020, Th1 & Th17 = 0.015,
547 All three = 0.004, log-linear model.

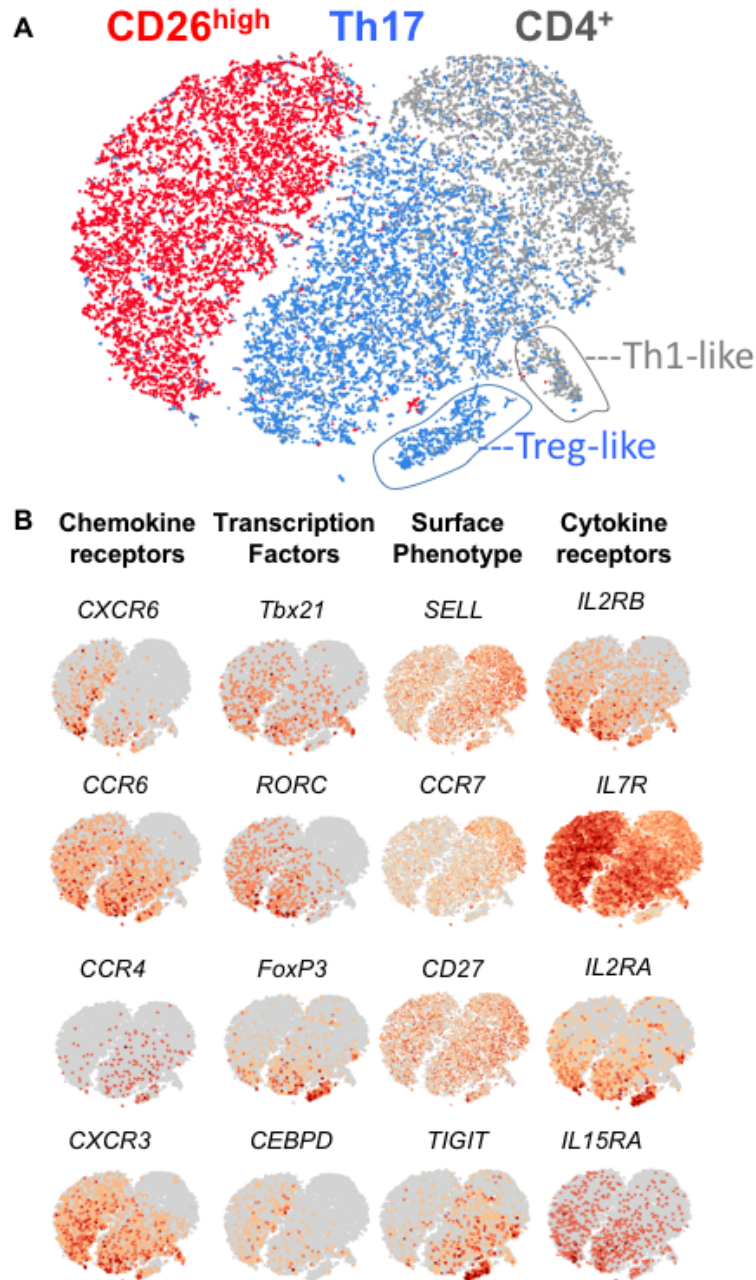
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554 **Figure 3: CD4⁺CD26^{high} T cells are distinguished from Th17 cells via single-cell sequencing.**

555 Total CD4⁺, CD26^{high} and Th17 cell subsets were sorted from the peripheral blood of healthy

556 donors and ~3000 cells assayed by single cell RNA sequencing. **A)** Data were analyzed by t-

557 Distributed Stochastic Neighbor Embedding (t-SNE). **B)** t-SNE plot overlaid with mRNA

558 expression of chemokine receptors, transcription factors, memory markers and cytokine
559 receptors. Representative of 3 healthy donors.

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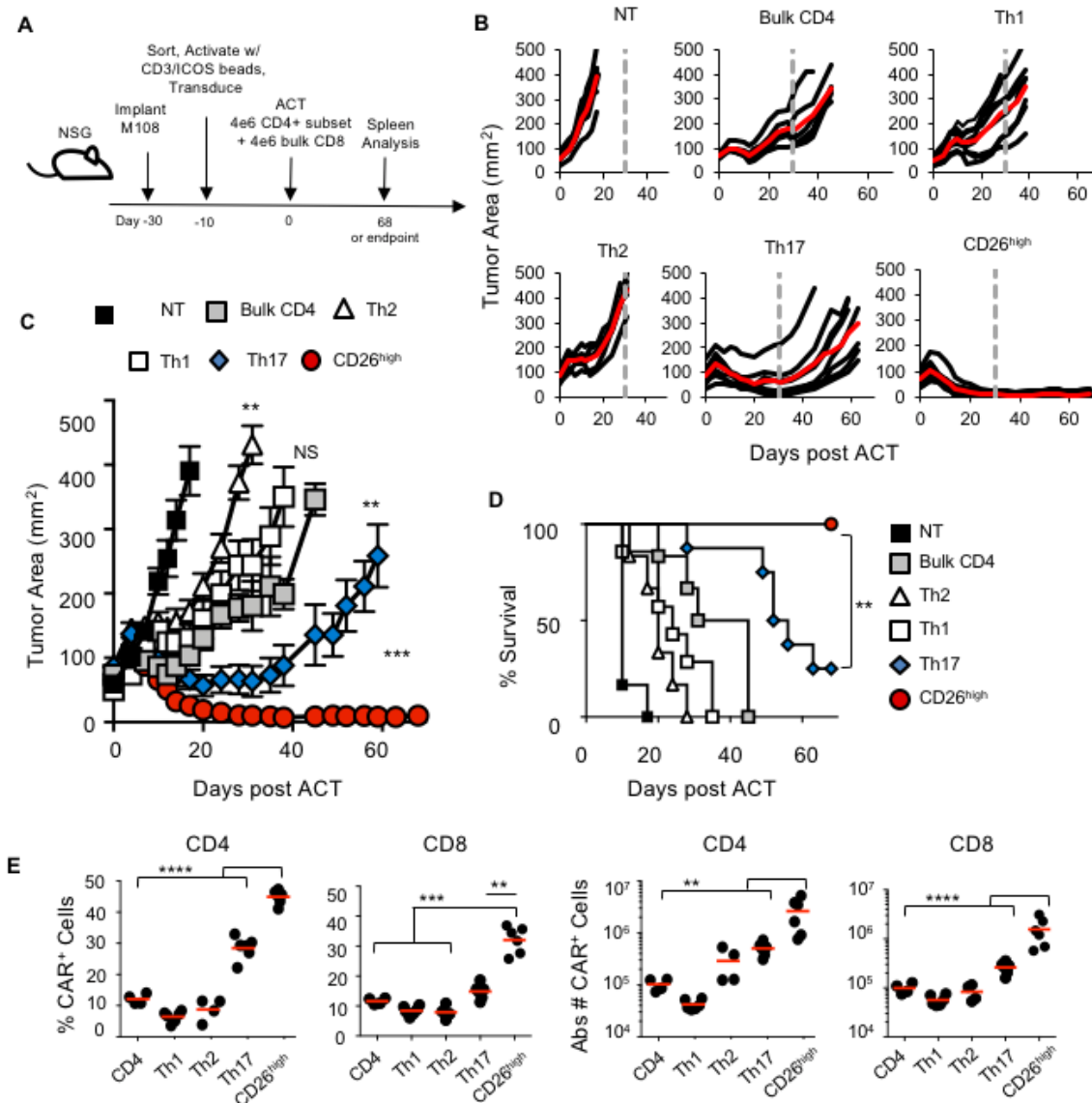
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567 **Figure 4. Human CD26^{high} T cells ablate large human tumors and persist relative to other**

568 **CD4⁺ T cell subsets.** **A)** ACT schematic. Th1 (CXCR3⁺), Th2 (CCR4⁺), Th17 (CCR4⁺/CCR6⁺),

569 CD26^{high} or bulk CD4⁺ cells were sorted from normal donor PBL and expanded with

570 α CD3/ICOS bead at a 1 bead:10 T cell ratio. Cells were transduced with a 1st generation

571 mesothelin-specific CD3 ζ CAR and expanded with IL-2. NSG mice bearing mesothelioma were

572 treated with 4×10^6 transduced, sorted CD4⁺ cells + 4×10^6 transduced CD8⁺ cells and 50,000 IU

573 IL-2 was given to each mouse daily for 3 days. **B)** Single tumor curves overlaid with average

574 curve (red) and C) average tumor curves of 6-9 mice/group. All groups were significantly
575 different from NT, $P < 0.005$. CD4 vs. Th1 NS; CD4 vs. Th2, $P = 0.0015$ (**); CD4 vs. Th17, P
576 $= 0.0035$ (**); CD4 vs. CD26^{high}, $P = 0.0003$ (***); Th17 vs. CD26^{high}, $P = 0.008$ (**);
577 polynomial regression. D) The percentage of mice surviving with tumor size below the 200mm²
578 threshold. E) Spleens were analyzed by flow for the percentage and total number of
579 CD3⁺CAR⁺CD4⁺ or CD8⁺ cells at day 68 (Th17 and CD26^{high}) or group endpoint (CD4, Th1,
580 Th2). n=4-6 mice/group. Compared to CD26^{high} **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$;
581 ANOVA, Tukey post-hoc comparisons.

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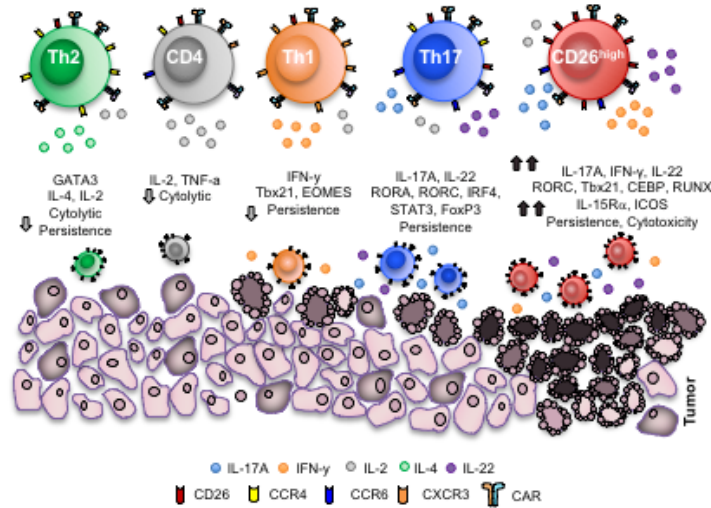
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597 **Figure 5. CD4⁺CD26^{high} T cells possess distinct antitumor and molecular properties relative**
598 **to other helper subsets.** CD26^{high} T cells have been described herein for use in adoptive T cell
599 transfer therapy. These cells produce heightened levels of cytokines including IL-17, IFN- γ , IL-
600 22, IL-2 and can co-secrete these cytokines. CD26^{high} T cells have a distinct chromatin landscape
601 with accessible regions near *RORC*, *Tbx21*, *CEBP*, and *RUNX* transcription factors, and have a
602 unique transcriptional signature. These cells are cytotoxic, multi-functional and inflammatory.
603 Overall, CD26^{high} T cells persist and regress tumors to a remarkably greater extent than other
604 CD4⁺ T cells *in vivo* and represent a novel CD4⁺ helper population with potent antitumor
605 properties.

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618 and **B)** Heat map of log₂-fold change in expression of genes with the highest or lowest
619 expression in CD26^{high} T cells. **C)** T cell subsets were sorted from peripheral blood of normal
620 human donors based on surface chemokine receptor expression (Th1 (CXCR3⁺CCR6⁻), Th2
621 (CCR4⁺CXCR3⁻) Th17 (CCR4⁺CCR6⁺), CD26^{high} (top 5%)). DNA was isolated, TCRβ
622 sequences were expanded using an immunoSEQ kit and subsequently sequenced. Data shown is
623 the graphical representation of TCR overlap between indicated T helper subsets. Representative
624 from 4 donors.

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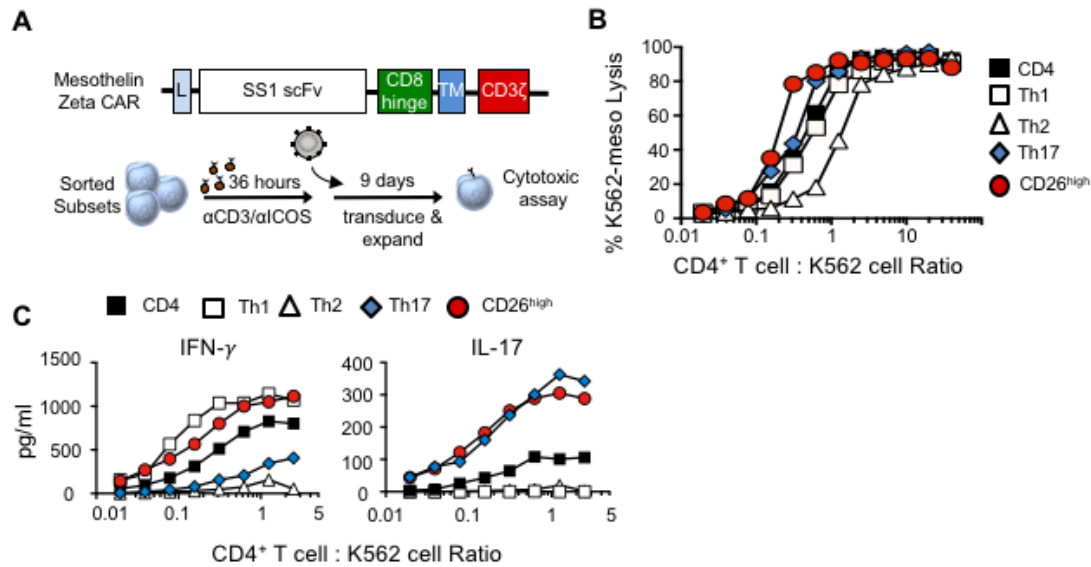
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641 **Figure S2. CD26^{high} cells are cytotoxic and polyfunctional *in vitro* when engineered with a**

642 **chimeric antigen receptor. A) Transduction method. $\alpha\text{CD3}/\text{ICOS}$ -stimulated CD4⁺ T cell**

643 **subsets were genetically engineered with a 1st generation mesothelin-specific CAR. Cells were**

644 **expanded for 6 days and analyzed by flow cytometry for CAR expression prior to use. B)**

645 **Percentage of K562-meso cells that were lysed by effector CD4⁺ T cell subsets. C) Cytokine**

646 **secretion determined by ELISA. Representative of 3 experiments.**

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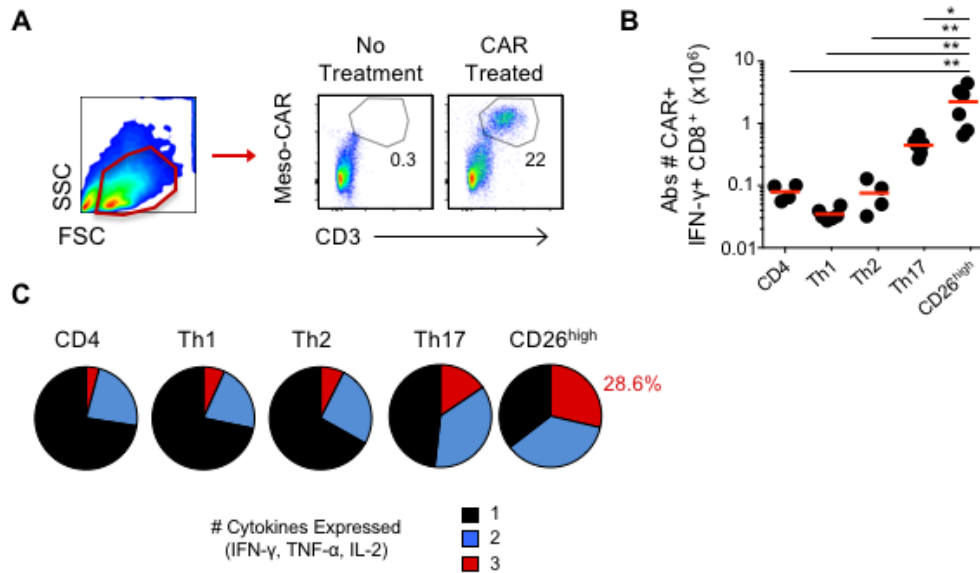
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657 **Figure S3: Human CD26^{high} T cells improve function of co-transferred CD8⁺ meso-CAR T**

658 **cells. A-C)** Sorted Th1, Th2 Th17, CD26^{high} or CD4⁺ cells were transferred into mesothelioma-

659 bearing NSG mice as described in Figure 3D. **A)** Representative flow cytometry gating for CAR

660 T *in vivo*. **B)** Total number of splenic IFN- γ -producing CD8⁺meso-CAR⁺ cells. n=4-6

661 mice/group. Compared to CD26^{high} *, $P < 0.05$; **, $P < 0.01$; ANOVA, Tukey post-hoc

662 comparisons. **C)** Simultaneous intracellular cytokine production in spleen CAR⁺CD8⁺ cells.

663 Average of 4-6 mice/group.

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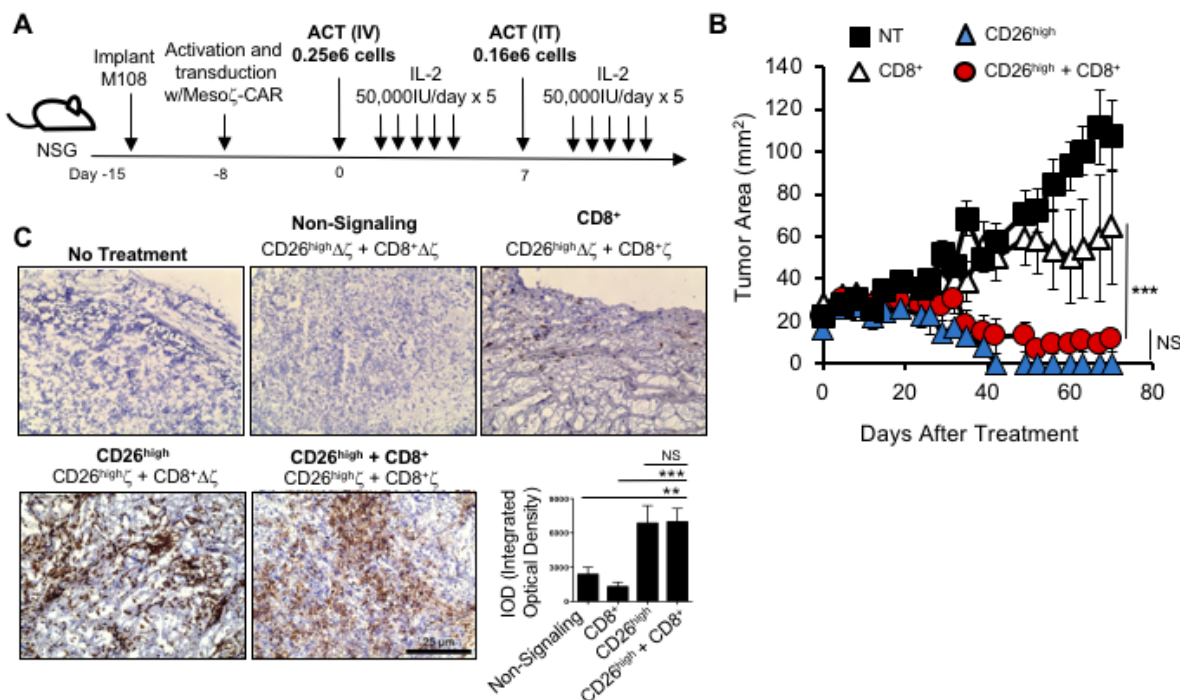
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672 **Figure S4. Human CD26^{high} meso-CAR T cells do not require CD8⁺ CAR T cells for**

673 **antitumor responses. A-B)** Mesothelioma-bearing NSG mice were treated with CD26^{high} T cells

674 co-infused with or without CD8⁺ T cells all engineered with 1st-gen-meso-CAR. Two infusions

675 of cells were given one week apart (250,000 cells i.v.; 160,000 cells i.t.). 5-6 mice/group. All

676 groups were significantly different, $P < 0.001$, except CD8⁺ + CD26^{high} vs. CD26^{high}, $P < 0.43$.

677 **C)** Immunohistochemistry staining of M108 from NSG mice treated with 1.45 × 10⁶ CD26^{high} and

678 CD8⁺ T cells transduced with a 1st-gen-Meso-CAR having either full-length CD3 ζ signaling or a

679 non-signaling truncated version ($\Delta\zeta$). Staining of human CD45 and hematoxylin on day 84 post-

680 transfer (x10; 3 or 4 mice/group; average IOD from 10 images). Compared to CD26^{high} **, $P <$

681 0.01; ***, $P < 0.001$; ANOVA.