1   **PD-1 expression during acute infection is repressed through a LSD1- Blimp-1 axis[1]**

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28   **Running Title:** LSD1 regulates PD-1

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30 **Key Points**

31   1. LSD1 suppress PD-1 expression following acute infection or transient induction.

32   2. Blimp-1 binding to the Pdcd1 locus is required to recruit LSD1.

33   3. LSD1 is required to fully remethylate the PD-1 proximal promoter region.

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36 **Abstract:**

37 During prolonged exposure to antigens, such as chronic viral infections, sustained T cell receptor

38 (TCR) signaling can result in T cell exhaustion mediated in part by expression of Programmed

39 cell death-1 (PD-1) encoded by the *Pdcd1* gene. Here, dynamic changes in histone H3K4

40 modifications at the *Pdcd1* locus during ex vivo and in vivo activation of CD8 T cells, suggested

41 a potential role for the histone H3 lysine 4 demethylase LSD1 in regulating PD-1 expression. CD8

42 T cells lacking LSD1 expressed higher levels of *Pdcd1* mRNA following ex vivo stimulation, as

43 well as increased surface levels of PD-1 during acute but not chronic infection with lymphocytic

44 choriomeningitis virus (LCMV). Blimp-1, a known repressor of PD-1, recruited LSD1 to the *Pdcd1*

45 gene during acute but not chronic LCMV infection. Loss of DNA methylation at *Pdcd1*'s promoter

46 proximal regulatory regions is highly correlated with its expression. However, following acute

47 LCMV infection where PD-1 expression levels return to near base line, LSD1-deficient CD8 T

48 cells failed to remethylate the *Pdcd1* locus to the levels of wild-type cells. Finally, in a murine

49 melanoma model, the frequency of PD-1 expressing tumor infiltrating LSD1-deficient CD8 T cells

50 was greater than wild-type. Thus, LSD1 is recruited to the *Pdcd1* locus by Blimp-1, downregulates

51 PD-1 expression by facilitating the removal of activating histone marks, and is important for

52 remethylation of the locus. Together, these data provide insight into the complex regulatory

53 mechanisms governing T cell immunity and the regulation of a critical T cell checkpoint gene.

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Introduction

Programmed cell death-1 (PD-1) is an immunoinhibitory protein that is expressed on lymphocytes following the engagement of their antigen specific receptor (1). During the course of an acute infection, PD-1 expression is transient on the surface of CD8 T cells, peaking during the height of an infection and returning to near baseline levels in the resulting memory T cell pool (1-5). During chronic exposure to antigen, such as that from a chronic viral infection or in certain cancers, PD-1 expression is sustained at high levels and a state of T cell exhaustion is induced that is characterized by severe curtailment in effector functions, including the ability to proliferate, produce cytokines, and carry out cytotoxic responses (6). T cell exhaustion is in part mediated by signaling through PD-1's intracellular tyrosine immunoinhibitory domains following PD-1's surface engagement with its ligands PD-L1 or PD-L2 on target cells (1, 7-9). Although antibody blockade of PD-1/PD-L1 interactions can temporarily reinvigorate immune function from the exhausted state (1), PD-1 remains stably expressed on T cells during a chronic infection (10), and is expressed even upon removal of the cell from a chronic stimulatory environment (11). The stability of PD-1 expression and inhibition of effector functions across generations of cell division suggests that an epigenetic program stably regulates the transcriptional state of the *Pdcd1* locus.

PD-1 is encoded by the *Pdcd1* gene. In CD8 T cells, *Pdcd1* is regulated by the direct actions of transcription factors and epigenetic mechanisms (2, 10, 12). Upon T cell receptor engagement, *Pdcd1* is directly activated by a combination of transcription factors (NFATc1 and AP-1) that bind to a series of promoter proximal elements termed Conserved Regions (CR)-B and CR-C (12, 13), respectively. NFAT also binds to sites at -3.7 and +17.1 kb with respect to the transcription start site (14). Additional transcription factors appear to sustain *Pdcd1* during chronic infection and include the binding of NUR77 and FOXN1 to sites located at -23 kb and CR-C, respectively (15-18). Cytokine stimulation that results in STAT3 and STAT4 activation can further induce or sustain *Pdcd1* expression in mouse CD8 T cells by binding to the distal -3.7 and +17.1 sites (14). Following the cessation of the TCR signaling (e.g., via antigen/viral clearance), *Pdcd1* is silenced through the binding of B lymphocyte induced maturation protein-1 (Blimp-1) to a region between CR-C and CR-B (19). Blimp-1 binding results in the eviction of NFATc1 from CR-C (19). In addition to these mechanisms, epigenetic regulation through DNA methylation occurs across CR-C and near CR-B in CD8 T cells in both mice and humans (2, 10). In naïve CD8 T cells, CpGs in the above DNA regions are consistently methylated. Upon CD8 T cell activation, the methylation is lost in a time course that parallels *Pdcd1* expression. In an acute infection setting, the locus is remethylated as the infection is cleared and PD-1 levels return to the baseline as

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89 mentioned above. By contrast, during chronic infection, DNA methylation is permanently lost and
90 is not regained (2, 10).

91   In a similar manner, the accumulation and removal of activating and repressing histone
92 modifications correlate completely with *Pdcd1* expression in mouse CD 8 T cells. For example,
93 both H3K27^{ac} and H3K9^{ac} activation modifications at CR-B and CR-C correlate with *Pdcd1*
94 expression when driven by ex vivo TCR stimulation (19, 20), and H3K4^{me1} is enriched when the
95 above stimulation is coupled with IL-6 and IL-12 (STAT3/STAT4) treatment (14). However, as
96 expression wanes during ex vivo stimulation, the repressive modifications H3K9^{me3}, H3K27^{me3},
97 and H4K20^{me3} appear at CR-B and CR-C (19). Using the EL4 T cell line, exogenous expression
98 of the transcriptional repressor Blimp-1 induced the appearance of all three of these repressive
99 modifications at the *Pdcd1* locus and subsequently silenced PD-1 expression (19). Surprisingly,
100 Blimp-1 is also expressed during a chronic infection in exhausted CD8 T cells where PD-1 levels
101 are at their highest, yet fails to repress PD-1 (21). The molecular mechanism for how Blimp-1
102 could function to repress *Pdcd1* exclusively following acute inflammation is not fully clear.

103   As a repressor, Blimp-1 is known to recruit additional transcriptional repressors that result
104 in silencing the local chromatin environment (22-24). Along with histone deacetylases HDAC1
105 and HDAC2 and the histone methyltransferase G9a, Blimp-1 can recruit the lysine-specific
106 demethylase 1a (LSD1) encoded by *Kdm1a* (23-25). LSD1 catalyzes the removal of mono and
107 dimethylation modifications of H3K4 that are associated with transcriptional activation (26-28),
108 thereby facilitating an epigenetic state of gene silencing. Given the ability of Blimp-1 to repress
109 genes through recruitment of histone modifiers such as LSD1, we set out to test the hypothesis
110 that LSD1 contributes to the regulation of *Pdcd1* in a Blimp-1-dependent manner. Indeed, we
111 found that Blimp-1 was necessary to recruit LSD1 to the *Pdcd1* locus, and that when bound, LSD1
112 actively down-regulates PD-1 transcription and expression. Furthermore, we found that Blimp-1
113 was bound to the *Pdcd1* locus in both acute and chronic settings; however, LSD1 was only
114 recruited following an acute stimulation, correlating with removal of proximal H3K4$^{me1/me2}$
115 modifications and appearance of a repressive epigenetic profile concurrent with *Pdcd1* silencing
116 and DNA remethylation of the locus following acute viral infection. We also found that greater
117 frequency of PD-1 expressing tumor infiltrating CD8 T cells in LSD1-deficient compared to LSD1-
118 sufficient mice in a melanoma model. Thus, LSD1 and Blimp1 together are responsible for
119 resetting the epigenetic programming of the *Pdcd1* locus to a resting state.

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121 **Material and Methods:**

122 ***Animals***

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123   C57BL/6J mice were obtained from Jackson laboratories. *Kdm1a*^{fl/fl} mice (29) (provided by D.

124   Katz at Emory University) were crossed to mice containing a Granzyme B promoter-driven Cre

125   recombinase (B6;FVB-Tg(GMB-cre)1Jcb) (provided by J. Jacob at Emory University) (30), and

126   subsequently back-crossed to the C57BL/6 mouse line for 4-5 generations. *Prdm1*^{fl/fl} mice were

127   provided by K. Calame (Columbia University) and similarly crossed to Granzyme B-cre mice (19).

128   Equal numbers of male and female mice were used in all experiments. The numbers of animals

129   used in each experiment are provided in the figure legends. All experiments were performed in

130   accordance with approved protocols by the Emory University Institutional Animal Care and Use

131   Committee.

132

133   ***Virus Infection***

134   Viral stocks of lymphocytic choriomeningitis virus (LCMV) strains Armstrong and Clone-13 were

135   generated as previously described (31) and kindly provided by Dr. Rafi Ahmed (Emory University).

136   Mice were infected with 2×10^5 pfu of LCMV Armstrong intraperitoneally or with 2×10^6 pfu LCMV

137   Clone-13 intravenously as described (32). Viral titers were determined by plaque assay using

138   Vero cells (ATCC CCL-81) as previously described (32, 33)

139

140   ***Cell isolation and ex vivo cell activation***

141   For analysis or ex vivo cell culture, CD8 T cells were isolated from single-cell splenocyte

142   suspensions using the Miltenyi CD8a+ T cell isolation kit (Miltenyi Biotec, Inc. Cat.130-104-075)

143   according the manufacture's protocol. Where indicated, LCMV tetramer specific cells (see below)

144   were further purified by FACS on a BD FACSAria II (Emory SOM Flow Cytometry Core) following

145   infection as indicated and biochemically/molecularly analyzed immediately.   For some

146   experiments, isolated cells were cultured ex vivo in RPMI supplemented with 5% FBS, 5% bovine

147   calf serum, 4.5 g/l glucose, 1.0 mM sodium pyruvate, 10 mM HEPES and 100 U/ml

148   Penicillin/streptomycin. For ex vivo activation, isolated CD8 T cells were stimulated using

149   Dynabeads Mouse T-activator CD3/CD28 kit (Thermo Fisher Scientific (Gibco) Cat. 11453D)

150   according to the manufacture's protocol at a ratio 2:1 beads/cell for the indicated time (24-96

151   hours).

152

153   ***Flow cytometry***

154   Cells were stained for flow cytometry in FACS buffer (PBS, 1% BSA, 1 mM EDTA) for 30 m, and

155   subsequently fixed using 1% paraformaldehyde for 30 m. Events were collected on a BD LSR II

156   and analyzed using FloJo 9 software. Antibodies used to stain cells included: CD4 PerCP-Cy5.5

157   (clone RM4.5); CD8 FITC (clone 53-6.7); CD44 APC-Cy7 (clone IM7); CD62L Alexa Fluor 700

158   (clone MEL-14); CD69 PE-Cy7 (Clone H1.2F3); CD127 BV510 (Clone SB/199); PD-1 PE (clone

159   RMP1-30). Biotinylated H-2D^b MHC tetramers specific for LCMV peptides for gp33 var C41M

160   (KAVYNFATM), gp276 (SGVENPGGYCL), and np396 (FQPQNGQFI) were obtained from the

161   NIH Tetramer Core facility at Emory University and subsequently tetramerized to streptavidin-

162   APC (Prozyme) following their protocols (tetramers.yerkes.emory.edu).

163

Quantitative Real-time PCR

165   RNA was isolated from at least three independent preparations of cells using the RNAeasy kit

166   (Qiagen, Inc.), and cDNA was prepared from RNA libraries using SuperScript II reverse

167   transcriptase (Life Technologies Co). RT-PCR was used to quantitate mRNA levels in technical

168   duplicates, and values were normalized using 18s ribosomal RNA as previously described (34).

169

Chromatin Immunoprecipitation.

171   Chromatin immunoprecipitation (ChIP) assays were performed as previously described (19, 35).

172   Briefly, purified cell populations were crosslinked for 10 minutes in 1% formaldehyde, and

173   subsequently lysed in cell lysis buffer (5mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40). Chromatin

174   was extracted using nuclei lysis buffer (50 mM TRIS pH 8.1, 10 mM EDTA, 1% SDS) and

175   sonicated to an average length of 400-600 bp. Chromatin (5 µg) was used for

176   immunoprecipitation reactions on Protein A beads with 0.5 µg of polyclonal antibodies for H3K4^{me1}

177   (Millipore Sigma Cat. 07-436), H3K4^{me2} (Millipore Sigma Cat. 07-030), H3K4^{me3} (Millipore Sigma

178   Cat. 07-473), H3K27^{ac} (Millipore Sigma. Cat. 07-360), IgG (Millipore Sigma, Cat. 12-370), Blimp-

179   1 (Rockland Cat. 600-401-B52) and LSD1 (Santa Cruz Cat. SC-271720). Precipitates were

180   quantitated by quantitative PCR and calculated as a percent of input. (Supplemental Figure 1).

181

DNA methylation analysis

183   The DNA methylation content of the CR-B associated region was determined by clonal bisulfite

184   sequencing as previously described (2). Briefly, genomic DNA purified from CD8 T cells and

185   bisulfite converted using the EpiTect Bisulfite Kit as per the manufacturer's instructions (Qiagen,

186   Inc.). Bisulfite-converted DNA was PCR amplified and cloned with the TOPO TA cloning kit (Life

187   Technologies). Clones were isolated and the plasmid DNA regions were sequenced and changes

188   in CpG DNA methylation were determined. Data were aligned *in silico* using the R / Bioconductor

189   Biostrings package and custom scripts as previously described (36). A Fisher exact test was

190   used to determine significance.

191

192 **Results:**

193 **Pdcd1 *histone modifications differ between acute and chronic infection***

194 As previously shown (1, 2), infection with LCMV Armstrong induces an acute infection that results

195 in CD8 T cells expressing high surface levels of PD-1 on day 5 post-infection, but this ultimately

196 decreases to near naïve T cell levels 8 days following infection when virus is no longer detectable

197 (Fig.1A). Conversely, infection with the chronic LCMV strain Clone-13 results in PD-1 surface

198 (Fig. 1A) and mRNA levels (2) that remain elevated during the course of the infection. To gain

199 additional insight into epigenetic mechanisms that may be driving the above changes and

200 differences in PD-1 expression between acute and chronic infections, we examined activating

201 and repressive histone modifications at key regulatory elements (37) across the *Pdcd1* locus (Fig.

202 1B). At day 8, splenic CD8 T cells were isolated and analyzed by chromatin immunoprecipitation

203 (ChIP). We observed that the -3.7 and 17.1 regions were enriched for the active H3K4 mono-

204 methylated histone mark (H3K4^{me1}) in antigen specific CD8 T cells of chronically infected mice

205 compared to both acutely infected and naïve mice (Fig. 1C). Another active histone mark,

206 H3K4^{me2}, was also enriched in chronically infected mice at both the CR-B and CR-C regulatory

207 regions of the *Pdcd1* locus. H3K27^{ac} enrichment, a mark associated with active

208 promoters/enhancer regions (38) was higher at all sites in CD8 T cells from Clone-13 infected

209 mice compared to those from Armstrong infected or naïve mice. ChIP for the repressive histone

210 marks H3K9^{me2} and H3K27^{me3} were enriched at both CR-B and CR-C but only in Armstrong

211 infected mice (Fig.1C). Taken together, these data demonstrate that at day 8 post infection, CD8

212 T cells from chronically infected mice have active histone marks in the *Pdcd1* locus whereas the

213 acutely infected mice have an enrichment for repressive marks. Moreover, these histone

214 modifications correspond with PD-1 expression on CD8 T cells from chronically and acutely

215 infected mice (Fig. 1A, C).

216 The above data raise the question as to whether active histone marks examined appear

217 at an earlier timepoint during an *in vivo* response to an Armstrong infection that would correlate

218 with the increased surface expression. To test this, cells were isolated at day 5 following LCMV

219 Armstrong infection, a time point in which PD-1 expression was high (Fig. 1A) and enough

220 activated CD8 T cells (CD44^{hi} CD62L^{low}) could be isolated for ChIP. In these experiments,

221 H3K4^{me2} and H3K27^{ac} were enriched over control naïve CD8 T cells at all four key regulatory

222 elements; whereas H3K4me1 was only significantly enriched at -3.7 and 17.1 (Fig 1D). Thus,

223 active histone modifications at key regulatory elements are associated with PD-1 expression and

224 during the course of an acute infection, these marks are replaced by repressive histone

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225   modifications as expression is silenced. This raises the question of how the dynamics of histone

226   modifications at the *Pdcd1* locus are regulated.

227

228   ***LSD1 represses PD-1 following ex vivo stimulation***

229   LSD1 encoded by *Kdm1a* is responsible for catalyzing the removal of both H3K4^{me2} and H3K4^{me1}

230   marks to an unmethylated state, a process that has been dubbed enhancer decommissioning as

231   it can lead to gene silencing (26, 27). To test the hypothesis that LSD1 is actively recruited and

232   responsible for down regulation of *Pdcd1* gene expression following an acute infection, we

233   crossed an *Kdm1a$^{fl/fl}$* mouse (29) with the *Cre^{GranzB}* mouse (30). The *Kdm1a$^{fl/fl}$* allele allows for

234   *Cre*-mediated deletion of LSD1's amine oxidase catalytic domain (29). The *Cre^{GranzB}* allele is

235   induced in CD8 T cells upon T cell activation (19, 30) leading to a conditional deletion of LSD1 in

236   activated CD8 T cells. Using the *Kdm1a$^{fl/fl}$Cre^{GranzB}* mouse (designated LcKO henceforth), we first

237   determined whether an LSD1 deficiency could alter the kinetics of *Pdcd1* expression in an *ex vivo*

238   activation system. Here splenic CD8 T cells from littermate *Kdm1a$^{fl/fl}$Cre$^−$* controls (termed WT)

239   and LcKO mice were purified and stimulated in culture using αCD3/CD28 beads over a 4-day

240   period and expression of *Pdcd1* mRNA was measured by qRT-PCR at daily intervals. CD8 T

241   cells from LcKO animals displayed significantly higher levels of *Pdcd1* mRNA compared to WT

242   littermate controls at 24 hours, the peak of *Pdcd1* expression by this method of stimulation (Fig.

243   2A). Moreover, whereas WT *Pdcd1* mRNA levels decayed over the 96 h time course, *Pdcd1*

244   mRNA levels remained significantly higher in the LcKO CD8 T cells at all time points after initial

245   stimulation. Additionally, qRT-PCR for *Kdm1a* mRNA levels showed that the LcKO animals had

246   a significant reduction in LSD1 mRNA compared to WT controls, confirming efficient deletion of

247   the floxed *Kdm1a* alleles following stimulation (Fig. 2B). The lag phase prior to complete deletion

248   of *Kdm1a* may account for the slight, although significantly less than in WT, decrease in *Pdcd1*

249   expression after peak. These results demonstrate that LSD1 acts as a negative regulator of PD-

250   1 expression in CD8 T cells following ex vivo stimulation.

251

252   ***LSD1 represses PD-1 following acute viral infection***

253   To test if PD-1 expression is modulated by LSD1 during an acute viral infection, WT and LcKO

254   mice were infected with LCMV Armstrong and PD-1 expression on LCMV antigen-specific CD8 T

255   cells was monitored by flow cytometry. At day 8 post infection, when viral loads have been cleared

256   and WT mice have down-regulated surface PD-1 expression on LCMV antigen-specific CD8 T

257   cells (Fig. 3A, blue), LcKO CD8 T cells retained high levels of PD-1 expression (Fig. 3A red).

258   Previously (19), we found that conditional deletion of Blimp-1, a transcriptional repressor that has

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259 been suggested to interact with LSD1 (23), produced a similar phenotype to LcKO mice, which is

260 reconfirmed here (Fig. 3A) using *Cre^{GranzB}Prdm1^{fl/fl}* (**BcKO**) mice. The similar phenotype suggests

261 that these enzymes may operate along the same pathway, in line with the hypothesis that Blimp-

262 1 recruits LSD1 in order to mediate *Pdcd1* repression. By 28 days post infection with Armstrong,

263 LSD1 knockout antigen-specific CD8 T cells no longer showed an increase in PD-1 expression,

264 unlike cells from Blimp-1 knockout mice, which still showed some level of PD-1 expression over

265 their littermate (*Cre^−*) controls (Fig. 3A).   This suggests that while LSD1 is an important

266 determinant in the pathway responsible for decreasing PD-1 expression it does not account for

267 the entire level of PD-1 repression mediated by Blimp-1.

268 To determine if LSD1 affected PD-1 expression during a chronic LCMV infection, antigen

269 specific cells were isolated from WT and LcKO mice following LCMV Clone-13 infection and

270 analyzed by flow cytometry. At day 8, WT and LcKO CD8 T cells expressed moderate levels of

271 PD-1 (Fig. 3B), while at day 28 post infection, no difference in MFIs were observed between WT

272 and LcKO (Fig. 3, bottom). During chronic infection, BcKO CD8 T cells at day 8 had an overall

273 mean fluorescent intensity that was higher, but the histogram patterns overlap with WT cells to a

274 significant degree. Thus, at this early stage, Blimp-1 may not be contributing to *Pdcd1* regulation.

275 At the chronic infection time point (day 28), BcKO CD8 T cells expressed slightly lower levels of

276 PD-1, which was in agreement with previous reports (21). The differential roles performed by

277 Blimp-1 and LSD1 at day 28 of a chronic infection may indicate that their activities may be

278 dissociated under these conditions and at these late time points.

279

280 ***Blimp-1 recruits LSD1 to* Pdcd1**

281 To determine if LSD1 interacts directly with the *Pdcd1* locus concurrent with PD-1 down-

282 regulation, and whether it is recruited there by Blimp-1, ChIP was performed on *ex vivo*

283 αCD3/CD28 stimulated splenic CD8 T cells isolated from LcKO and BcKO animals, as well as the

284 corresponding *Cre^−* controls (WT). At day 4, when PD-1 expression has nearly returned to

285 baseline levels (Fig. 2A), LSD1 was bound to the CR-B region in WT mice (Fig. 4A). This indicates

286 a direct interaction with the locus and suggests that LSD1 itself directly represses *Pdcd1*

287 transcription. However, LSD-1 binding was not observed in BcKO mice concurrent with a loss of

288 Blimp-1 at the *Pdcd1* locus (Fig. 4A, B), indicating that Blimp-1 is necessary to recruit LSD1 to

289 the region.   Importantly, LSD1 binding was minimally observed at the locus in LcKO cells,

290 indicating that the conditional deletion was efficient (Fig. 4A). However, Blimp-1 was still bound

291 in LcKO mice (Fig. 4B), indicating that Blimp-1 is capable of independently interacting with *Pdcd1*.

292   These data demonstrate that LSD1 interacts directly with the *Pdcd1* locus, corresponding with

293   suppression of *Pdcd1* expression *ex vivo*, and requires Blimp-1.

294

295   ***LSD1 interacts with Pdcd1 following an acute but not chronic infection***

296   Blimp-1 binds directly to *Pdcd1* following an acute infection, and acts as a transcriptional

297   repressor (19). However, during a chronic infection, Blimp-1 is expressed at even higher levels,

298   and was correlated with maximal PD-1 expression (21). To determine if Blimp-1 interacted directly

299   with *Pdcd1* during chronic inflammation *in vivo*, ChIP was performed on virus-specific CD8 T cells

300   from naïve, day 8 acutely-infected (Armstrong), or day 8 chronically-infected (Clone-13) mice. As

301   we previously reported, Blimp-1 was bound to *Pdcd1* following acute infection (19). LSD1 was

302   also bound, correlating with its repressive role (Fig. 5A). Blimp-1 was also bound at day 8 during

303   a chronic infection (Fig. 5A). Importantly, in this setting, LSD1 was not recruited to the region

304   even though Blimp-1 was bound. To rule out the possibility of differential expression levels of

305   LSD-1 between viral infections, we analyzed previously published (39) RNA microarray data and

306   observed no differences (Fig. 5B). This suggests that although Blimp-1 interacts with the *Pdcd1*

307   locus in CD8 T cells during a chronic infection setting, it fails to down-regulate PD-1 levels

308   because LSD1 is not recruited.

309

310   ***LSD1 is responsible for the decommissioning of Pdcd1 enhancer elements.***

311   As LSD1 is responsible for removing the histone marks H3K4^{me1} and H3K4^{me2}, we set out to

312   determine if LcKO mice possessed increased levels of these marks at the *Pdcd1* locus. To test

313   this, LcKO and *Cre*$^-$ control (WT) mice were infected with LCMV Armstrong for 8 days. Antigen

314   specific (pooled gp33, gp276, np396) CD8 T cells were isolated by FACS and subjected to ChIP

315   for H3K4^{me1} and H3K4^{me2} at key regulatory regions across the *Pdcd1* locus. CD8 T cells from

316   LcKO mice showed enrichment for H3K4^{me1} at the -3.7 and +17.1 regions compared to WT cells

317   (Fig. 5C). Enrichment for H3K4^{me2} was also observed in the LcKO CD8 T cells at the CR-B, CR-

318   C, and -3.7 regulatory elements. The increased levels of these active histone marks correlated

319   with the elevated levels of PD-1 expressed by LcKO CD8 T cells and mirrors the histone pattern

320   observed in the PD-1 expressing cells at day 8 during chronic infection (Fig. 1C). Together, these

321   results demonstrate that LSD1 is responsible for removing the activating histone marks H3K4^{me1}

322   and H3K4^{me2} from the *Pdcd1* locus and suggest that LSD1 downregulates PD-1 expression by

323   facilitating the removal of active histone marks after being recruited to locus by Blimp-1 during an

324   acute viral infection.

325

Epigenetic silencing of the locus following acute infection is enforced by LSD1

We previously showed that during the course of LCMV Armstrong infection antigen specific CD8 T cells initially lose CpG methylation at a region near CR-B (2). As the infection wanes and PD-1 levels return to near baseline, DNA methylation in that region reappears. Thus, loss of CpG methylation at the *Pdcd1* locus is correlated with gene expression, and reappearance of methylation is concurrent with gene silencing. Since *de novo* DNA methylation is dependent on an H3K4^{me0} methylation state (40, 41) and LSD1 has been shown to catalyze the removal of H3K4^{me1} and H3K4^{me2} (Fig. 5C), as well as being important for global DNA methylation (42), we sought to determine if remethylation of the *Pdcd1* was altered in LcKO mice during an acute infection. In accordance with the remethylation observed in WT animals at day 8 after LCMV Armstrong infection (2), WT control mice showed a relative abundance of CpG methylation across the *CR-B* region of *Pdcd1* (Fig. 6). In contrast, LSD1-deficient CD8 T cells, which have elevated levels of PD-1 expression at this time (Fig. 3), showed minimal remethylation of the locus with a majority of alleles analyzed exhibiting either no methylation or only a handful of methylated CpGs across the region (Fig. 6). This suggests that the inability to remove H3K4 methylation in LSD1-deficient CD8 T cells inhibits the acquisition of DNA methylation, which leads to a failure to fully silence PD-1, thereby providing a further mechanism for retaining prolonged gene expression.

Increased frequency of PD-1 expressing CD8 T cells in the absence of LSD1 during melanoma

In order to investigate whether LSD1 deletion had an effect on PD-1 expression and disease progression in another model, we employed the B16 melanoma model (43). LcKO and *Cre*$^{-}$ littermate controls were injected with 1x10^{6} tumor cells and mice were checked for weight and tumor size daily. Compared to each other, LcKO and WT mouse weights mice did not statistically differ throughout the course of the experiment; however, tumor growth appeared to be statistically lower in the mice with LSD-deficient CD8 T cells (Fig. 7A). Examination of the tumor infiltrating CD8+ T cells (TILs) showed that the mice which had LSD1-deficient cells had a higher frequency of PD-1^{hi} cells compared to control animals, suggesting that LSD1 deficiency plays a role in the expression of PD-1 in a non-viral inflammatory model (Fig. 7B).

Discussion:

Multiple recent studies have highlighted the important effects of dynamic epigenetic regulation in driving immune responses (44-47). Here, we demonstrate that LSD1 is a novel epigenetic repressor of PD-1 expression. *Ex-vivo* stimulated LSD1-deficient CD8 T cells displayed increased

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360 levels of PD-1 expression compared to WT cells. LCMV-specific CD8 T cells also displayed
361 increased PD-1 expression levels at day 8 following acute infection, but not chronic. Blimp-1 was
362 identified as necessary to recruit LSD1 to *Pdcd1* regulatory regions using LSD1 and Blimp-1
363 conditional knockout mice. Although Blimp-1 associates with *Pdcd1* in both acute (PD-1
364 silencing) and chronic (PD-1 permissive) inflammatory environments, LSD-1 is only recruited to
365 the locus in the acute infection, providing a mechanistic epigenetic switch for the differential
366 expression of PD-1 in the two infectious systems. Furthermore, the association of LSD1 with
367 *Pdcd1* during resolution of an acute infection correlates with and is necessary for the
368 disappearance of the active histone modifications targeted by LSD1: H3K4^{me1} and H3K4^{me2}.
369 Additionally, the actions of LSD1 and the removal these histone marks correlate with the
370 remethylation of the CR-B upstream, regulatory region and the silencing of PD-1 protein
371 expression.

372   The results from this work also show that in a melanoma cancer model, the tumor
373 infiltrating CD8 T cells from the LcKO mice displayed higher PD-1 levels compared to wild-type
374 controls. This matches the observation seen in the acute viral infection where activated CD8 T
375 cells deficient in LSD1 expressed higher levels of PD-1. Despite the increased presence of PD-
376 1^{hi} cells, the overall health (as measured by weight loss) of the conditional knockout animals was
377 not significantly different than in the wild-type mice. However, LcKO mice had smaller tumors
378 across the time course. This could be due to LSD1 controlling other genes that also show
379 enhanced expression when LSD1 is deleted in this model. In line with this, deletion of LSD1 in
380 plasmablasts resulted in the upregulation of 471 genes compared to wild-type cells (28).
381 Pharmacological inhibition of epigenetic modifiers, including LSD1 are effective in treating some
382 cancers as a method to directly inhibit genes aberrantly expressed in the cancer itself (48). Thus,
383 although the model used in our research does not affect LSD1 within the tumor cells, the higher
384 PD-1 on TILs and potential subsequent cellular exhaustion could complicate this treatment
385 strategy.

386   Blimp-1, a protein associated with B cell maturation into antibody-secreting plasma cells,
387 is a critical inhibitor of *Pdcd1* transcription following acute T cell stimulation (19). Increases in
388 Blimp-1 mRNA and protein following antigen clearance are associated with concurrent decreases
389 in PD-1. Paradoxically, if antigen persists and stimulation through the TCR continues, Blimp-1
390 levels nonetheless increase further (21). Whereas in an acute infection the CD8 T cells from
391 BcKO mice showed increased PD-1, suggesting an inhibitory function, the same deletion resulted
392 in modestly lower levels of PD-1 in a chronic infection, suggesting Blimp-1 acted as an activator.
393 In this study, Blimp-1 was shown to be bound at *Pdcd1* in both infection modalities, thereby

13

394   precluding the possibility that the changing function of Blimp-1 was mediated indirectly through

395   binding to other target genes. Instead, recruitment of LSD1, which was dependent on the

396   presence of Blimp-1, was found to be unique to acute infections.

397   Many mechanisms could potentially explain the failure of Blimp-1 to recruit LSD1 in a

398   chronic infection setting. Splice variants of Blimp-1 have been shown both to be involved in

399   different timing of expression (49) and to have alternative functions (50). These include variants

400   that alter Blimp-1's ability to recruit additional transcription factors while preserving its ability to

401   bind DNA (21, 51). Other biochemical mechanisms could also be involved, including

402   posttranslational modifications of either Blimp-1 or LSD1. Furthermore, other factors binding to

403   locus could sterically hinder or aid LSD1 recruitment. Another mechanism explaining the

404   differential binding of LSD1 during acute and chronic infection could be expression levels of LSD1,

405   however analysis of published transcriptomics data sets (39) suggest that this is not the case.

406   For *Pdcd1*, LSD1 provides an important mechanistic link between the dynamics of histone

407   code modifications, DNA methylation, and gene expression of this critical immune regulatory

408   locus. Dynamic molecular events at the *Pdcd1* locus are mirrored in chromatin accessibility

409   patterns and DNA methylation patterns that change as CD8 T cells differentiate from naïve to

410   effector, memory, and exhausted cells at both *Pdcd1*, as well as many other T cell expressed

411   genes (2, 10, 15, 45-47). While this study focused on the effects of epigenetics on determining

412   the expression of a single immune-related gene, LSD1 is most likely to have additional

413   consequences on regulating CD8 T cell differentiation and gene expression. Irrespective of

414   LSD1's additional roles in modulating CD8 T cell gene expression, the work presented in this

415   study establishes a clear mechanism for LSD1 to differentially regulate the expression levels of

416   PD-1 during acute and chronic viral infections.

417

418   **Acknowledgments**

424

425   **Abbreviations**

426 Blimp-1, B lymphocyte induced maturation protein-1; ChIP, Chromatin immunoprecipitation

427 assay; CR-B, Conserved region B; CR-C, Conserved region C; LCMV, Lymphocytic

428 choriomeningitis virus; LSD1, Lysine specific demethylase 1; PD-1, Programmed cell death 1.

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607

608 **Figure legends:**

609

610 **Figure 1. Activating histone marks are dynamically regulated and correlate with Pdcd1**

611 **expression.** C57Bl/6 mice were infected with either LCMV Armstrong or Clone-13 for 5 or 8

612 days. (**A**) Representative flow cytometry plots showing the surface PD-1 levels on CD8 T cells.

613 Plots were gated on CD8 T cells and show combined LCMV gp33, gp276 and np396 tetramer

614 positive cells (day 8) or total activated CD8 T cells defined as $CD44^{hi}$ and $CD62L^{low}$ (day 5). Bar

615 graphs represent combined averages of 3 mice. (**B**) Schematic highlighting regulatory elements

616 of the *Pdcd1* locus where ChIP assays were performed. (**C**) ChIP assay for both activating and

617 repressive histone marks on CD8 T cells isolated from day 8 Armstrong or Clone-13 infected

618 mice. (**D**) ChIP on activated $CD44^{hi}$ $CD62L^{low}$ isolated from day 5 infected Armstrong mice and

619   naïve uninfected mice for activating histone marks. Data represent three independently isolated

620   biological replicates. Two tailed Student's *t* tests were used to determine significance in these

621   experiments. *, P < 0.05 **, P < 0.01 ***, P < 0.001.

622

623   **Figure 2. LSD1 acts as a repressor of *Pdcd1* expression in *ex vivo* stimulated CD8 T cells.**

624   CD8 T cells were isolated from *Kdm1a^{fl/f}Cre^-* (WT) and *Kdm1a^{fl/fl}Cre^{GranzB}* (LcKO) by magnetic

625   separation and then stimulated for up to 96 hours using αCD3/CD28 beads at a 2:1 bead:cell

626   ratio. Every 24 hours, cells were collected for RNA and analyzed by qRT-PCR. (**A**) qRT-PCR

627   showing *Pdcd1* expression. (**B**) qRT-PCR showing *Kdm1a* (LSD1) expression and that it was

628   efficiently deleted from LcKO CD8 T cells. Data represent three independently isolated biological

629   replicates. A two tailed Student's *t* test was used to determine significance at each time point. *,

630   P < 0.05 **, P < 0.01 ***, P < 0.001.

631

632   **Figure 3. LSD1 represses PD1 during acute viral infection**. LcKO, BcKO and WT control mice

633   were infected with LCMV Armstrong or Clone-13 for 8 or 28 days. Cells were isolated and subject

634   to flow cytometry to assay surface PD-1 expression. Plots show naïve (N) CD8 T cells gated as

635   CD8$^+$CD44^{low} CD62L^{hi}; and LCMV-specific cells gated as CD8$^+$, CD44^{hi} and gp33 tetramer

636   positive (Tet+). Data represent three independently isolated biological replicates. A two tailed

637   Student's *t* test was used to determine the significance at each time point. *, P < 0.05 **, P < 0.01

638   ***, P < 0.001.

639

640   **Figure 4. Blimp-1 recruits LSD1 to the *Pdcd1* locus.** CD8 T cells from LcKO, BcKO, and WT

641   control mice were isolated by magnetic separation and then stimulated with αCD3/CD28 beads

642   for 96 hours. Chromatin was prepared from collected cells and then subject to ChIP. (**A**) Binding

643   of LSD1 to the CR-B region of the *Pdcd1* locus. (**B**) Binding of Blimp-1 to site 2 of the *Pdcd1*

644   locus. An α-IgG antibody was used as a control for ChIP experiments. Data represent three

645   independently isolated biological replicates. A two tailed Student's *t* test was used to determine

646   statistical significance. **, P < 0.01 ***, P < 0.001.

647

648   **Figure 5. LSD1 is recruited to the *Pdcd1* locus only during an acute infection.** (**A**) C57BL/6

649   mice were infected with either LCMV Armstrong or Clone-13 for 8 days. CD8 T cells were isolated

650   by magnetic separation and subject to ChIP to assess LSD1 and Blimp-1 binding. Naïve CD8 T

651   cells were also magnetically isolated (See Methods) from uninfected mice were used as a control,

652   as was an α-IgG antibody. Blimp-1 binding was assessed at the Blimp-1 binding site (Fig 1B) of

19

653   the *Pdcd1* locus and LSD1 binding was assessed at CR-B. (B) Microarray RNA expression data

654   from (39) was analyzed for *Kdm1a* expression (LSD1). (**C**) LcKO and littermate control WT mice

655   were infected with LCMV Armstrong. At day 8, gp33-, gp276-, and np396-specific CD8 T cells

656   were isolated, pooled, and subjected to ChIP with α-H3K4^{me1} and H3K4^{me2} antibodies at the

657   indicated regions of the *Pdcd1* locus. Three independent biological replicates were assayed. A

658   two tailed Student's *t* test was used to determine statistical significance between samples. *, P

659   < 0.05 ***, P < 0.001.

660

661   **Figure 6. LSD1 deficient CD8 T cells fail to remethylate the *Pdcd1* locus during acute**

662   **infection.** LcKO and and *LSD1$^{fl/f}$Cre$^-$* (WT) control mice were infected with LCMV Armstrong for

663   8 days. CD8 T cells from these mice or naïve, uninfected *Cre-* mice were isolated by MACS, and

664   tetramer-specific cells were sorted by FACS. DNA from each population was bisulfite converted

665   and PCR amplified. 8 clones from each mouse were sequenced, and incomplete sequences

666   were discarded. At each CpG site across the CR-B region, the presence of DNA methylation

667   within each clone is indicated by a closed circle and unmethylated CpG sites are indicated by an

668   open circle. Frequency of methylation at each site across all clones is indicated in the

669   corresponding bar graph. Data are combined from 2 biological replicates. Statistical significance

670   was determined using a Fisher's Exact Test and the P values are indicated.

671

672   **Figure 7. Percent PD-1 positive cells is increased in tumors from mice with LSD1-deficient**

673   **CD8 T cells.** LcKO and *LSD1$^{fl/f}$Cre$^-$* (WT) control were injected with 1x10^6 tumor cells and

674   analyzed at days 16 or 17 post inoculation. (**A**) Percent starting weight and tumor diameter

675   (inches) from mice during the 16 day time course. (**B**) At the end of the experiment, cells were

676   collected from the spleen and tumor and subjected to flow cytometry. Activated CD8 T cells

677   (CD44^{hi} CD62L^{low}) were gated on for their percentage of PD-1^{hi} cells in both the spleen and tumor

678   (TIL). The summary graph in B shows the average of two independent experiments where the

679   end points were day 16 and 17 post tumor cell inoculation. Significance was calculated in A using
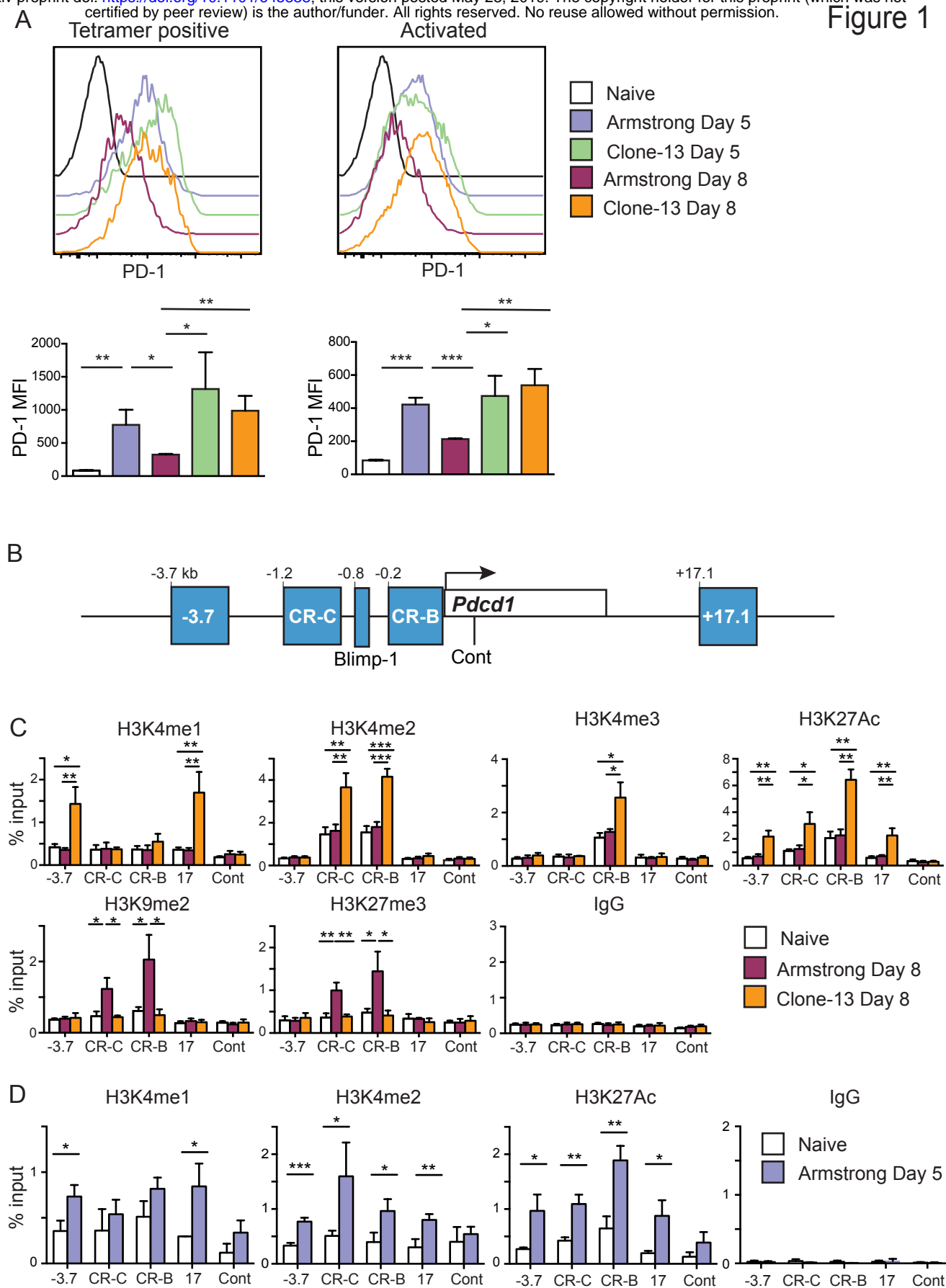
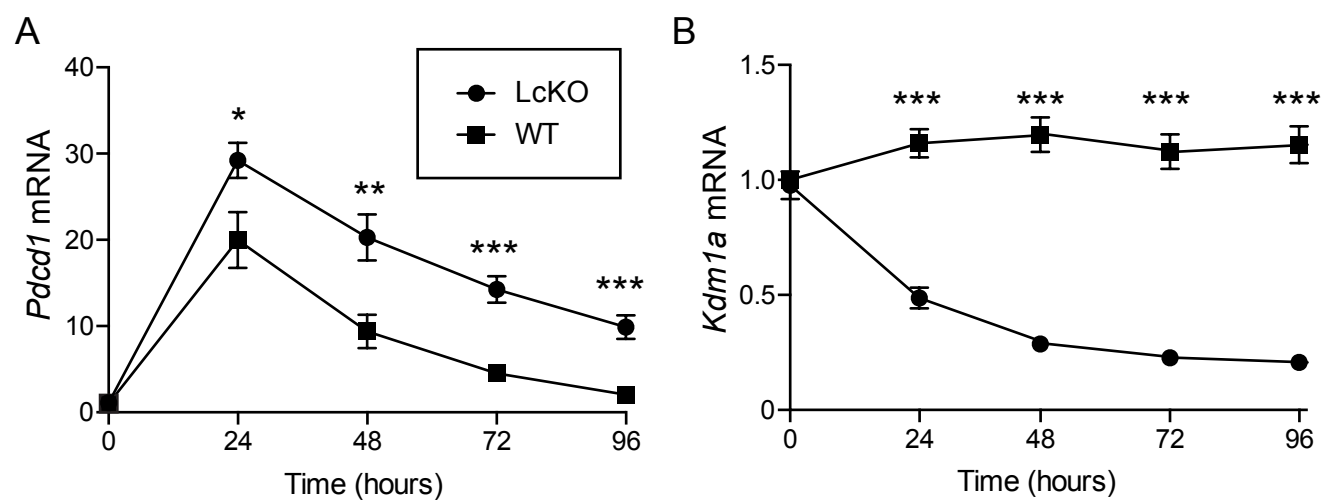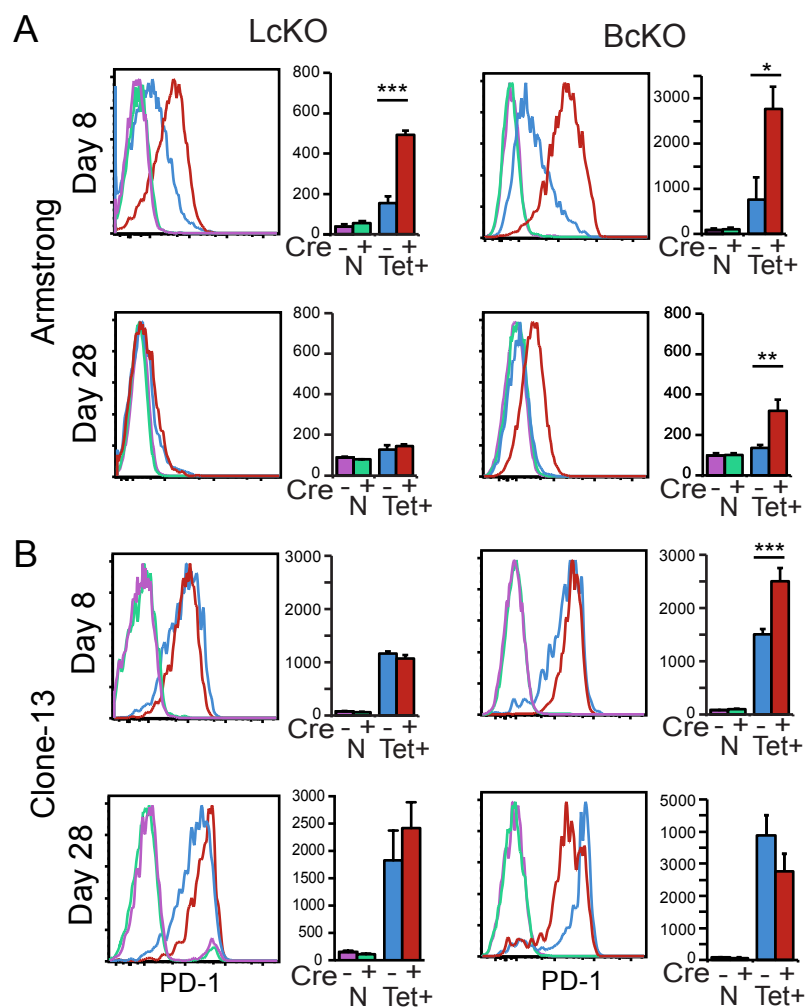680   a two-way ANOVA (P = 0.015) and a Student's *t* test in B. *, P < 0.05.
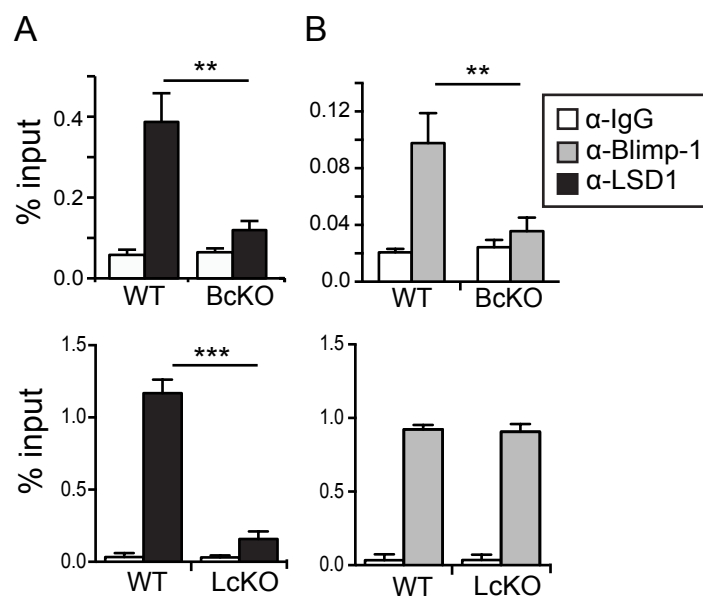
Figure 1

Figure 2

Figure 3

Figure 4
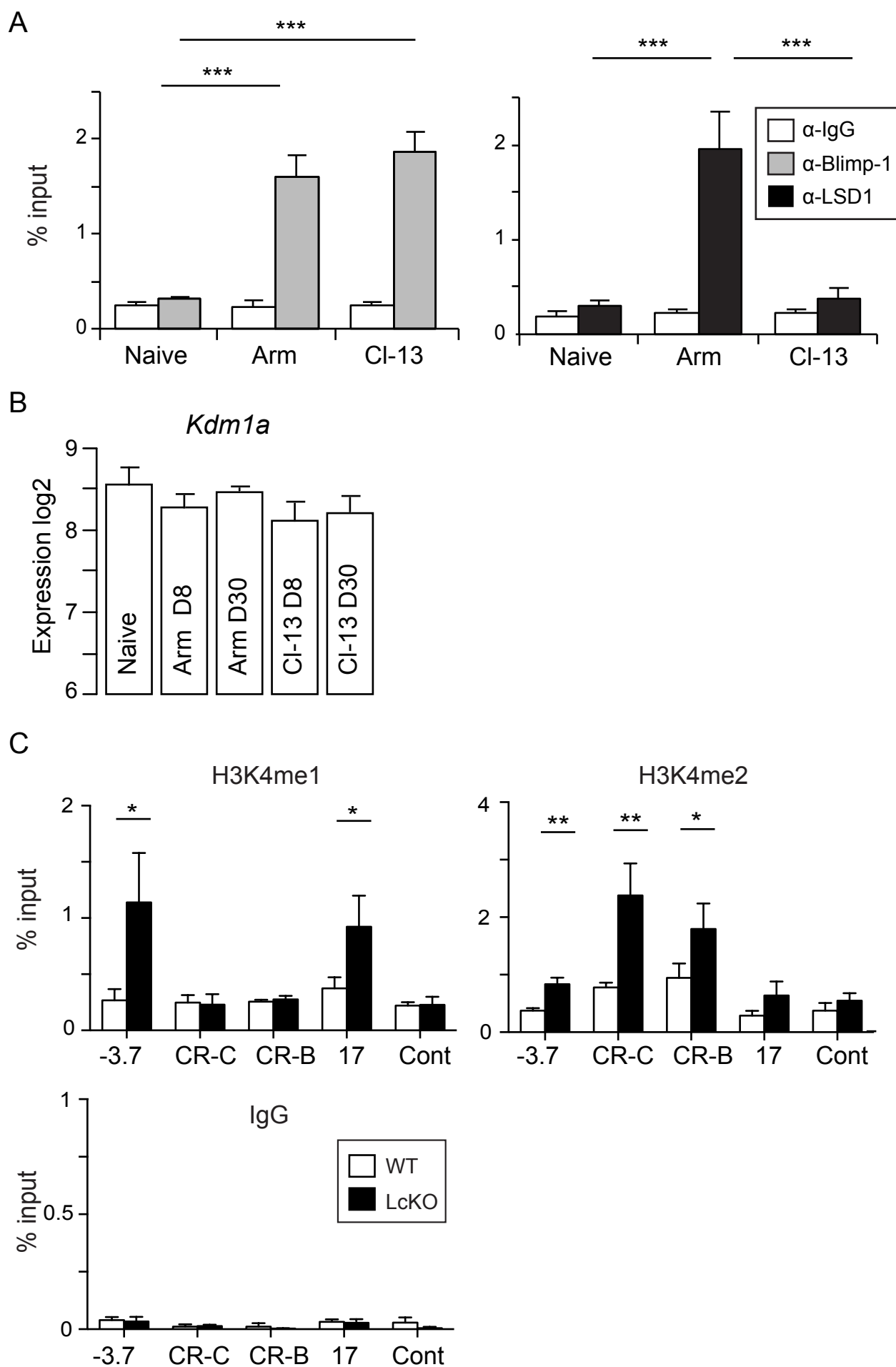
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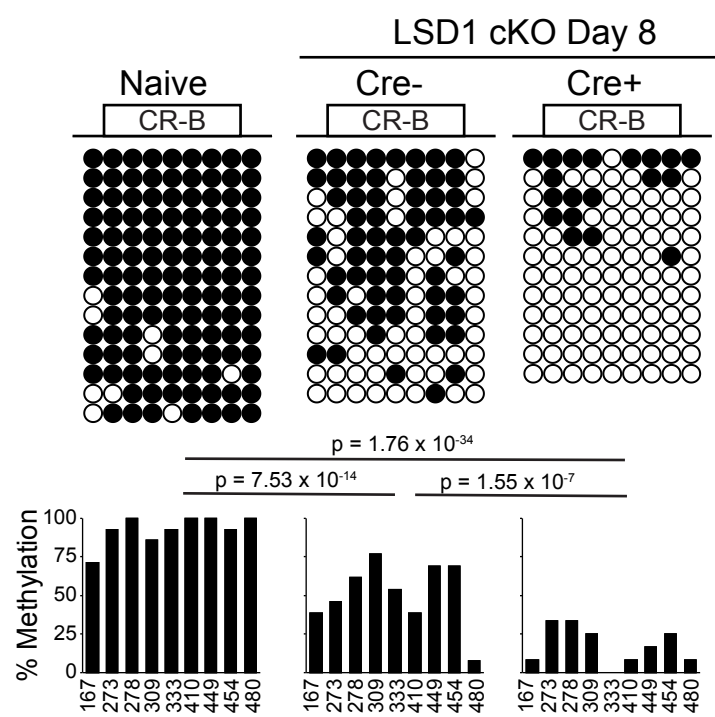
Figure 6

Figure 7
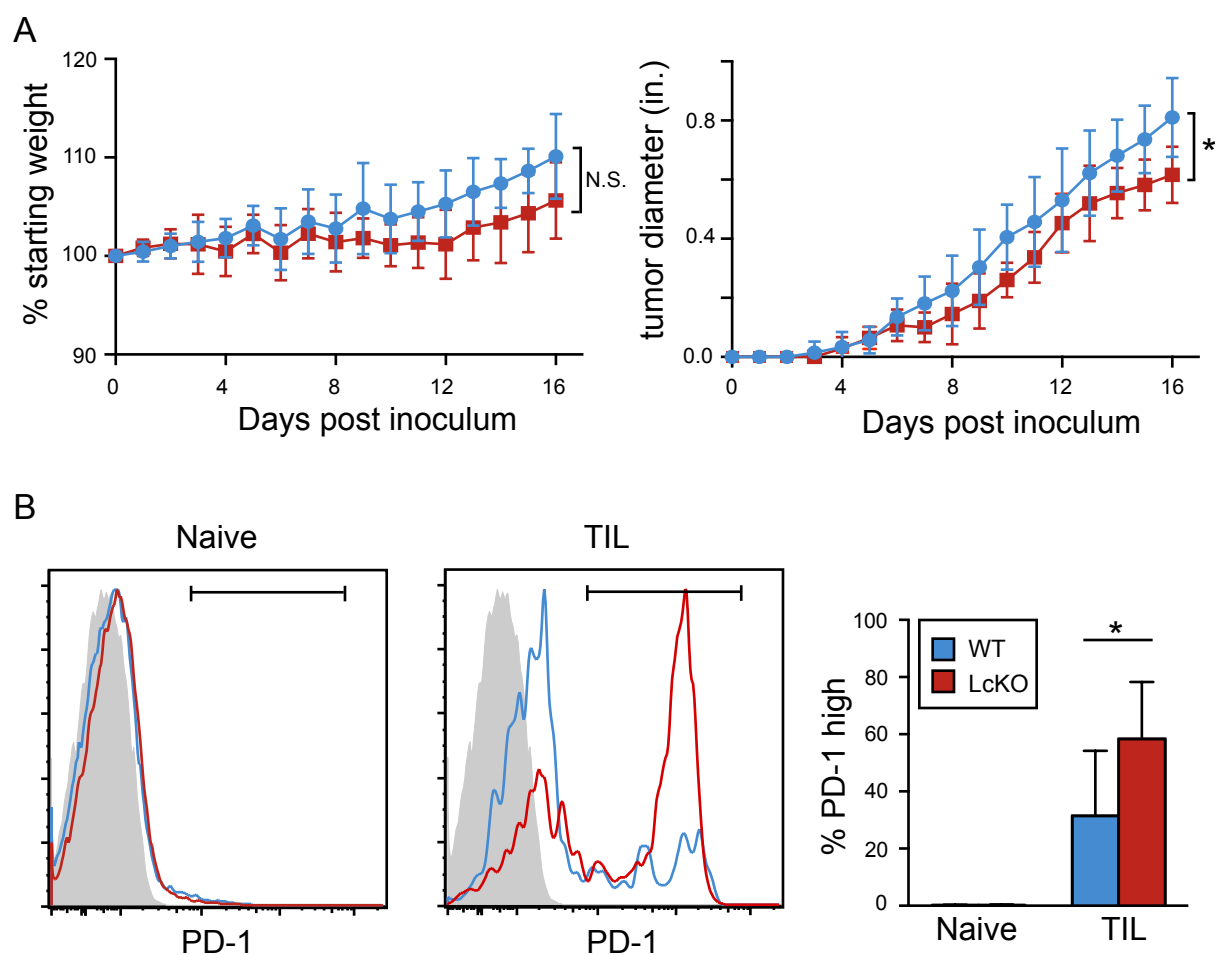
Supplemental Table 1. Primers used

ChIP Primers	Primer sequence 5'-3'
CR-B forward	GGCAGTGTCGCCTTCAGTAGC
CR-B reverse	CCACCTCTAGTTGCCTGTTCTC
CR-C forward	CCTCACCTCCTGCTTGTCTCTC
CR-C reverse	GTGAGACCCACACATCTCATTGC
-3.7 forward	AGGGTGAGCAGGCGAGAGCAA
-3.7 reverse	AGCACAGGGAAGAAGCTGTTGGG
+17 forward	GGAGGGGATAGGCGCTGGGT
+17 reverse	TCTGGGCCAAGCCATCCGGT
Control forward	CAGAGGCCACTCTTGACTCC
Control reverse	AAGGCTCCCTGGAGGAGATA
qPCR Primers	
PD-1 forward	CGTCCCTCAGTCAAGAGGAG
PD-1 reverse	GTCCCTAGAAGTGCCCAACA
18S forward	GTAACCCGTTGAACCCCATT
18S reverse	CCATCCAATCGGTAGTAGCCG
LSD1 forward	CAGCCTGTTTCCCAGACATC
LSD1 reverse	GCTGGATCTTTGGGTTGTCC