

1 **Induction and transcriptional regulation of the co-inhibitory gene module in T cells**

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24

25 **Abstract**

26 Expression of co-inhibitory receptors, such as CTLA-4 and PD-1, on effector T cells is a
27 key mechanism for ensuring immune homeostasis. Dysregulated co-inhibitory receptor
28 expression on CD4⁺ T cells promotes autoimmunity while sustained overexpression on
29 CD8⁺ T cells promotes T cell dysfunction or exhaustion, leading to impaired ability to
30 clear chronic viral infections and cancer^{1,2}. Here, we used RNA and protein expression
31 profiling at single-cell resolution to identify a module of co-inhibitory receptors that
32 includes not only several known co-inhibitory receptors (PD-1, Tim-3, Lag-3, and
33 TIGIT), but also a number of novel surface receptors. We functionally validated two
34 novel co-inhibitory receptors, Activated protein C receptor (Procr) and Podoplanin
35 (Pdpn). The module of co-inhibitory receptors is co-expressed in both CD4⁺ and CD8⁺ T
36 cells and is part of a larger co-inhibitory gene program that is shared by non-responsive T
37 cells in multiple physiological contexts and is driven by the immunoregulatory cytokine
38 IL-27. Computational analysis identified the transcription factors Prdm1 and c-Maf as
39 cooperative regulators of the co-inhibitory module, which we validated experimentally.
40 This molecular circuit underlies the co-expression of co-inhibitory receptors in T cells
41 and identifies novel regulators of T cell function with the potential to regulate
42 autoimmunity and tumor immunity.
43

44 We used single-cell RNA-seq (scRNA-Seq) to analyze co-inhibitory and co-
45 stimulatory receptor expression in 588 CD8⁺ and 316 CD4⁺ tumor-infiltrating
46 lymphocytes (TILs) from B16F10 melanoma³. We found that PD-1, Tim-3, Lag-3,
47 CTLA-4, 4-1BB, and TIGIT strongly co-vary in CD8⁺ TILs. CD4⁺ TILs showed a similar
48 pattern with the additional co-expression of ICOS, GITR, and OX40 (**Fig. 1a, top**).
49 Single-cell mass cytometry (CyTOF) confirmed the surface co-expression of these
50 receptors (**Fig. 1a, bottom, Supplementary Table Information 1**). Expression of PD-1,
51 Lag-3, Tim-3, and TIGIT was tightly correlated on both CD8⁺ and CD4⁺ TILs (**Fig. 1a,**
52 **bottom**). Clustering analysis (t-SNE⁴, **Methods**) showed two groups of CD8⁺ TILs
53 (clusters 1 and 2) (**Fig. 1b, Extended Data Fig. 1a,c**) where PD-1, Lag-3, Tim-3, and
54 TIGIT were mainly expressed in cluster 1 cells (**Fig. 1b, Extended Data Fig. 1c**) as were
55 LILRB4 (**Extended Data Fig. 1a**), and co-stimulatory receptors of the TNF-receptor
56 family, 4-1BB, OX-40, and GITR. In contrast, ICOS and CD226 were less restricted to
57 cluster 1 (**Extended Data Fig. 1a**). We further observed two discrete clusters of CD4⁺
58 TILs (clusters 3 and 4) wherein PD-1, Tim-3, Lag-3, and TIGIT co-expression was
59 restricted to cluster 3 (**Fig. 1b, Extended Data Fig. 1c**).

60 The co-expression of co-inhibitory receptors on CD8⁺ and CD4⁺ T cells suggests
61 a common trigger. One candidate is IL-27, a heterodimeric member of the IL-12 cytokine
62 family that suppresses autoimmunity⁵, induces IL-10-secreting Type 1 regulatory (Tr1)
63 cells^{6,7}, and induces expression of Tim-3 and PD-L1 on CD4⁺ and CD8⁺ T cells^{8,9}.
64 Activation of CD4⁺ and CD8⁺ T cells in the presence of IL-27 induced Tim-3 (Havcr2),
65 Lag-3, and TIGIT at mRNA (**Fig. 1c**) and protein levels (**Extended Data Fig. 2a**).
66 Expression of Tim-3, Lag-3, and TIGIT was reduced in IL-27R-deficient T cells, whereas

67 PD-1 (*Pdcd1*) expression was unaffected by IL-27 *in vitro* (**Fig. 1c, Extended Data Fig.**
68 **2a**).

69 CyTOF analysis showed that loss of IL-27ra resulted in loss of cells in cluster 1 of
70 CD8⁺ TILs and cluster 3 of CD4⁺ TILs (**Fig. 1d**, p-value= 5×10^{-23} and 6.8×10^{-7} for CD8⁺
71 and CD4⁺ respectively, hypergeometric test, **Extended Data Fig. 1b,c,d**), indicating a
72 key role for IL-27 in driving co-inhibitory receptor co-expression in both CD4⁺ and CD8⁺
73 T cells *in vivo*. Although PD-1 expression wasn't dependent on IL-27 *in vitro*, it was
74 dependent on IL-27R signaling *in vivo*. In line with the induction of IL-10 by IL-27⁵⁻⁷, we
75 observed reduced IL-10 in IL27ra KO CD8⁺ TILs (**Extended Data Fig. 2b**).

76 scRNA-seq of CD8⁺ and CD4⁺ TILs from WT and IL27ra KO mice (**Fig. 1e**,
77 **Extended Data Fig. 3a,b; Methods**) revealed distinct clusters of CD8⁺ (cluster 5) and
78 CD4⁺ (cluster 4) TILs that highly expressed the co-inhibitory receptors PD-1, Tim-3,
79 Lag-3, and TIGIT. Expression of these genes was decreased in CD8⁺ TILs from IL27ra
80 KO mice, while only Tim-3 and Lag-3 were decreased in CD4⁺ TILs from IL27ra KO
81 mice (**Fig. 1e**). Thus, IL-27 drives a module of co-inhibitory receptors that are strongly
82 co-expressed *in vivo* together with IL-10.

83 The co-inhibitory receptor module could be part of a larger IL-27-driven
84 inhibitory gene program. We analyzed the mRNA profiles of CD4⁺ and CD8⁺ T cells
85 stimulated in the presence or absence of IL-27. IL-27 induced similar expression
86 programs in CD4⁺ and CD8⁺ T cells (**Extended Data Fig. 4a,b**). We identified 1,201
87 genes with IL-27-dependent expression (**Methods**). We compared the IL-27-driven gene
88 program to the gene signatures for four different states of T cell non-responsiveness:
89 CD8⁺ T cell exhaustion in both cancer³ and chronic viral infection¹⁰ and antigen-

90 specific¹¹ or non-specific (anti-CD3 antibody¹²) CD4⁺ T cell tolerance. We found
91 significant overlap with all of these signatures (**Methods, Extended Data Fig. 4c-f**).

92 Projection of the IL-27/CD8⁺ cancer T cell exhaustion overlap signature onto the
93 single-cell profiles of CD8⁺ TILs marked a distinct subset of cells (**Fig. 2a**, panel I). This
94 subset scored highly for the overlap signatures between the IL-27-driven gene program
95 and each of the other three states of T cell non-responsiveness (**Fig. 2a**, panels II-IV).
96 The transcriptional program induced in IL27ra KO TILs was active in a complimentary
97 subset of TILs (**Methods, Fig. 2a** panel V). The control signature from cells stimulated
98 with IL-27 *in vitro* showed bimodal distribution and by itself did not detect the same
99 population of cells (**Fig. 2a** panel VI). From these analyses, we identified a co-inhibitory
100 gene module (272 genes) that is shared across multiple states of T cell non-
101 responsiveness (**Supplementary Information Table 2**). Within this module, we
102 identified a set of 57 genes encoding cell surface receptors and cytokines, including Tim-
103 3, Lag-3, TIGIT, and IL10 (**Fig. 2b**), which we further stratified by their expression in
104 cancer and chronic viral infections (**Fig. 2c**). Two surface molecules, Procr (protein C
105 receptor) and Pdpn (podoplanin) were highly expressed in the setting of cancer (**Fig. 2c**).
106 Activation of naïve CD4⁺ and CD8⁺ T cells *in vitro* in the presence of IL-27 induced the
107 expression of Procr and Pdpn (**Extended Data Fig. 5a**). *In vivo*, Procr and Pdpn
108 exhibited IL-27 dependent co-expression with PD-1 and Tim-3 on CD8⁺ TILs (**Extended**
109 **Data Fig. 5b**).

110 Procr⁺ CD8⁺ TILs exhibited an exhausted phenotype, producing less TNF α and
111 IL-2 and more IL-10 than Procr⁻ CD8⁺ TILs (**Extended Data Fig. 5c**). Growth of
112 B16F10 melanoma was inhibited in Procr hypomorph (Procr^{d/d})¹³ mice (**Fig. 2d**), and

113 Procr^{d/d} CD8⁺ TILs mice exhibited enhanced TNF α production, but no difference in IL-2,
114 IFN- γ , or IL-10 (**Fig. 2e**). Procr^{d/d} TILs exhibited a decreased frequency of Tim-3^{hi}PD-1^{hi}
115 CD8⁺ T cells suggesting that Procr signaling promotes a severely exhausted phenotype in
116 CD8⁺ T cells¹⁴ (**Fig. 2f**). Adoptive transfer of CD8⁺ T cells lacking Procr revealed a T
117 cell specific role for Procr in constraining tumor growth (**Extended Data Fig. 5d**).

118 Although Pdpn can limit CD4⁺ T cell survival in inflamed tissues¹⁵, its role in T
119 cell exhaustion is unknown. We observed a significant delay in B16F10 tumor growth in
120 mice with Pdpn deficiency in T cells (Pdpn cKO) (**Fig. 2g**). Pdpn-deficient CD8⁺ TILs
121 exhibited enhanced TNF α production but no significant difference in IL-2, IFN- γ , or IL-
122 10 (**Fig. 2h**). The frequency of Tim-3^{hi}PD-1^{hi} CD8⁺ TILs was decreased, indicating a
123 reduced accumulation of T cells with a severely exhausted phenotype in Pdpn cKO¹⁴
124 (**Fig. 2i**). Consistent with previous data¹⁵, Pdpn-deficient PD-1⁺Tim-3⁺ CD8⁺ TILs had
125 higher expression of IL-7Ra, indicating that Pdpn may limit the survival of CD8⁺ TILs in
126 the tumor microenvironment (**Extended Data Fig. 5e,f**).

127 We identified the transcription factor (TF) Prdm1 as a candidate regulator of the
128 co-inhibitory module. Prdm1 is induced *in vitro* by IL-27 in CD4⁺ and CD8⁺ T cells
129 (**Extended Data Fig. 6a**), is enriched in TILs with high expression of the IL-27 co-
130 inhibitory module (**Extended Data Fig. 3c-f** and **6b,c** and **Methods**), and is
131 overexpressed in exhausted CD8⁺ TILs (p-value= 0.0004, t-test, **Extended Data Fig. 6d**).
132 Network analysis based on profiling of naïve CD8⁺ T cells from mice with a T cell
133 specific deletion of Prdm1 (Prdm1 cKO) stimulated with IL-27, showed that Prdm1
134 regulates multiple genes in the IL-27 co-inhibitory module (**Extended Data Fig. 6e**, p-

135 value= 2.32×10^{-12} ; hypergeometric test; **Methods**). This was further supported by Prdm1
136 Chip-seq data¹⁶ (p-value= 2.9×10^{-8} respectively, hypergeometric test; **Fig. 6e; Methods**).

137 CD8⁺ TILs from B16F10 tumor-bearing Prdm1 cKO mice expressed lower levels
138 of Tim-3, PD-1, and Procr (**Fig. 3a**); however, there was no difference in tumor growth
139 compared to wild type (WT) controls (**Fig. 3b**), indicating that the reduction of co-
140 inhibitory receptor expression in Prdm1 cKO mice was insufficient to promote effective
141 anti-tumor immunity. We therefore examined whether other TFs may regulate the co-
142 inhibitory module and compensate for the absence of Prdm1. We analyzed CD8⁺ TILs
143 from Prdm1 cKO mice for the expression of genes from the IL-27-driven gene signature
144 and the signature for exhausted CD8⁺ TILs (**Methods; Supplementary Information**
145 **Table 3**). We found that only a few genes were upregulated in Prdm-1 cKO CD8⁺ T cells,
146 including one TF, c-Maf (p-value < 0.05) (**Fig. 3c**). Indeed, c-Maf is induced by IL-27, is
147 co-expressed with Prdm1 in T cells upon IL-27 stimulation (**Extended Data Fig. 6a**),
148 and can regulate IL-10 expression¹⁷ and T cell exhaustion¹⁸. Additionally, many genes
149 (226 genes, p-value 5.34×10^{-5} , hypergeometric test) in the co-inhibitory gene module
150 have a binding motif and a reported binding event for c-Maf within their promoter
151 regions¹⁹.

152 CD8⁺ TILs from c-Maf cKO mice exhibited decreased expression of multiple co-
153 inhibitory receptors (**Fig. 3d**). Interestingly, Prdm1 and c-Maf each impacted co-
154 inhibitory receptor expression only partially (**Fig. 3e**). As in the Prdm1 cKO mice, c-Maf
155 cKO mice did not show any differences in tumor growth relative to controls (**Fig. 3f**).
156 Notably, Prdm1 expression in c-Maf cKO TILs was similar to that in WT TILs,

157 indicating that Prdm1 might drive expression of the co-inhibitory gene module in the
158 absence of c-Maf.

159 We addressed whether Prdm1 and c-Maf could act cooperatively to regulate co-
160 inhibitory receptor expression. We found no evidence for a physical interaction between
161 Prdm1 and c-Maf (data not shown); therefore we examined whether they shared targets.
162 We combined the network analysis for Prdm1 (**Extended Data Fig. 6e**) with c-Maf
163 ChIP-seq data¹⁹ and c-Maf targets (**Methods**). We observed 121 genes in the co-
164 inhibitory module that are affected (RNAseq) or have a direct binding event (ChIP-Seq)
165 for both Prdm1 and c-Maf (**Fig. 4a**), but that are not affected in either individual
166 knockout. This is consistent, among other possibilities, with compensatory (*e.g.*, “OR”)
167 regulation²⁰. Examination of ATACseq^{21,22} and ChIP-seq data for PD-1, Tim-3, Lag-3
168 and TIGIT shows that Prdm1 and c-Maf can bind both overlapping and non-overlapping
169 sites in the loci of these receptors and can synergistically trans-activate Tim-3 expression
170 (**Extended Data Fig. 7**).

171 Mice with a T cell specific deletion in both Prdm1 and c-Maf (Prdm1/c-Maf
172 cDKO) showed normal development of CD4⁺ and CD8⁺ T cells in terms of frequency
173 and expression of memory/activation markers, although the frequency of Foxp3⁺ Treg
174 was increased (**Extended Data Fig. 8a**). CD4⁺ and CD8⁺ TILs from cDKO mice bearing
175 B16F10 melanomas exhibited a near absence of PD-1, Tim-3, Lag-3, TIGIT, Pdpn, and
176 Procr expression (**Fig. 4b; Extended Data Fig. 8b**). Moreover, cDKO CD8⁺ TILs
177 exhibited enhanced IL-2 and TNF α production (**Extended Data Fig. 8c**). In contrast to
178 singly deficient mice, cDKO mice showed significant control of B16F10 tumor growth
179 despite the increased frequency of Treg (**Fig. 4c**). We addressed whether Prdm1 and c-

180 Maf play a cell-intrinsic role in CD8⁺ and CD4⁺ T cells in controlling tumor growth by
181 using an adoptive transfer model. Although CD8⁺ T cells from cDKO were able to inhibit
182 tumor growth with decreased expression of co-inhibitory molecules, these effects were
183 stronger when Prdm1 and c-Maf were lacking in both CD4⁺ and CD8⁺ T cells (**Fig. 4d**;
184 **Extended Data Fig. 8d**). We examined the roles of Prdm-1 and c-Maf in tumor antigen-
185 specific T cell responses using the MC38-OVA tumor model. We observed a significant
186 reduction in tumor growth in mice receiving cDKO T cells as compared to mice receiving
187 WT T cells (**Extended Data Fig. 8e**). We also observed an increase in Ova-specific T
188 cells in the tumor draining lymph nodes and in OVA-specific IFN- γ and TNF-
189 α producing CD8⁺ T cells in both the tumor infiltrate and in the periphery in mice
190 receiving DKO T cells (**Fig. 4e,f**; **Extended Data Fig. 8f**). Lastly, we observed an
191 increase in CD8⁺ Ki67⁺ T cells in the periphery of mice receiving DKO T cells (**Fig. 4f**).

192 We tested for non-additive effects between Prdm1 and c-Maf by using a binomial
193 generalized linear model to compare the effect of single knockouts to the cDKO, and
194 found that 149 out of 940 differentially expressed genes (adj. p-value<0.05, likelihood
195 ratio test and FDR correction) between WT and cDKO CD8⁺ TILs have non-additive (i.e.
196 synergistic) effects (**Extended Data Fig. 9, Methods**).

197 Examination of the transcriptional signatures of cDKO CD8⁺ TILs showed
198 significant overlap with those of CD8⁺ Tim-3⁻PD-1⁻ TILs (**Fig. 4g**; p-value = 2.8×10^{-7}
199 one-sample Kolmogorov-Smirnov test, **Extended Data Fig. 10a-c**, p-value=0.008),
200 suggesting that loss of both c-Maf and Prdm1 increases the proportion of non-exhausted
201 CD8⁺ effectors that exist normally in tumors. We scored the individual scRNA-seq
202 profiles of CD8⁺ TILs for the cDKO 940 gene signature and found that expression of the

203 cDKO gene signature and the co-inhibitory gene module signature mark mutually
204 exclusive populations of TILs (**Extended Data Fig. 10e**). The cDKO signature showed
205 significant overlap with PD-1⁺CXCR5⁺CD8⁺ T cells, which may represent precursors for
206 functional effectors in chronic LCMV infection²³ (**Extended Data Fig. 10d,e**, p-value =
207 1×10^{-13} one-sample Kolmogorov-Smirnov test). Furthermore, the IL27ra KO TILs
208 signature also showed significant overlap with this PD-1⁺CXCR5⁺CD8⁺ T cell signature
209 (p-value < 2.2×10^{-16} one-sample Kolmogorov-Smirnov test, **Extended Data Fig. 10e**;
210 **Fig. 2a**). Collectively, our data indicate that loss of c-Maf and Prdm1 preferentially
211 results in loss of the co-inhibitory gene module expression and acquisition of a more
212 responsive effector T cell state.

213 In conclusion, we identified a co-inhibitory gene module, which is expressed in
214 multiple settings of both CD4⁺ and CD8⁺ T cell non-responsiveness, along with its
215 transcriptional regulators. The discovery of this module provides a basis for the
216 identification of novel co-inhibitory and co-stimulatory receptors that may play an
217 important role in T cell regulation.

218

219 **Acknowledgements**

220 We thank Mary Collins for insightful discussions, Deneen Kozoriz, Junrong Xia, and
221 Zoujia Chen for technical advice, Samantha Riesenfeld for computational advice, Nicole
222 Paul and Josh Keegan for CyTOF, and Leslie Gaffney for artwork. This work was
223 supported by grants from the National Institutes of Health, the American Cancer Society,
224 the Melanoma Research Alliance, the Klarman Cell Observatory at the Broad Institute,
225 and the Howard Hughes Medical Institute.

226

227 **Author Contributions**

228 N.C., A.M., P.R.B., A.C.A., O.R.R., A.R. and V.K.K. designed the experiment; N.C.,
229 A.M., S.K., J.N., C.B., P.R.B., J.D.B, and A.R. developed analytical tools; N.C., A.M.,
230 T.K., N.A, J.N., N.D.M., M.S.K., C.W., H.Z., T.L., Y.E. and P.R.B. performed
231 experiments; A.M. and M.S. performed computational analysis. N.C. and A.M. wrote the
232 original draft of the paper and P.R.B., A.C.A, A.R. and V.K.K. reviewed and edited the
233 paper; A.C.A., A.R., and V.K.K. supervised the project.

234

235 **Conflict of Interest**

236 A.C.A. is a member of the SAB for Potenza Therapeutics and Tizona Therapeutics.
237 V.K.K. has an ownership interest and is a member of the SAB for Potenza Therapeutics
238 and Tizona Therapeutics. A.C.A.'s and V.K.K.'s interests were reviewed and managed by
239 the Brigham and Women's Hospital and Partners Healthcare in accordance with their
240 conflict of interest policies. A.R. is an SAB member for Thermo Fisher and Syros
241 Pharmaceuticals and is a consultant for Driver Group.

242

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- 313

314 **Figure Legends**315 **Figure 1. Multiple co-inhibitory receptors are expressed as a module on CD4⁺ and**
316 **CD8⁺ T cells**

317 **a)** CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes (TILs) were harvested from WT mice
318 bearing B16F10 melanoma tumors. Top panels, co-expression analysis of co-inhibitory
319 and co-stimulatory receptor mRNA expression as determined by single-cell RNA-seq for
320 316 CD4⁺ and 588 CD8⁺ TILs. Bottom panels, protein expression by CyTOF for 23,656
321 CD4⁺ and 36,486 CD8⁺ TILs. Spearman correlation, followed by dendrogram ordering of
322 the matrix using Euclidian distance is shown. Data are from biologically independent
323 experiments. **b)** TILs from WT mice bearing B16F10 melanoma were analyzed using
324 CyTOF with a custom panel of antibodies against co-inhibitory and co-stimulatory cell
325 surface receptors^{2,24} (**Supplementary Information Table 1**). Data were analyzed using
326 vi-SNE. Polygons indicating clusters 1, 2 (in CD8⁺ T cells), 3 and 4 (in CD4⁺ T cells) are
327 shown. Individual panels show expression of the indicated markers. **c)** Naïve T cells from
328 either wild type (WT) or IL-27ra deficient (IL27ra KO) mice were stimulated with anti-
329 CD3/CD28 in the presence or absence of IL-27. Indicated co-inhibitory receptors
330 expression was examined by real-time PCR (qPCR) at 96hr (CD4) and 72hr (CD8). Data
331 are from biologically independent animals. mean \pm s.e.m is shown. **d)** vi-SNE plot
332 showing WT (red) and IL27ra KO (blue) cells. **e)** ScRNA-seq of TILs from mice bearing
333 B16F10 melanoma. Data were analyzed using t-SNE. Polygons indicating cluster 4 (in
334 CD4⁺ T cells, orange) and cluster 5 (in CD8⁺ T cells, blue) are shown. Individual panels
335 show expression of the indicated markers. Bar graphs show the mean signal intensity for
336 indicated co-inhibitory receptors from WT (CD4⁺ (n=849); CD8⁺ (n=1752)) and IL27ra

337 KO (CD4⁺ (n=628); CD8⁺ (n=541)) TILs for CyTOF (d) or WT (CD4⁺ (n=707); CD8⁺
 338 (n=825)) and IL27ra KO (CD4⁺ (n=376); CD8⁺ (n=394)) TILs for ScRNA-seq (e). Error
 339 bars indicate s.e.m. and *p < 0.05, **p < 0.01, ***p < 0.001; two-sided t-test.

340

341 **Figure 2. The IL-27-induced gene program overlaps with multiple signatures of T**
 342 **cell dysfunction and tolerance**

343 **a)** Panels I-VI, tSNE plots of the 588 CD8⁺ single-cell TILs (dots) harvested from WT
 344 mice bearing B16F10 melanoma. Cells are colored in each panel by their signature score
 345 that reflects the relative average expression of the genes in the overlap of the IL-27-
 346 induced gene program with the signatures for each of the indicated states of T cell non-
 347 responsiveness. Panel VI is a projection of a signature of the differentially expressed
 348 genes between CD8⁺ TILs from WT and IL27ra KO mice bearing B16 melanoma
 349 (**Methods**). The contour marks the region of highly scored cells based on cells with
 350 signature scores above the mean. **b)** Graphical representation of the overlap of 57 IL-27-
 351 induced cell surface receptors or cytokine genes with genes expressed in different states
 352 of T cell non-responsiveness. The width of the gray bars reflects the extent of overlap
 353 across states. Significance of the overlap genes between the IL-27 induced and each
 354 state of T cell non-responsiveness state were calculated using Wilcox GST and
 355 camera. **c)** Graphical representation of the selected overlap genes between the cancer
 356 exhaustion and the chronic viral exhaustion signatures. The shaded background reflects
 357 the ranking based on the extent of overlap with the T cell states depicted. **d)** WT (n=8)
 358 mice and Procr^{d/d} (n=7) or **e)** WT (n=5) and Pdpn cKO (n=5) mice were implanted with
 359 B16F10 melanoma. Data are from 3 biologically independent experiments. Mean tumor

360 size \pm s.e.m is shown. ****P<0.0001, repeated measures ANOVA, Sidak's multiple
 361 comparisons test. e and h) Summary of flow cytometry data for cytokine production in
 362 the indicated CD8⁺ TILs. f and i) Left panels, representative flow cytometry data for
 363 Tim-3 and PD-1 expression on the indicated CD8⁺ TILs. Right panels, summary data. e-i)
 364 *p < 0.05; **p < 0.01; ***p < 0.001, two-sided t-test.

365

366 **Figure 3. Prdm1 and c-Maf individually regulate co-inhibitory receptors on T cells**

367 a) Summary data of co-inhibitory receptor expression on CD8⁺ TILs from WT and
 368 Prdm1 cKO mice bearing B16F10 melanoma. Data are from biologically independent
 369 animals. mean \pm s.e.m is shown. *p<0.05, ***p<0.001, two-sided t-test. b) WT (n=5) and
 370 Prdm1 cKO (n=5) mice were implanted with B16F10 melanoma. Mean tumor size \pm
 371 s.e.m. is shown. Data are from 3 biologically independent experiments. c) Left panel,
 372 gene expression in CD8⁺ TILs from WT and Prdm1 cKO mice bearing B16F10
 373 melanoma was analyzed by n-counter code-set (**Supplementary Information Table 3**).
 374 Differentially expressed genes are shown as a heatmap. Right panel, expression of c-Maf
 375 in CD8⁺ TILs from WT and Prdm1 cKO mice as determined by qPCR. Data are from
 376 biologically independent animals. mean \pm s.e.m is shown. p = 0.03, two-sided t-test. d)
 377 Summary data of co-inhibitory receptor expression on CD8⁺ TILs from WT and c-Maf
 378 cKO. Data are from biologically independent animals. mean \pm s.e.m is shown. *p < 0.05,
 379 two-sided t-test. e) Frequency of co-inhibitory receptor expression of Prdm1 cKO (gray
 380 bar) and c-Maf cKO (open bar) CD8⁺ TILs relative to WT (filled bar). Data are from 3a
 381 and 3d, mean \pm s.e.m is shown. f) Left panel, WT (n=8) and c-Maf cKO (n=5) mice were
 382 implanted with B16F10 melanoma. Mean tumor size \pm s.e.m is shown. Data are from two

383 biologically independent experiments. Right panel, expression of Prdm1 in CD8⁺ TILs
384 from WT and c-Maf cKO mice as determined by qPCR.

385

386 **Figure 4. Prdm1 and c-Maf together regulate a co-inhibitory gene module that**
387 **determines anti-tumor immunity**

388 **a)** Network model based on coupling RNAseq gene expression data of naïve CD8⁺ T
389 cells from Prdm1 cKO or c-Maf cKO mice stimulated in the presence of IL-27 and
390 Prdm1 and c-Maf ChIPseq data. Up-regulated genes (green arrows), down-regulated
391 genes (red arrows), and c-Maf or Prdm1 binding events (gray arrows) are shown. **b)**
392 Summary data of indicated co-inhibitory receptors expression on CD8⁺ TILs from WT
393 and Prdm1/c-Maf cDKO bearing B16F10 melanoma. Data are from biologically
394 independent animals. mean \pm s.e.m is shown. **p < 0.01; ***p < 0.001, two-sided t-test.

395 **c)** WT (n=15) and cDKO (n=8) mice were implanted with B16F10 melanoma. Data
396 shown are from 3 biologically independent experiments. **d)** CD4⁺ or CD8⁺ T cells sorted
397 from cDKO mice or littermate controls were transferred into Rag1 KO mice at a 2:1
398 CD4:CD8 ratio followed by subcutaneous injection of B16-OVA (n=5, each condition).

399 Data are representative of 3 biologically independent experiments. c-d) Mean tumor size
400 \pm s.e.m is shown. *P<0.05, **P<0.01, ****P<0.0001, repeated measures ANOVA,

401 Sidak's multiple comparisons test. **e-f)** T cells were harvested from Rag1 KO mice that
402 received an adoptive transfer of CD4⁺ and CD8⁺ T cells from WT or cDKO mice (2:1
403 CD4:CD8 ratio) followed by subcutaneous injection of MC38-OVA (**Extended Data**

404 **Fig. 8e**). **e)** The frequency of IFN- γ and TNF- α CD8⁺ TILs after OVA-peptide
405 stimulation, **f)** the frequency and expression of Ki67⁺ cells on splenocytes (upper panel),

406 and the frequency of IFN- γ and TNF- α CD8⁺ splenocytes (lower panel) after OVA-
407 peptide stimulation. mean \pm s.e.m is shown. Data are from biologically independent
408 animals. *P<0.05, **P<0.01, two-sided t-test. **g)** 940 differentially expressed genes
409 between CD8⁺ TILs from WT and cDKO bearing B16F10 melanoma. (adj. P. value<0.05,
410 likelihood ratio test and FDR correction) (top panel) and their corresponding expression
411 pattern in PD-1⁺Tim-3⁺ CD8⁺, PD-1⁺Tim-3⁻ CD8⁺, and PD-1⁻Tim-3⁻ CD8⁺ TILs.

412

413

414 **Methods**415 **Mice**

416 C57BL/6 wild-type (WT), IL27ra KO, and Prdm1 fl/fl mice were obtained from the
417 Jackson Laboratory (Bar Harbor, ME). c-Maf fl/fl, Pdpn fl/fl mice and Procr delta/delta
418 mice were previously described^{13,15,26}. Pdpn fl/fl mice were initially obtained from
419 Christopher Buckley (University of Birmingham, Birmingham, UK) and crossed to
420 CD4Cre mice to obtain conditional deletion in T cell. CD4Cre mice were purchased from
421 Taconic (Hudson, NY). Prdm1 fl/fl and c-Maf fl/fl mice were crossed to CD4Cre mice to
422 generate doubly deficient T cell conditional knockout mice. All experiments were
423 performed in accordance to the guidelines outlined by the Harvard Medical Area
424 Standing Committee on Animals (Boston, MA).

425

426 **Tumor Experiments**

427 5×10^5 B16F10 melanoma cells (ATCC) were implanted into the right flank of C57BL/6
428 mice. Tumor size was measured in two dimensions using a caliper. TILs were isolated by
429 dissociating tumor tissue in the presence of 2.5 mg/ml collagenase D for 20 min before
430 centrifugation on a discontinuous Percoll gradient (GE Healthcare). Isolated cells were
431 then used in various assays of T cell function. For antigen specific analysis, we applied
432 adoptive transfer tumor experiments using T cells from Prdm1/c-Maf cDKO mice, CD4⁺
433 or CD8⁺ T cells sorted from cDKO mice or littermate controls were transferred into Rag1
434 KO mice at a 2:1 ratio (CD4: 1 million/mouse and CD8: 0.5 million/mouse) 2 days
435 before subcutaneous injection of B16-OVA or MC38-OVA tumor. B16-Ova was kind gift
436 from Kai Wucherpfennig (Dana-Farber Cancer Institute, Boston, MA) and MC38-Ova

437 was kind gift from Mark Smyth (QIMR Berghofer, Queensland Institute of Medical
438 Research, Brisbane Australia). For adoptive transfer tumor experiments using T cells
439 from Procr^{d/d} mice, CD4⁺ T cells from WT and CD8⁺ T cells from WT or Procr^{d/d} mice
440 were isolated by cell sorting (BD FACS Aria) and transferred into Rag deficient recipient
441 mice at a 2:1 ratio (WT CD4⁺: 1 million/mouse and WT or Procr^{d/d} CD8⁺: 0.5
442 million/mouse) 2 days before tumor implant. Although we did not blinding or
443 randomization, at least 5 animals of target gene knock out and control mice were used to
444 adequately power biological validation experiments throughout the article. All mice used
445 are C57BL/6 background, both male and female, 6-12 weeks of age, 15-25g. Each
446 experiment was performed using age, sex matched controls (**Supplementary**
447 **Information Table 5**).

448

449 **CyTOF**

450 Antibodies were labeled using MaxPar® Metal Labeling Kits (DVS) by The Longwood
451 Medical Area CyTOF Antibody Resource and Core. In some experiments, TILs were
452 enriched using Dynabeads FlowComp Mouse Pan T (CD90.2) Kit (Invitrogen). Cells
453 were washed and resuspended in CyTOF PBS (PBS + 0.05% sodium azide + 0.5% BSA)
454 and stained viability marker Rhodium (DVS) following the cocktail of antibodies against
455 cell-surface molecules for 30 min. Cells were washed again and resuspended in CyTOF
456 PBS with 4% paraformaldehyde. After 10 min fixation, cells were washed and barcoded
457 with Cell-ID intercalators (DVS). Before analysis, cells were resuspended in water with
458 beads and loaded to the CyTOF® Mass Cytometer (DVS). CyTOF data were recorded in
459 dual-count according to Fluidigm's recommended settings that calibrated on the fly,

460 combining pulse-count and intensity information. Data obtained as mass peaks for the
461 channels are processed according to cell event selection criteria. These criteria include
462 cell viability selection (Pt195), single-cell selection (Intercalator-Ir), and barcoding
463 selection (Pt194 and Pt198) to identify single-cell events from WT TILs and KO TILs for
464 further analysis.

465 To obtain clusters of cells similar in their protein expression patterns, cells were
466 clustered using k-means algorithm. Optimal cluster number was estimated using the
467 within groups sum of squared error (SSE) plot followed by gap statistics with
468 bootstrapping and first SE max method. These methods suggested 9 clusters as optimal in
469 the multidimensional space. Applying k-means clustering with (k=9) on our CyTOF data,
470 resulted in clear distinction between cluster 1 and 2 of the CD8⁺ TILs and cluster 3 and 4
471 of the CD4⁺ TILs. This separation could be further visualized by two-dimensional non-
472 linear embedding of the protein expression profiles using t-stochastic neighborhood
473 embedding (t-SNE⁴). The t-SNE plot can then be overlaid by k-means clustering results
474 to reflect a non-biased approach to the clusters or with intensity of the different markers.

475

476 **Flow Cytometry**

477 Single cell suspensions were stained with antibodies against CD4 (RM4-5), CD8 (53-
478 6.7), PD-1 (RMP1-30), Lag-3 (C9B7W), TIGIT (GIGD7), and Tim-3 (5D12), Procr
479 (eBio1560), and Pdpn (8.1.1.) were obtained from BioLegend (San Diego, CA). Fixable
480 viability dye eF506 (eBioscience) was used to exclude dead cells. For intra-cytoplasmic
481 cytokine staining, cells were stimulated with (PMA) (50ng/ml, Sigma-Aldrich, MO),
482 ionomycin (1µg/ml, Sigma-Aldrich, MO). Permeabilized cells were then stained with

483 antibodies against IL-2, TNF- α , IFN- γ or IL-10. All data were collected on a BD LSR II
484 (BD Biosciences) and analyzed with FlowJo software (Tree Star).

485

486 ***In vitro* T cell differentiation**

487 CD4⁺ and CD8⁺ T cells were purified from spleen and lymph nodes using anti-CD4
488 microbeads and anti-CD8a microbeads (Miltenyi Biotech) then stained in PBS with 0.5%
489 BSA for 15 min on ice with anti-CD4, anti-CD8, anti-CD62L, and anti-CD44 antibodies
490 (all from Biolegend, CA). Naïve CD4⁺ or CD8⁺ CD62L^{high}CD44^{low} T cells were sorted
491 using the BD FACSAria cell sorter. Sorted cells were activated with plate bound anti-
492 CD3 (2 μ g/ml for CD4 and 1 μ g/ml for CD8) and anti-CD28 (2 μ g/ml) in the presence of
493 rmIL-27 (25ng/ml) (eBioscience). Cells were harvested at various time points for RNA,
494 intracellular cytokine staining, and flow cytometry.

495

496 **Real-time PCR**

497 Total RNA was extracted using RNeasy columns (Qiagen). Reverse transcription of
498 mRNA was performed in a thermal cycler (Bio-Rad) using iScript™ cDNA Synthesis Kit
499 (Bio-Rad). Real-time PCR was performed in the Vii7™ Real-Time PCR system (Applied
500 Biosystems) using the primers for Taqman gene expression (Applied Biosystems). Data
501 was normalized to the expression of ACTB.

502

503 **Nanostring RNA analysis**

504 **Expression profiling of TILs.** We analyzed gene expression in CD8⁺ TILs from Prdm1
505 or c-Maf cKO mice bearing B16F10 melanoma collected on day 14 after tumor

506 implantation, using a custom nanostring code-set of 397 genes representing both the IL-
507 27-driven gene signature (245 genes) and the dysfunctional CD8⁺ TIL gene signature
508 (245 genes) (**Supplementary Information Table 3**). Expression values were normalized
509 by first adjusting each sample based on its relative value to all samples. This was
510 followed by subtracting the calculated background (mean.2sd) from each sample with
511 additional normalization by housekeeping geometric mean, where housekeeping genes
512 were defined as: Hprt, Gapdh, Actin and Tubb5. Differentially expressed genes were
513 defined using the function that fits multiple linear models from the Bioconductor package
514 limma in R²⁷ with p-value<0.05.

515

516 **Microarray processing and analysis**

517 Naïve CD4⁺ and CD8⁺ T cells were isolated from WT or IL27ra KO mice, and
518 differentiated *in vitro* with or without IL-27. Cells were collected at 72 hours for CD8⁺
519 and 96 hours for CD4⁺ and Affymetrix GeneChip Mouse Genome 430 2.0 Arrays were
520 used to measure the resulting mRNA levels at these time points. Individual .CEL files
521 were RMA normalized and merged to an expression matrix using the
522 ExpressionFileCreator of GenePattern with default parameters²⁸. Gene-specific intensities
523 were then computed by taking for each gene j and sample i the maximal probe value
524 observed for that gene. Samples were then transferred to log-space by taking
525 $\log_2(\text{intensity})$.

526 Differentially expressed genes were annotated as genes with FDR-corrected
527 ANOVA <0.05 computed between the CD4 with or without IL-27 stimulation (CD4⁺
528 IL27 and Th0) subpopulations (1,202 genes). 468 genes were differentially expressed

529 between WT CD8⁺ T cells stimulated in the presence or absence of IL-27 (p-value<0.05).
530 234 genes were shared between these two differentially expressed gene lists (p-value =
531 2.25×10^{-157} , hypergeometric test, background=16,618 (union of genes expressed)). A list
532 of 972 cell surface/cytokines genes of interest that include: cytokines, adhesion,
533 aggregation, chemotaxis and other cell surface molecules (**Supplementary Information**
534 **Table 4**) composed using GO annotation in Biomart was used to generate the gene subset
535 in **Fig. 2b** and **c**.

536

537

538 **RNAseq gene expression profiling of tumor infiltrating cells**

539 Tumor infiltrating CD8⁺ T cells were isolated from WT, IL27ra KO, Prdm1 cKO, c-Maf
540 cKO, and Prdm1/c-Maf cDKO tumor bearing mice via FACS sorting on a FACSAria (BD
541 Biosciences). Tumor infiltrating CD8⁺ T cells were processed using an adaptation of the
542 SMART-Seq 2 protocol²⁹, using 5uL of lysate from bulk CD8⁺ T cells as the input for
543 each sample during RNA cleanup via SPRI beads (~2,000 cells lysed on average in RLT).

544 RNA-seq reads were aligned using Tophat³⁰ (mm9) and RSEM-based
545 quantification³¹ using known transcripts (mm9), followed by further processing using the
546 Bioconductor package DESeq in R³². The data was normalized using TMM
547 normalization. The TMM method estimates scale factors between samples that can be
548 incorporated into currently used statistical methods for DE analysis. Post-processing and
549 statistical analysis was carried out in R³¹. Differentially expressed genes were defined
550 using the differential expression pipeline on the raw counts with a single call to the

551 function DESeq (adjusted p value<0.1). Heatmap figures were generated using pheatmap
552 package³³.

553

554 **Single-cell RNA-seq**

555 CD4⁺ and CD8⁺ TILs from WT or IL27ra KO mice bearing B16 melanomas were sorted
556 into 96-well plates with 5 µl lysis buffer comprised of Buffer TCL (Qiagen) plus 1% 2-
557 mercaptoethanol (Sigma). Plates were then spun down for one minute at 3000rpm and
558 immediately frozen at -80°C. Cells were thawed and RNA was isolated with 2.2x
559 RNAClean SPRI beads (Beckman Coulter Genomics) without final elution³⁴. The beads
560 were then air-dried and processed immediately for cDNA synthesis. Samples were then
561 processed using the Smart-seq2 protocol³⁵, with minor modifications applied to the
562 reverse transcription (RT) step (MSK and AR., in preparation). This was followed by
563 making a 25µl reaction mix for each PCR and performing 21 cycles for cDNA
564 amplification. Then 0.25 ng cDNA from each cell and ¼ of the standard Illumina
565 NexteraXT reaction volume were used in both the tagmentation and final PCR
566 amplification steps. Finally, libraries were pooled and sequenced (50 x 25 paired-end
567 reads) using a single kit on the NextSeq500 5 instrument. All CD4⁺ TILs (WT and IL27ra
568 KO) single-cell RNA-seq data was generated as part of this study. CD8⁺ TILs single-cell
569 data includes WT CD8⁺ TILs data from³ and WT and IL27ra KO CD8⁺ single-cell data
570 generated as part of this study.

571

572 **Single-cell RNA-seq data preprocessing and expression**

573 Initial preprocessing was performed as described in³. Briefly, paired reads were mapped
574 to mouse annotation mm10 using Bowtie³⁶ (allowing a maximum of one mismatch in
575 seed alignment, and suppressing reads that had more than 10 valid alignments) and TPMs
576 were computed using RSEM³¹, and $\log_2(\text{TPM}+1)$ values were used for subsequent
577 analyses.

578 Next, we filtered out low quality cells and cell doublets, maintaining for
579 subsequent analysis the cells that had (1) 1,000-4,000 detected genes (defined by at least
580 one mapped read), (2) at least 200,000 reads mapped to the transcriptome, and (3) at least
581 50% of the reads mapped to the transcriptome, ending with a total of 707 CD4⁺ and 825
582 CD8⁺ WT TILs and 376 CD4⁺ and 394 CD8⁺ IL27ra KO TILs. We restricted the genes
583 considered in subsequent analyses to be the genes expressed at $\log_2(\text{TPM}+1) \geq 2$ in at
584 least twenty percent of the cells.

585 After removal of low quality cells the data was normalized using quantile
586 normalization followed by PCA analysis. PCs 1-10 were chosen for subsequent analysis
587 due to a drop in the proportion of variance explained following PC10. We used tSNE⁴ to
588 visualize single-cells in a two-dimensional non-linear embedding.

589

590 **Single-cell RNA-seq clustering and differential expression analysis**

591 For the coupled dataset of WT and IL27ra KO TILs we followed the analysis described in
592 ³⁷. We performed batch correction using ComBat³⁸ and the batch-corrected expression
593 matrix was then reduced using PCA, PCs 1-13 were chosen for subsequent analysis due
594 to a drop in the proportion of variance explained following PC13. Next, we cluster the
595 cells based on their PC scores using the Louvain-Jaccard method using 40 nearest

596 neighbors, and the 13 PCs^{25,39}; 11 clusters were detected. We then compared the
597 composition of each cluster in terms of total number and percentage of WT and IL27ra
598 KO cells and found cluster 5 to be enriched for WT CD8 TILs cells (p-value= 0.0357,
599 one sample t-test, **Extended Data Fig. 3c,d**). Projecting the IL-27 co-inhibitory gene
600 module onto the single-cell RNA-seq data highlighted clusters 4 and 5 (CD4 and CD8
601 respectively) (**Extended Data Fig. 3e**), further showing that in addition to the decrease in
602 the expression of the co-inhibitory receptors: PD-1, Tim-3, Lag-3 and TIGIT (**Fig. 1e**), a
603 significant decrease in the total IL-27 co-inhibitory gene module signature score is
604 observed with lack of IL-27 signaling (p-value=0.01, t-test, **Extended Data Fig. 3f**).
605 Last, we searched for differentially expressed genes between clusters 4/5 and the
606 rest of the clusters using a nonparametric binomial test³⁷.

607

608 **Signature analysis of other states of T cell non-responsiveness**

609 Given that orthogonal approaches were used to generate the various signatures, we first
610 addressed the robustness of each signature prior to the comparative analysis. First, to
611 address some of the concerns regarding the definition of these signatures we sub-sampled
612 the genes in each of the signatures and observed the resulting changes by projection on
613 the single-cell data. These changes were quantified by randomly selecting decreasing
614 subsets of genes from each signature (100%, 90% ... 30%) and calculating the average
615 silhouette width of the cells that scored high for the different generated signatures, based
616 on Euclidian distance between the principal component values used to generate the tSNE
617 plot. This analysis shows that the signatures are relatively resilient to this procedure up
618 to 60% of the original signature (**Extended Data Fig. 4e**).

619 Second, we calculated a signature p-value per cell. The p-value is calculated by
620 generating random sets of signatures that are composed of genes with a similar average
621 and variance expression levels as the original signature. This was followed by comparing
622 the generated scores to the score obtained from the original signature. Cells that had a
623 statistically significant score (adjusted p-value<0.05) were marked by '+' (**Extended**
624 **Data Fig. 4f**).

625 For viral exhaustion: Microarray dataset¹⁰ was downloaded, followed by RMA. A
626 signature of viral exhaustion was defined as the genes that are differentially expressed
627 between chronic and acute viral infection on day 15 and day 30. Genes were ranked
628 based on a *t*-test statistic and fold change, each gene rank was then adjusted for multiple
629 hypotheses testing using false discovery rate (FDR). A threshold of fold change>1.1 and
630 FDR<0.2 was applied.

631 For antigen-specific tolerance: Data¹¹ were downloaded. Two groups were
632 defined, group 1 that includes the PBS and 0.008 µg treated samples (treatment number
633 1) versus group 2 - 80 µg (treatment number 5 and 6). After Log2 transformation and
634 quantile normalization, the Limma package was used to estimate the fold changes and
635 standard errors by fitting a linear model for each gene for the assessment of differential
636 expression. Genes with p value < 0.05 were selected: 1,845 genes were upregulated of
637 which 88 were defined as cytokine and cell surface molecules^{27,40,41}.

638 For antigen non-specific tolerance: Data¹² was downloaded. Robust Multi-array
639 Average (RMA) and quantile normalization were applied for background correction and
640 normalization using the ExpressionFileCreator module of GenePatterns. Differentially
641 expressed genes were defined using signal-to-noise ratio (SNR), following FDR

642 correction. Differentially expressed genes were identified as genes having a FDR<0.2
643 between mRNA expression profiles of naïve CD4⁺ or CD4⁺ GFP/IL-10⁺ T cells isolated
644 from the spleen or cLNs of B6NOD.F1^{IL10:GFP} mice following nasal treatment with anti-
645 CD3 which attenuates the of progressive phase of EAE.

646 For cancer: Data³ was obtained. Briefly, mRNA samples from CD8⁺Tim-3⁻PD-1⁻
647 (DN) TILs, CD8⁺Tim-3⁻PD-1⁺(SP), and CD8⁺Tim-3⁺PD-1⁺ (DP) TILs were measured
648 using Affimetrix GeneChip Mouse Genome 430 2.0 Arrays, expression values were RMA
649 normalized, corrected for batch effects using ComBat³⁸ and gene-specific intensities were
650 then computed by using the maximal prob intensity per gene, values were transferred to
651 log-space by taking log₂(intensity). Differentially expressed genes were defined as genes
652 with either an FDR-corrected t-test p-value smaller or equal to 0.2 computed between the
653 DN and DP subpopulations and a fold-change of at least 1.5 between the two
654 subpopulations.

655 The IL-27 co-inhibitory gene module was defined as a union of the overlap
656 between the IL-27-driven gene program (1,201 genes see **Methods**: Microarray
657 processing and analysis) and each of the four different states of T cell non-responsiveness
658 mentioned above (272 genes, **Supplementary Information Table 2**).

659 For IL27ra KO signature: mRNA samples from FACS sorted CD8⁺ TILs from
660 WT and IL27ra KO mice bearing B16 melanomas were measured an adaptation of the
661 SMART-Seq 2 protocol²⁹ (see **Method: RNA expression profiling of tumor infiltrating**
662 **cells**). Differentially expressed genes were defined as genes with either an FDR-corrected
663 t-test p-value smaller or equal to 0.2 computed between the WT and IL27ra KO and a
664 fold-change of at least 1.5 between the two subpopulations. IL27ra KO signature was

665 defined as 929 differentially expressed genes in IL27ra KO CD8⁺ TILs compared to WT
666 CD8⁺ TILs.

667

668 **Single-cell gene signature computation**

669 As an initial step, the data was scaled (z-score across each gene) to remove bias towards
670 highly expressed genes. Given a gene signature (list of genes), a cell-specific signature
671 score was computed by first sorting the normalized scaled gene expression values for
672 each cell followed by summing up the indices (ranks) of the signature genes. For gene-
673 signatures consisting of an upregulated and downregulated set of genes, two ranking
674 scores were obtained separately, and the down-regulated associated signature score was
675 subtracted from the up-regulated generated signature score. A contour plot was added on
676 top of the tSNE space, which takes into account only those cells that have a signature
677 score above the mean to further emphasis the region of highly scored cells.

678

679 **Network construction**

680 Networks were generated using Cytoscape version 3.2.1⁴². The network model is based
681 on coupling *in vitro* RNAseq gene expression data of naïve CD8⁺ T cells from KO
682 (Prdm1 or c-Maf) and WT controls stimulated in the presence of IL-27 and previously
683 published ChIP-seq data for c-Maf and predicted Prdm1 binding sites by motif scan.
684 More specifically, differentially expressed genes between WT control and KO were
685 defined using the function that fits multiple linear models from the Bioconductor package
686 limma in R²⁷ with FDR<0.05. We used published c-Maf ChIP-seq data¹⁹ and Prdm1
687 ChIP-seq data¹⁶. In addition, potential Prdm1 binding sites were detected using FIMO

688 (MEME suite - <http://meme-suite.org/doc/fimo.html>). Association to gene promoters was
 689 based on the following thresholds (upstream=5000, downstream=500 of TSS) and the
 690 overlap with the co-inhibitory module was found to be significant (p-value= 0.009 hyper
 691 geometric, background of 20,000 genes). In the network presentation, we visualize all the
 692 genes that are part of the IL-27 inhibitory module (**Extended Data Fig. 6e** and **Fig. 4a**).

693

694 **Data availability**

695 Sequence data that support the findings of this study have been deposited in GEO
 696 with the accession codes XXX

697

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- 746
- 747 **Extended Data Figure Legends**

748

749 **Extended Data Figure 1. CyTOF analysis of co-inhibitory and co-stimulatory**
 750 **receptor co-expression in TILs. a)** TILs were harvested from B16F10 melanoma tumor-
 751 bearing WT and IL27ra KO mice from Fig. 1b and analyzed using CyTOF (5000 cells
 752 from each). CyTOF data were analyzed using vi-SNE. Applying k-means clustering with
 753 (k=9) on the CyTOF data resulted in clear distinction between clusters 1, 2, 3 and 4.
 754 Polygons indicating clusters 1, 2 (in CD8⁺ T cells), 3 and 4 (in CD4⁺ T cells) are shown.
 755 Individual panels show expression of the indicated markers. **b)** Pie charts show the
 756 distribution of WT or IL27ra KO CD8⁺ and CD4⁺ TILs in clusters 1 and 2 (C1 and C2) of
 757 CD8⁺ TILs and clusters 3 and 4 (C3 and C4) of CD4⁺ TILs as defined in **Fig. 1d. c)**

758 Independent data of WT and IL27ra KO TILs samples from that shown in **Fig. 1** (5000
759 cells from each). Applying k-means clustering with (k=7) on the CyTOF data resulted in
760 clear distinction between clusters 1, 2, 3 and 4. Polygons indicating clusters 1, 2 (in CD8⁺
761 T cells), 3 and 4 (in CD4⁺ T cells) are shown. **d)** vi-SNE plot highlighting the distribution
762 of cells from WT (blue) and IL27ra KO (red) in CD8⁺ TILs clusters 1 and 2 and CD4⁺
763 TILs clusters 3 and 4. Pie charts show the distribution of WT or IL27ra KO CD8⁺ and
764 CD4⁺ TILs in each cluster.

765

766 **Extended Data Figure 2. IL-27 induces multiple co-inhibitory receptors on CD4⁺**
767 **and CD8⁺ T cells.**

768 **a)** Naïve T cells from WT or IL27ra KO mice were stimulated *in vitro* with anti-
769 CD3/CD28 in the presence or absence of IL-27. Expression of co-inhibitory receptors
770 was determined by flow cytometry. Representative data of 3 biologically independent
771 experiments are shown. **b)** Expression of PD-1, Tim-3, Lag-3, TIGIT, and IL-10 on CD8⁺
772 TILs obtained from WT and IL27ra KO mice bearing B16F10 melanoma was determined
773 by flow cytometry. Thy1.1-IL-10 reporter mice crossed with WT and IL27ra KO mice
774 were used for IL-10 expression analysis. Representative data of 3 biologically
775 independent experiments are shown.

776

777 **Extended Data Figure 3. Single-cell RNA-seq expression analysis of WT and IL27ra**
778 **KO TILs.**

779 **a)** TILs were harvested from B16F10 melanoma tumor-bearing WT (707 and 825 for
780 CD4⁺ and CD8⁺ respectively) and IL27ra KO (376 and 394 for CD4⁺ and CD8⁺

781 respectively) mice as in **Fig. 1e**. t-SNE plot shows the presence of WT and IL27ra KO
782 CD4⁺ and CD8⁺ TILs as indicated. **b**) Clustering using the Louvain-Jaccard method (40
783 nearest neighbors and 13 principal components²⁵). **c**) The composition of each cluster in
784 terms of total number (c) and percentage (d) of WT (red) and IL27ra KO (blue) cells. P-
785 values (*p-value<0.05, **p-value<0.01, ***p-value<0.001) were calculated using one
786 sample t-test. **e**) Projection of the IL-27 co-inhibitory module signature on the single-cell
787 RNA-seq data. The contour plot marks the region of highly expressing cells by taking
788 into account only those cells that have an expression value above the mean. **f**) Violin and
789 box plots displaying the distribution of the IL-27 co-inhibitory module signature score
790 compared between WT (72 and 98 for CD4⁺ and CD8⁺ respectively) and IL27ra KO (85
791 and 77 for CD4⁺ and CD8⁺ respectively) cells in clusters 4 and 5 (CD4⁺ and CD8⁺
792 respectively, *p-value=0.01, one-sided t-test. The lower and upper hinges in the boxplot
793 correspond to the first and third quartiles and the horizontal line corresponds to the
794 median).

795

796 **Extended Data Figure 4. Overlap of the IL-27-induced gene program with**
797 **signatures from four states of T cell impairment/tolerance/dysfunction.**

798 **a**) Pearson correlation between WT CD4⁺ and CD8⁺ T cells for the 1,201 genes that were
799 differentially expressed between WT CD4⁺ T cells stimulated in the presence or absence
800 of IL-27 (FDR<0.05). **b**) Expression profile of 118 differentially expressed genes (from
801 (a)) encoding cell surface receptors and cytokines are shown as a heatmap. **c**) The IL-27-
802 induced gene program (1,201 genes) was compared to T cell signatures obtained from
803 four states of T cell non-responsiveness. Number of overlapping genes between the IL-27

804 gene program and each signature is depicted. P values (**p < 0.01) were determined
805 by hypergeometric test: Nasal anti-CD3 – 4.7×10^{-21} , Cancer – 1.2×10^{-33} , antigen-specific
806 tolerance – 4×10^{-14} and Viral exhaustion – 1.7×10^{-26} . **d)** p-value statistics for the
807 significance of the overlap between the IL-27-induced gene program (1,201) and genes
808 induced in other states of T cell non-responsiveness using wilcoxGST and camera. **e)**
809 Gene signatures from (c) were sub-sampled and projected onto the CD8⁺ single-cell TILs
810 data. Changes were quantified by randomly selecting decreasing subsets of genes from
811 each signature and calculating the average silhouette width of cells that scored high for
812 the different generated signatures based on Euclidian distance between the principal
813 component values used to generate the tSNE plot. The lower and upper hinges in the
814 boxplot correspond to the first and third quartiles and the horizontal line corresponds to
815 the median (**Methods**). **f)** Panels I-V, tSNE plots of the 588 CD8⁺ single-cell TILs (dots)
816 harvested from WT mice bearing B16F10 melanoma tumor. Cells are colored in each
817 panel by their signature score. The score reflects the relative average expression of the
818 genes in the overlap of the IL-27 gene signature with the signatures for each of the
819 indicated states of T cell non-responsiveness. Panel VI is a projection of a signature of the
820 differentially expressed genes between CD8⁺ TILs from WT and IL27ra KO mice bearing
821 B16 melanomas (**Methods**). The contour plot marks the region of highly scored cells by
822 taking into account only those cells that have a signature score above the mean score.
823 Cells that had a statistically significant score (adjusted p-value<0.05) were marked by ‘+’
824 (**Methods**).
825

826 Extended Data Figure 5. Characterization of the role of Pdpn and Procr in CD8⁺**827 TILs**

828 **a)** Pdpn and Procr protein and mRNA expression was determined in T cells from WT and
829 IL27ra KO stimulated with anti-CD3/CD28 in the presence or absence of IL-27. CD4⁺
830 cells were analyzed at 96hr and CD8⁺ cells at 72hr. Data are representative flow
831 cytometry and qPCR data from biologically independent animals. mean \pm s.e.m is shown.

832 **b)** Representative flow cytometry data of 3 independent experiments showing Pdpn and
833 Procr expression in PD-1⁺Tim-3⁺ CD8⁺ and PD-1⁻Tim-3⁻ CD8⁺ TILs obtained from WT
834 and IL27ra KO mice bearing B16F10 melanoma. **c)** TILs from WT mice bearing B16F10
835 melanoma were stimulated with PMA and Ionomycin. Cytokine production in Procr⁺ or
836 Procr⁻ CD8⁺ TILs is shown. Thy1.1-IL-10 reporter mice were used for IL-10 expression
837 analysis. Data are from biologically independent animals. mean \pm s.e.m is shown. *p <

838 0.05; **p < 0.01, paired t-test. **d)** 5x10⁵ CD8⁺ T cells from wild type or Procr^{d/d} mice
839 were transferred along with 1x10⁶ wild type CD4⁺ T cells to Rag1 KO mice (N=5). On
840 day 2, 5x10⁵ B16F10 cells were implanted. Mean tumor size \pm s.e.m is shown. *P<0.05,
841 repeated measures ANOVA, Sidak's multiple comparisons test. **e)** TILs were obtained

842 from WT and Pdpn cKO mice bearing B16F10 melanoma and stained for the expression
843 of IL-7Ra. Representative flow cytometry data from 3 independent animals. **f)** Summary
844 data of IL-7Ra expression are from biologically independent animals. mean \pm s.e.m is
845 shown. *p < 0.05, one-sided t-test.

846

847 Extended Data Figure 6. Prdm1 is a candidate regulator of the co-inhibitory
848 module.

849 **a)** Log2 fold change RNA levels between naïve CD4⁺ or CD8⁺ T cells simulated with or
 850 without IL-27. Data are from two independent experiments. Shown are transcription
 851 factors that are part of the IL-27 co-inhibitory module (Differentially expressed
 852 transcription factors were annotated as genes with FDR-corrected ANOVA <0.05). **b)**
 853 Transcription factors that are both in the IL-27 co-inhibitory module and are also
 854 overexpressed in clusters 4 and 5 in the single-cell data (clusters that were enriched for
 855 the IL-27 signature, **Extended data Fig. 3e,f**). Differentially expressed genes between
 856 clusters 4/5 and the rest of the clusters were determined using binomcount.test (binomial
 857 distribution, **Methods**). Log effect corresponds to log proportion of expressing cells and
 858 p-value is calculated by the probability of finding n or more cells positive for the gene in
 859 clusters 4/5 given the fraction in the rest of the clusters. **c)** tSNE plot of **Fig. 1e**. showing
 860 the expression of Prdm1 in WT (707 and 825 for CD4⁺ and CD8⁺, respectively) and
 861 IL27ra KO (376 and 394 for CD4⁺ and CD8⁺, respectively) cells. **d)** Normalized RNA
 862 expression levels of Prdm1 in PD-1⁻Tim-3⁻ (n=3) and PD-1⁺Tim-3⁺ (n=3) CD8⁺ TILs
 863 (mean ± s.e. is shown, ***p = 0.0004, two-sided t-test). **e)** Network model based on
 864 RNAseq gene expression data of naïve CD8⁺ T cells from Prdm1^{fl/fl} (WT) or
 865 CD4^{cre}Prdm1^{fl/fl} (Prdm1 cKO) mice stimulated in the presence of IL-27 and actual
 866 binding events (ChIPseq) data for Prdm1¹⁹. Green arrows designate genes up-regulated
 867 by Prdm1, red arrows designate genes down-regulated by Prdm1, and dashed gray arrows
 868 mark binding events.

869

870 **Extended Data Figure 7. Genomic tracks surrounding the co-inhibitory molecules**

871 Lag3 **(a)**, Pd-1 **(b)**, Tigit **(c)** and Tim-3 **(d)** with overlay of Chipseq data of Prdm1¹⁶ and

872 c-Maf¹⁹ and ATACseq data of naïve CD4⁺ cells induced with IL27 for 72h and ATACseq
873 data of CD8⁺ T cells 27 days following chronic viral infection²². Regions of binding sites
874 common to both Prdm1 and c-Maf are indicated by the dotted rectangles. e) Luciferase
875 activity in 293T cells transfected with pGL4.23 luciferase reporters for depicted
876 enhancers of Tim-3 together with empty vector (control), constructs encoding Prdm1, c-
877 Maf, or both. Firefly luciferase activity was measured 48h after transfection and is
878 presented relative to constitutive Renilla luciferase activity.

879

880 **Extended Data Figure 8. Immune characterization of Prdm1 cKO, cMaf cKO, and**
881 **Prdm1/c-Maf cDKO before and after tumor challenge.**

882 **a)** Analysis of steady-state immune system in WT, c-Maf cKO, Prdm1 cKO, and
883 Prdm1/c-Maf cDKO. Summary data for CD4, CD8, Foxp3, CD44, CD62L, and CD69
884 expression in spleen from WT, c-Maf cKO, Prdm1 cKO and Prdm1/c-Maf cDKO mice.
885 Data are from biologically independent animals. mean \pm s.e.m is shown. *p < 0.05; **p
886 <0.01; ****p < 0.0001, one-way ANOVA and Tukey's multiple comparisons test. **b)** co-
887 inhibitory receptor expression in CD4⁺ TILs from Prdm1/c-Maf cDKO mice. Top panels,
888 representative flow cytometry data from 3 independent experiments for TILs from WT
889 and Prdm1/c-Maf cDKO stained for PD-1, Tim-3, TIGIT, Pdpn, and Procr expression.
890 Bottom panels show summary data. Data are from biologically independent animals.
891 mean \pm s.e.m is shown *p < 0.05, two-sided t-test. **c)** Top panels, representative flow
892 cytometry data from 3 independent experiments showing cytokine production from CD8⁺
893 TILs from WT and cDKO bearing B16F10 melanoma. Bottom panels, summary data.
894 Data are from biologically independent animals. mean \pm s.e.m is shown. *p < 0.05, two-

895 sided t-test. **d)** Co-inhibitory receptor expression on CD8⁺ TILs sorted from B16-OVA-
896 bearing Rag1 KO mice that were transferred with Prdm1/c-Maf cDKO (n=4) or wild type
897 (n=4) CD4⁺ and CD8⁺ T cells as indicated. Data are from biologically independent
898 animals. mean \pm s.e.m is shown. *P<0.05, one-way ANOVA and Tukey's multiple
899 comparisons test. **e)** Rag1 KO mice were transferred with either wildtype or cDKO CD4⁺
900 and CD8⁺ (2:1 CD4:CD8 ratio) followed by subcutaneous injection of MC38-OVA. Mean
901 tumor size \pm s.e.m is shown. ****P<0.0001, repeated measures ANOVA, Sidak's
902 multiple comparisons test. On Day 14 post tumor implantation mice were sacrificed and
903 TILs, spleen and draining Lymph nodes were harvested. **f)** The frequency of antigen
904 specific CD8⁺ T cells in the dLN of mice in (e).

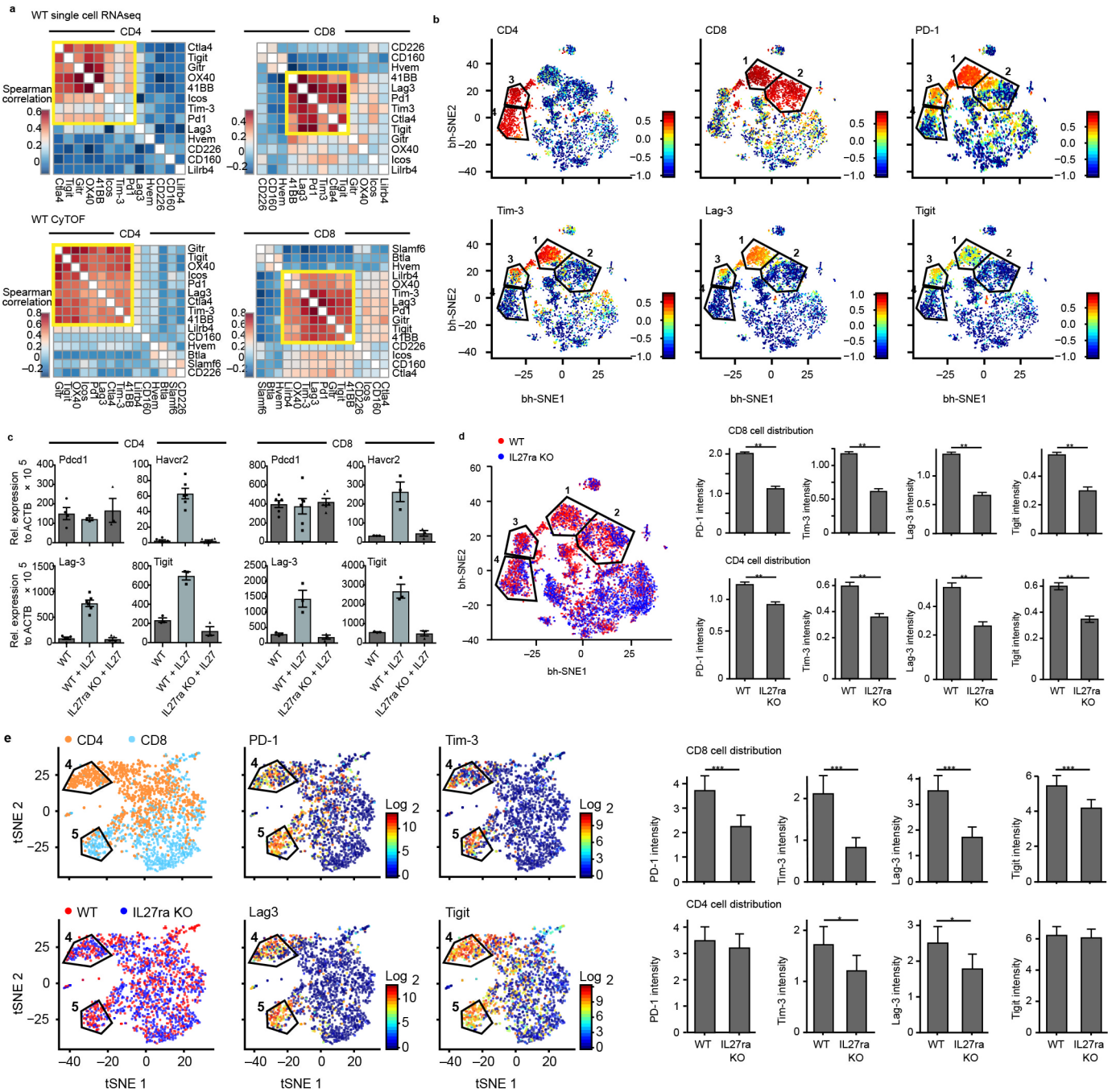
905

906 **Extended Data Figure 9. Examination of additive and non-additive (synergistic)**
907 **effects of Prdm1 and c-Maf.**

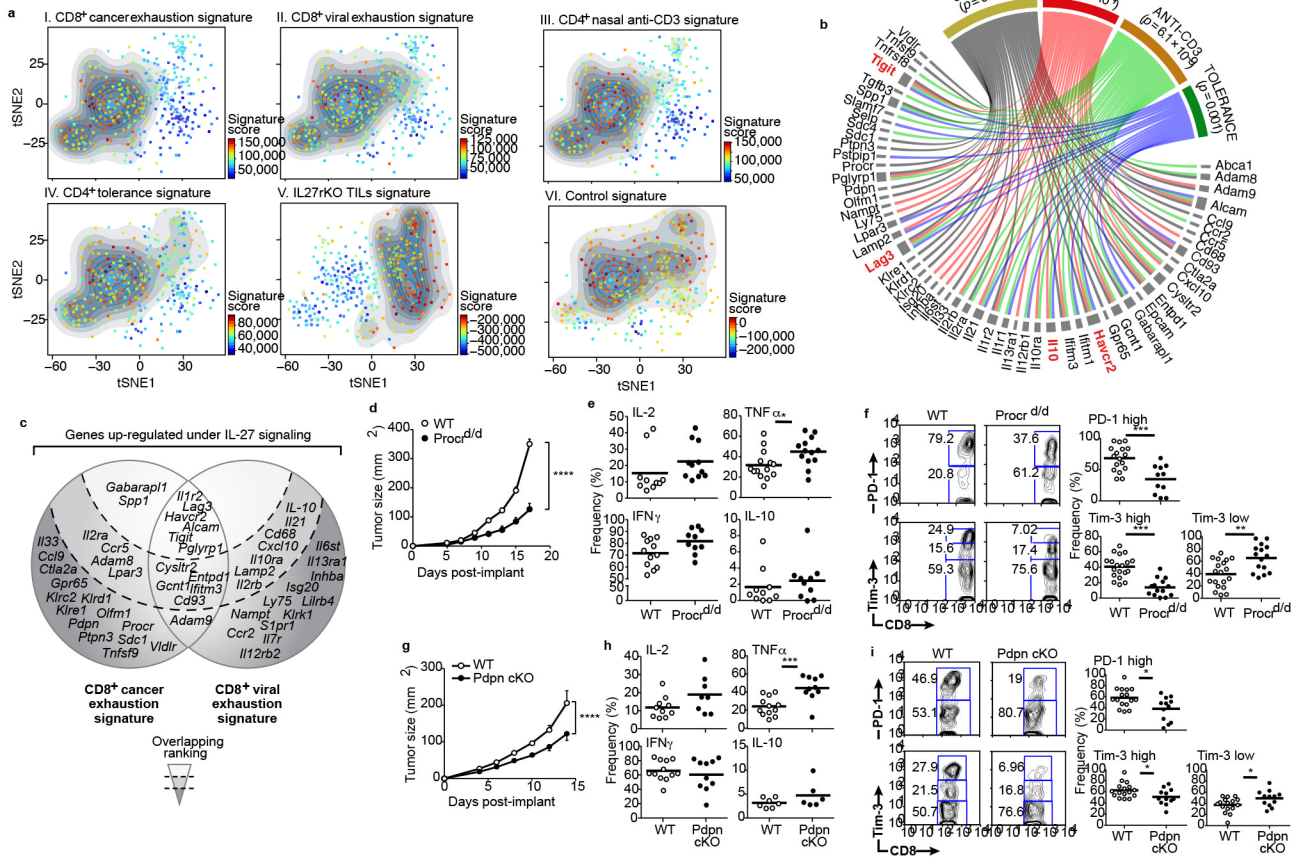
908 **a)** A Heatmap showing all 940 DE genes between WT (n=5) and cDKO (Prdm1/c-Maf,
909 n=4) and their expression in single KO (Prdm1 control n=7, Prdm1 KO n=3, cMaf
910 control n=4 and cMaf KO n=3) mice. The red markings on the top of the heatmap
911 indicate genes on whose expression the two knockouts have a statistically significant (p-
912 value<0.05) non-additive effect in the cDKO (149 out of 940 DE genes). **b)** Volcano plot
913 of the same analysis as in (a) for global gene expression. Genes whose expression in the
914 two single knockouts have a statistically significant (p-value<0.05) non-additive effect in
915 the cDKO (1144 out of 12,906 genes) and had abs (coefficient)>1 (779 out of 1144) are
916 shown in orange.

917

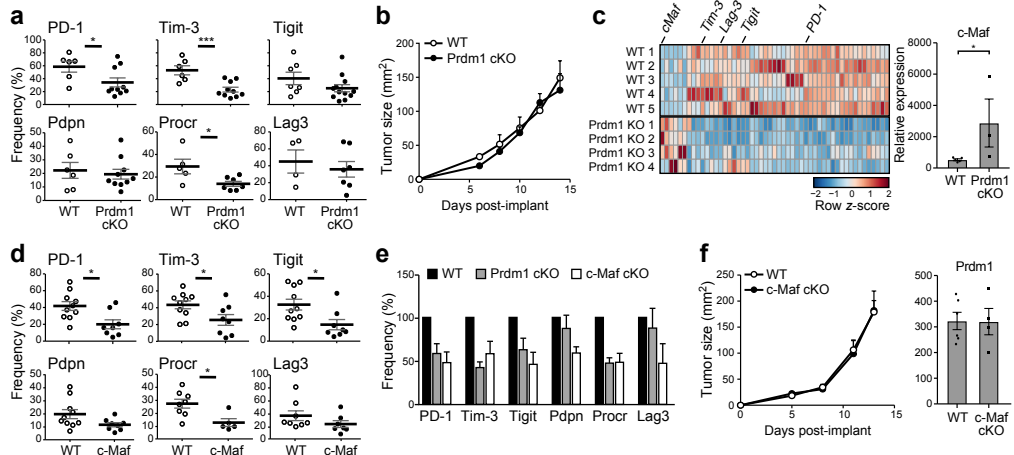
918 **Extended Data Figure 10. Comparison of gene expression between Prdm1/c-Maf**
919 **cDKO TILs and CD8⁺ TILs populations from wild type mice. a)** Barcode enrichment
920 plot displaying two gene sets in a ranked gene list. The ranked gene list was defined as
921 fold change in gene expression between Prdm1/c-Maf cDKO and WT CD8⁺ TILs. The
922 three gene sets consist of differentially expressed genes between: PD-1⁺Tim-3⁺ CD8⁺
923 (DP, n=3) and PD-1⁻Tim-3⁻ CD8⁺ (DN, n=3) TILs, PD-1⁺Tim-3⁺ CD8⁺ (DP) TILs and
924 Memory CD8⁺ (n=3), and PD-1⁺Tim-3⁻ CD8⁺ (SP, n=3) and PD-1⁻Tim-3⁻ CD8⁺ (DN)
925 TILs. **b)** This analysis was followed by four statistical tests (one-sample Kolmogorov-
926 Smirnov test, mean-rank gene set test (wilcoxGST), hypergeometric, and competitive
927 gene set test accounting for inter-gene correlation) for enrichment of these signatures in
928 the cDKO expression profile. **c)** WT versus cDKO volcano plot. Green indicates genes
929 that were up-regulated in the PD-1⁻Tim-3⁻ CD8⁺ (DN) TILs and red indicates genes that
930 were up-regulated in the PD-1⁺Tim-3⁺ CD8⁺ (DP) TILs. **d)** WT versus cDKO volcano
931 plot. Red indicates genes that were up-regulated in PD-1⁺CXCR5⁺CD8⁺ T cells and green
932 indicates genes that were up-regulated in PD-1⁺CXCR5⁻CD8⁺ T cells in chronic LCMV
933 infection²³. **e).** A tSNE plot of the 588 CD8⁺ TILs harvested from WT mice bearing
934 B16F10 melanoma tumors, colored by the relative signature score for the co-inhibitory
935 module (272 genes, **Supplementary Information Table 2**), the cDKO signature (shown
936 in (g)), and the PD-1⁺CXCR5⁺CD8⁺ T cell signature from chronic virus infection²³. The
937 contour plot marks the region of highly scored cells by taking into account only those
938 cells that have a signature score above the mean.
939
940

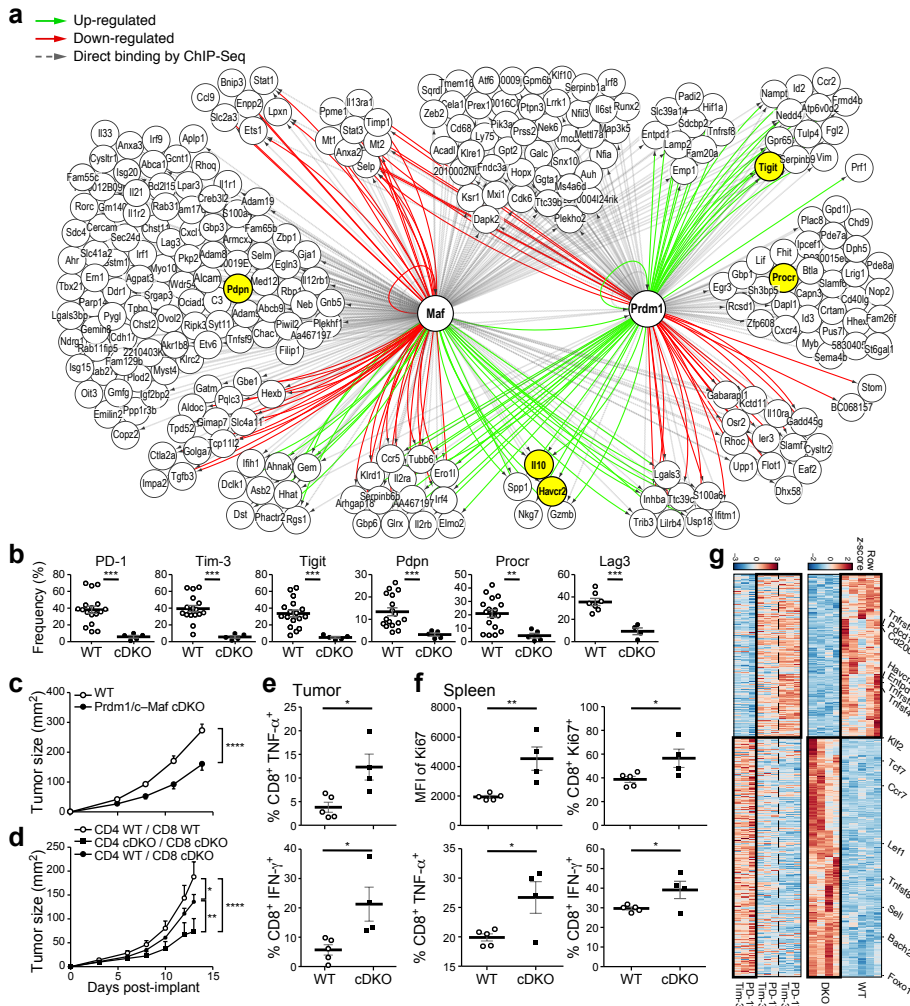


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170mm X 117mm





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