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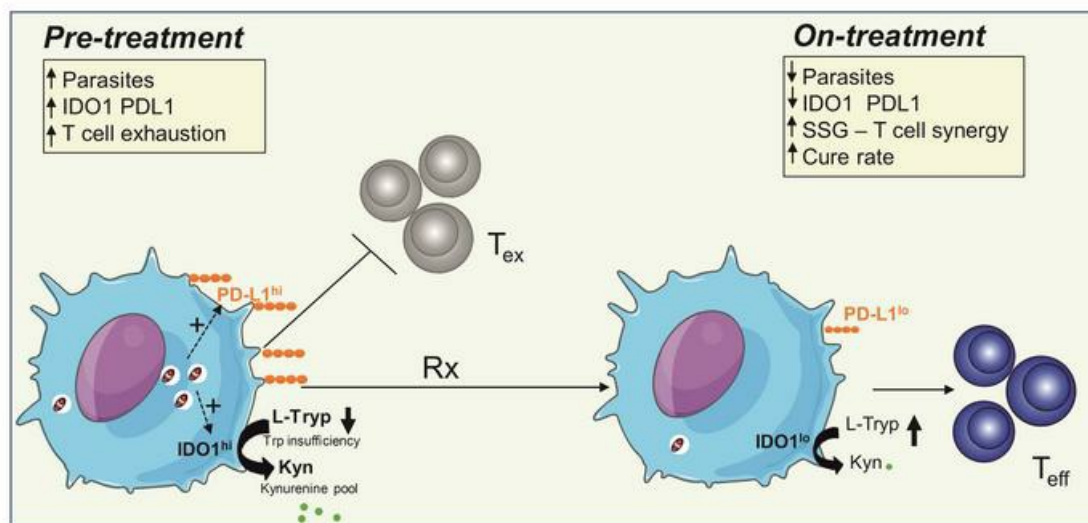
Early reduction in PD-L1 expression predicts faster treatment response in human cutaneous leishmaniasis

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1 **Early reduction in PD-L1 expression predicts faster treatment response in human**
2 **cutaneous leishmaniasis.**

3

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36 **Abstract**

37 **Cutaneous leishmaniasis (CL) is caused by *Leishmania donovani* in Sri Lanka.**
38 **Pentavalent antimonials (e.g. sodium stibogluconate; SSG) remain first line drugs for**
39 **CL with no new effective treatments emerging. We studied whole blood and lesion**
40 **transcriptomes from Sri Lankan CL patients at presentation and during SSG**
41 **treatment. From lesions but not whole blood, we identified differential expression of**
42 **immune-related genes, including immune checkpoint molecules, after onset of**
43 **treatment. Using spatial profiling and RNA-FISH, we confirmed reduced expression of**
44 **PD-L1 and IDO1 proteins on treatment in lesions of a second validation cohort and**
45 **further demonstrated significantly higher expression of these checkpoint molecules on**
46 **parasite-infected compared to non-infected lesional CD68⁺ monocytes / macrophages.**
47 **Crucially, early reduction in PD-L1 but not IDO1 expression was predictive of rate of**
48 **clinical cure (HR = 4.88) and occurred in parallel with reduction in parasite load. Our**
49 **data support a model whereby the initial anti-leishmanial activity of antimonial drugs**
50 **alleviates checkpoint inhibition on T cells, facilitating immune-drug synergism and**
51 **clinical cure. Our findings demonstrate that PD-L1 expression can be used as a**
52 **predictor of rapidity of clinical response to SSG treatment in Sri Lanka and support**
53 **further evaluation of PD-L1 as a host directed therapeutic in leishmaniasis.**

54

55

56

57 **Introduction**

58 Every year, approximately 600,000 – 1 million new cases of cutaneous leishmaniasis (CL)
59 occur, with a broad global distribution, often leading to stigma and reduced life chances and
60 placing a burden on health services (1-3). Treatment options for CL have changed little in
61 over 70 years, since pentavalent antimonial drugs were first introduced, and there are limited
62 new treatments on the horizon (3). Sri Lanka is endemic for CL, with the first autochthonous
63 case being reported in 1992 (4). Sri Lankan CL is caused by *Leishmania donovani*
64 zymodeme MON-37 parasite (5-7), usually associated with visceral leishmaniasis in other
65 endemic countries. Current treatment for CL in Sri Lanka involves weekly intra-lesional or
66 daily intra-muscular administration of sodium stibogluconate (SSG), with or without
67 cryotherapy, based on the site and size of the lesion and response to treatment. Cure often
68 takes many months, and some patients may fail to respond completely or withdraw from
69 treatment (8).

70

71 Most of our understanding of the host immune response in CL stems from experimental
72 models, and human disease is much less understood (9). Immune checkpoint molecules have
73 been implicated in disease progression in pre-clinical models (10-17), but their role in human
74 CL has not been explored. It is widely proposed that immune-drug synergy is required for
75 effective treatment and that host directed therapy (HDT) may have a future role in patient
76 management (18-20), but few validated targets have emerged. Here, we searched for early
77 correlates of treatment response that might be used to stratify patient response. Our results
78 indicate an intimate relationship between intracellular parasitism and immune checkpoint
79 molecule expression, with PD-L1 emerging as a promising target for HDT in Sri Lanka.

80 **Results and Discussion**

81 We first conducted a targeted transcriptomic analysis of the lesion site in a test cohort of 6
82 patients with typical homogeneous nodulo-ulcerative CL lesions (3 females, 3 males; mean
83 age \pm standard deviation, 34 ± 11 years; (Supplemental Figures 1-3 and Supplemental Table
84 1). Principal component analyses of lesion transcriptomic data showed separation of pre- and
85 on-treatment samples in most patients (Figure 1A) and 120 differentially expressed genes
86 were identified (DEGs; FDR adjusted p-value <0.01 ; Figure 1B). In contrast, no DEGs were
87 identified by RNA-seq in whole blood (Supplemental Figure 4) suggesting that unlike CL
88 caused by *L. braziliensis* (21), CL due to *L. donovani* in Sri Lanka is not accompanied by an
89 overt systemic immune response.

90

91 Following treatment, the majority of DEGs in dermal lesions were downregulated (87%;
92 105/120) suggesting a reduction in inflammation following treatment (105 downregulated, 15
93 upregulated; Figure 1B and Supplemental Table 3). Genes for cellular functions and
94 regulation, chemokines, membrane receptors, T cell function and regulation were amongst
95 the top 20 DEGs (Figure 1C). Further, STRING analysis (22) identified Lymphocyte
96 migration (GO: 0002687, FDR= $1.06E-14$; including interferon inducible chemokines like
97 *CXCL9*, *CXCL10*, *CXCL11*, *CCL19*, *CCL8*) and regulators of immune response (GO:
98 0002684, FDR= $1.94E-11$; including *IDO1*, *LAG3* and *CD274/PDL1*) as highly enriched
99 pathways (Figure 1D). Transcripts of inflammatory mediators including *CXCL10*, *GZMB*,
100 *CCL2* and *CCR7* (receptor for *CCL19*), previously shown to be associated with other forms
101 of murine (23-25) or human CL (26-28) were also downregulated with initiation of treatment
102 (Supplemental Table 3).

103

104 We next conducted multiplexed antibody digital spatial profiling (29) for 59 immune targets,
105 selecting regions of interest (ROIs) based on expression of CD3⁺ and/or CD68⁺
106 (Supplemental Figure 5 and Figure 2, A-F). The t-SNE dimensional reduction on a total of
107 33 regions of interest (ROIs) analysed from three patients (P4, P6 and P7) (Figure 2G)
108 indicated a considerable degree of inter-patient heterogeneity in pre-treatment lesional protein
109 profiles, but with clear discrimination for each patient between pre- and on-treatment ROIs.
110 Upon treatment, IDO1 and PD-L1 as well as PD-1 were selectively reduced in expression
111 (Figure 2, H and I). STRING analysis of all discoveries based on FDR (5%) also indicated
112 significant enrichment in GO: 002684, as well as a pathway associated with regulation of T
113 cell activation (GO: 0050863; Supplemental Figure 6, A-B).

114

115 As IDO1 and PD-L1 have been targeted in cancer immunotherapy and hold promise for drug
116 re-purposing, we next sought to further validate these findings using quantitative IHC in an
117 independent cohort of CL patients (5 females, 18 males; mean age \pm standard deviation, 44 \pm
118 11 years; time to diagnosis 7.76 \pm 8.2 months; Supplemental Figures 7 and 8 and
119 Supplemental Table 4) sampled at baseline and after 4 weeks of treatment. Using an
120 accepted cut-off of >5% of cells being positive (30), all patients (n=23) expressed IDO1
121 (Histochemical (H)-score (31) median = 81.2; range 16 - 165) and 20/23 patients had a
122 reduction in the abundance of IDO1⁺ cells on treatment (H-score median = 32; range 1 – 171;
123 p=0.0023; Figure 2J). All patients were PD-L1 positive at presentation (n=23; H-score
124 median = 82.8; range 12-164) and 20/23 patients exhibited a reduction in the number of PD-
125 L1 expressing cells on treatment (Figure 2J; H-score median = 36.7; range 12.3-36.7;
126 p=0.0008). Collectively, these data indicate that IDO1 and PD-L1 are highly expressed in the
127 lesions of Sri Lankan CL patients and reduction in expression of these two checkpoint
128 molecules represents an early response to SSG.

129 Though in vitro studies have indicated that intracellular parasitism by *Leishmania* could
130 impact on the expression of immune checkpoint molecules (32-34), this has not been
131 established in situ during human disease. To address this question, we combined IHC with
132 RNA-FISH (35) to identify *Amastin* transcripts (as a surrogate for viable amastigotes) with a
133 bespoke StrataQuest image analysis pipeline (Supplemental Figure 9, A-F). In 7 patients
134 studied that were *Amastin*⁺ at presentation (Supplemental Methods, Supplemental Table 5),
135 PD-L1 expression co-localised with CD68⁺ macrophages (Figure 3A, Supplemental Figure
136 10C) and parasitized cells were both PD-L1⁺ and PD-L1⁻ (Figure 3A). We binned the
137 *Amastin*⁺ PDL1⁺ and *Amastin*⁻ PDL1⁺ cells based on PD-L1 mean fluorescent intensity
138 (Figure 3, B-D) and found that cells containing abundant *Amastin* transcripts expressed more
139 PD-L1 than cells with less or no *Amastin* transcripts (Figure 3, B-E, Supplemental Figure 9,
140 G-L and Supplemental Figure 10). To independently corroborate this observation, we
141 showed that a Sri Lankan strain of *L. donovani* was also capable of inducing up-regulation of
142 PD-L1 expression on human monocyte-derived macrophages in vitro (Supplemental Figure
143 11, A-F) , as previously described for *L. major* (34). Similarly, IDO1 extensively co-localised
144 with CD68⁺ cells (Supplemental Figure 11A) and both IDO1⁺CD68⁺ and IDO1⁻CD68⁺ cells
145 were infected (Supplemental Figure 11B). Using a similar gating strategy (Supplemental
146 Figure 11C-H; n=3 patients), we found that cells with abundant *Amastin* transcripts expressed
147 more IDO1 than those with fewer or no *Amastin* transcripts (Supplemental Figure 11, I-K).
148 These data show that, although a notable population of uninfected CD68⁺ cells contribute to
149 PD-L1 and IDO-1 expression within CL lesions, intracellular parasitism leads to heightened
150 expression of these checkpoint molecules in lesional monocytes and macrophages.
151
152 Finally, we tested whether reduction in IDO1 or PD-L1 expression early during therapy could
153 be used as a prognostic marker for treatment response. Patients with the greatest reduction in

154 PD-L1 expression (i.e. greater than the geomean of the pre-treatment: on-treatment
155 expression ratio; n=12 patients) (Figure 4, A-B) cured earlier than those that had lower or no
156 reduction in PD-L1 expression (p=0.015). Patients with lower PD-L1 expression after 4
157 weeks of treatment (i.e. lower than the geomean of on-treatment expression; n=12 patients)
158 also cured faster (p=0.0045; Figure 4B). We assessed the association of PD-L1 with disease
159 cure rate using univariate Cox Proportional Hazard regression (Supplemental Figure S13A;
160 Hazard Ratio (HR) = 3.96, p=0.008). Upon adjustment for age and gender of the participants,
161 HR increased to 4.88 (p= 0.007; Figure 4D), indicating that patients that maximally reduced
162 PD-L1 expression upon treatment were about 5 times more likely to cure earlier. Conversely,
163 patients remaining parasite PCR⁺ at 4 weeks post treatment had a significantly longer cure
164 time (Figure 4E) and higher PD-L1 expression (Figure 4F). Surprisingly, reduction in IDO1
165 expression, calculated as either pre-treatment: on-treatment expression ratio or IDO1
166 expression at 4 weeks (n=12 vs 11), did not correlate with cure rate (Supplemental Figure 13,
167 B and C). Thus, the relationship between declining PD-L1 expression and rate of cure
168 (Figure 4, E-F) appears selective.

169

170 We conclude that expression of IDO1 and PD-L1 immune checkpoint molecules is a
171 common feature of Sri Lankan CL and that intracellular parasitism is associated with
172 heightened expression of these immunoregulatory proteins in lesional macrophages. Tissue
173 expression of both IDO1 and PD-L1 reduces significantly within 2-4 weeks of treatment
174 onset and well in advance of clinical cure, and a reduction in PD-L1 is associated with a more
175 rapid therapeutic response. The elevated expression of negative immune regulators on
176 macrophages at the lesion site, as shown here, has clear parallels with tumour-associated
177 macrophages (36) and extends our understanding of how *Leishmania* parasites influence the
178 function of their host cell during human disease (37). Though longitudinal sampling of the

179 same macrophage population was not possible, it seems likely that reduction of PD-L1
180 expression is facilitated by the leishmanicidal action of SSG, suggesting a model for drug-
181 immune synergy whereby early rounds of SSG treatment reduce intracellular parasite burden
182 leading to reduced checkpoint inhibition and re-engagement of T cell effector function. Our
183 data, together with strong pre-clinical evidence of an inhibitory role of PD-L1 in various
184 forms of leishmaniasis (10, 12, 38) supports the candidacy of PD-L1 blockade as an adjunct
185 HDT in Sri Lankan CL. In addition, our data suggest the possibility that changes in PD-L1
186 expression early after treatment could be considered as a biomarker to trigger drug tapering
187 or drug cessation.

188

189

190 **Methods**

191 Information is provided in Supplemental methods.

192 *Study approval*

193 The study was conducted in accords with the principles of the Declaration of Helsinki and
194 was approved by the Ethical Review Committee of the Faculty of Medical Sciences,
195 University of Jayewardenepura (Ref: 780/13 & 52/17) and the Department of Biology,
196 University of York. Written informed consent, including for lesion photographs, was
197 received from participants prior to inclusion in this study.

198

199

200

201 **Author Contributions**

202 NSD, SS, VS, NM, BS, MMDO, LR, SM and SR conducted experiments. NSD and SF
203 performed data analysis. NSD and PMK wrote the manuscript. PW, MC, HG, RW, DL, PMK
204 and SR were involved in conceptualisation and securing funding and PMK and SR
205 supervised the study. The order of the co-first authors was determined by their relative
206 contribution to this study.

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215 decision to publish.

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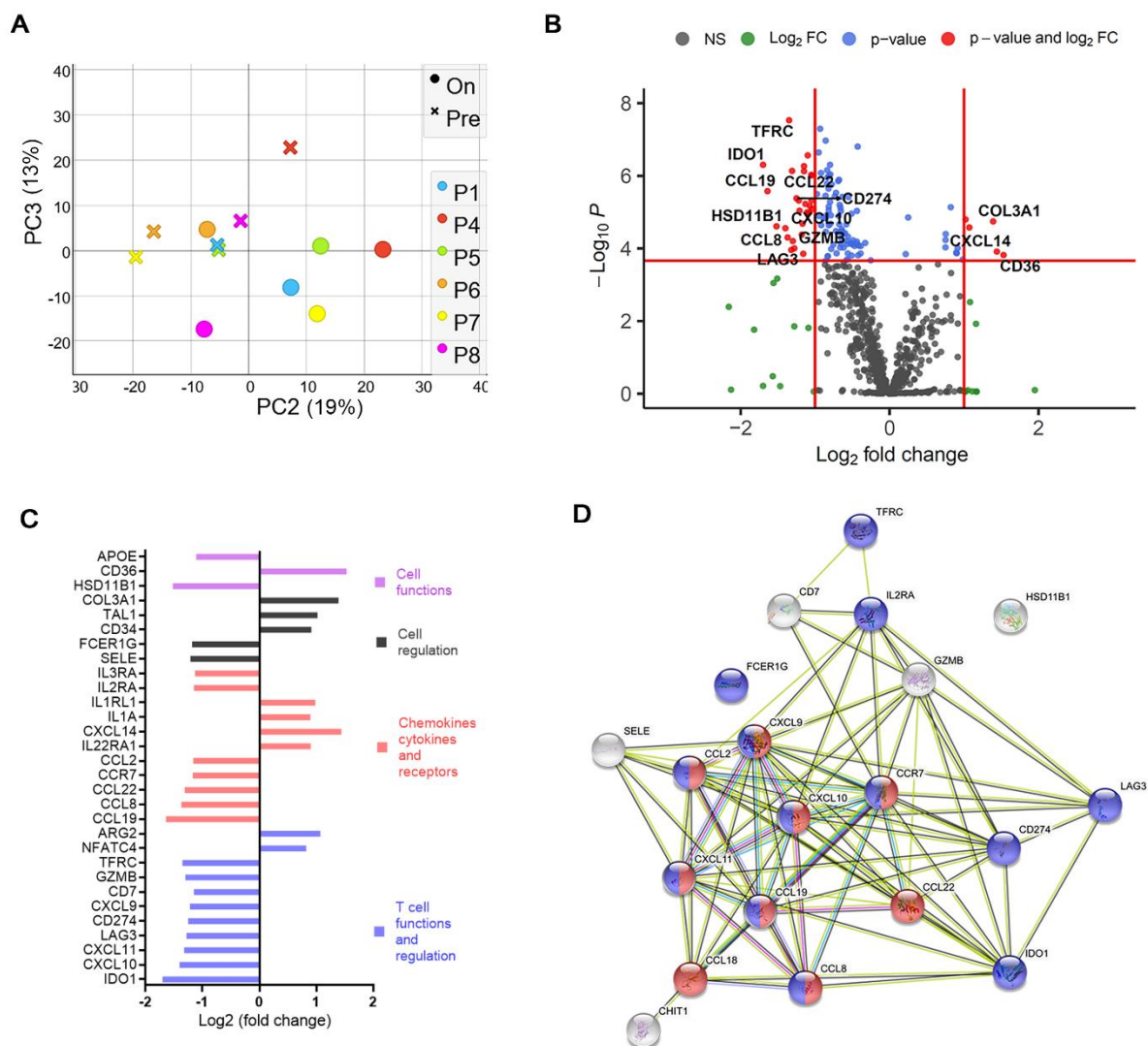
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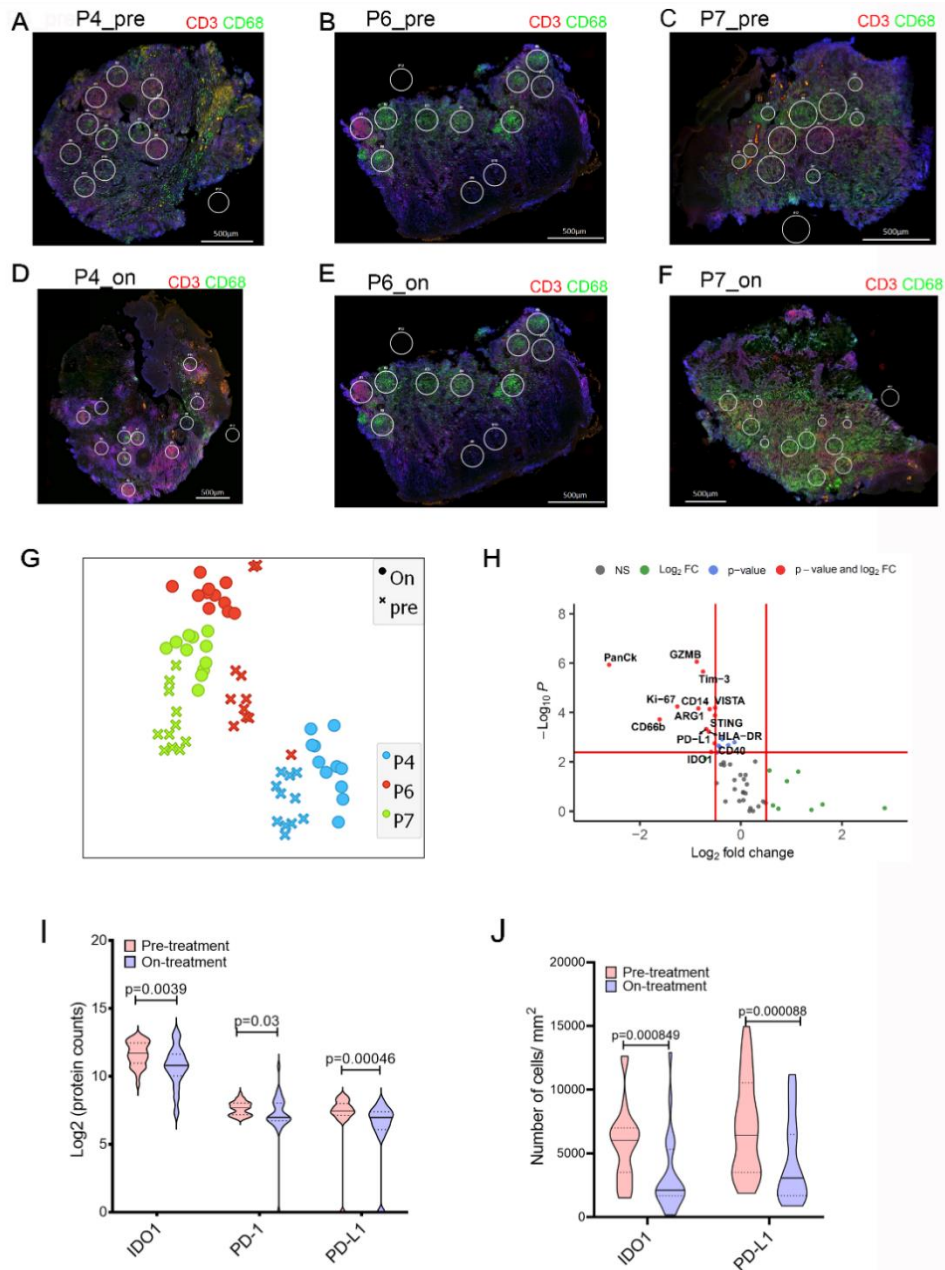
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324
 325 **Figure 1. Differential expression and network analysis of genes regulated by drug**
 326 **treatment in lesions of Sri Lankan CL patients.**

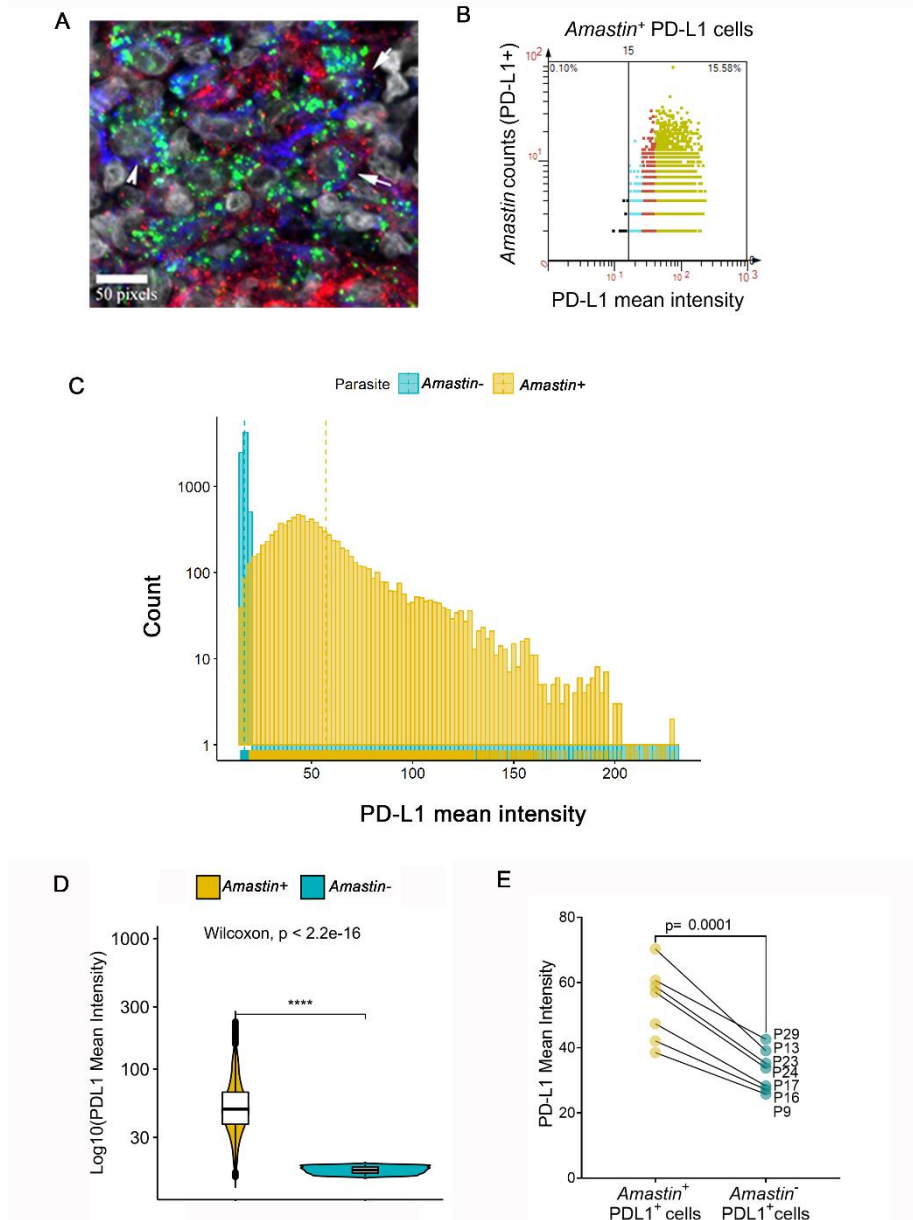
327 Immune-targeted tissue transcriptomics was conducted on tissue sections from test cohort
 328 patients comparing transcriptomes at presentation and on treatment. (A) Principal component
 329 analysis was performed to show differences between pre- and on-treatment transcriptome of
 330 each patient based on 770 gene nCounter PanCancer Immunology Panel (n=6) (B)
 331 Differentially expressed genes comparing pre-treatment biopsies with biopsies taken after
 332 two weeks on treatment (SSG). Cut off (red line) drawn at equivalent of adjusted p-value
 333 =0.01 and Log (Fold change) of 1(C) Top 30 genes that changed in expression on SSG
 334 treatment. (D) STRING protein-protein interaction network (22)(<https://string-db.org>)
 335 analysis of genes listed in **Supplemental Table 3** down-regulated on SSG treatment.
 336 Pathways represent GO: 0072676, Lymphocyte migration (red spheres) and GO: 0002684,
 337 positive regulation of immune system process (blue spheres). Top 20 genes are shown
 338 (Log2fold change ≥ 1.15) for clarity.



339
340 **Figure 2. Digital Spatial Profiling (DSP) of CL lesions.**

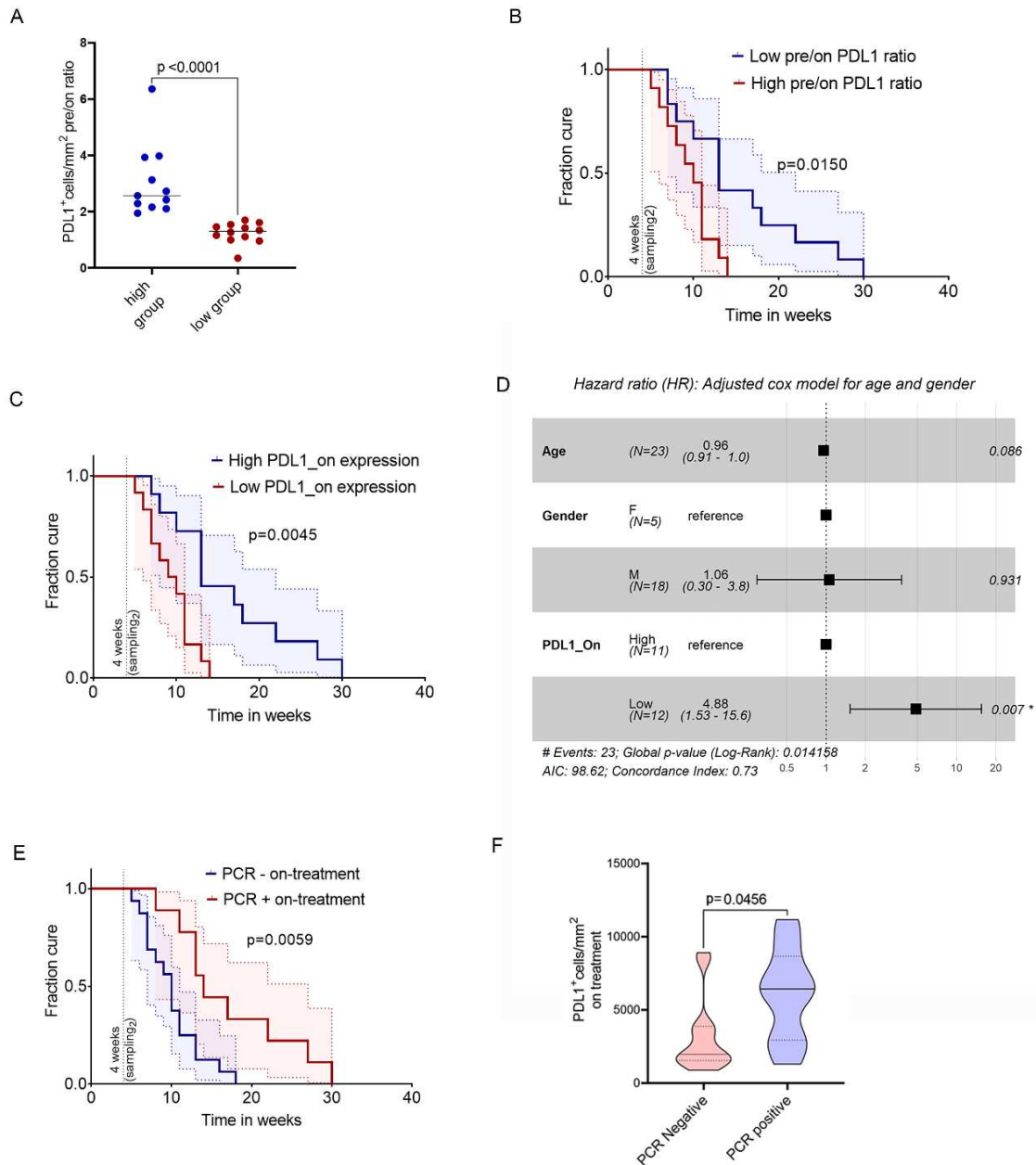
341
342 DSP was performed on tissue sections from test cohort individuals comparing ROIs from pre
343 and on-treatment biopsies. (A-F) ROIs on CD3⁺ and/or CD68⁺ rich areas from pre and on-
344 treatment biopsies from patients P4, P6 and P7 (CD68, green; CD3, red; Syto13, blue). 20x
345 magnification; scale bar, 500µm (G) t-SNE plot based on 20 PCA loadings coloured on
346 patient ID. (H) Differential protein expression analysis comparing pre-treatment to on-
347 treatment ROIs. Red lines indicate adjusted p value cut off of 1% (Mann-Whitney test with
348 FDR correction based on Benjamini, Krieger, and Yekutieli two stage set-up method) and
349 and Log₂FC = 0.5 (n=33 ROIs) (I) IDO1, PD-1 and PD-L1 expression in pre- and on-
350 treatment ROIs. Mann Whitney rank test (n=33 ROIs). (J) Immunohistochemistry (IHC) was
351 performed on sections from patients pre and on-treatment from the validation cohort and
352 quantitated using StrataQuest (see Methods) (n=23). Wilcoxon matched-pairs signed rank
353 test. Dotted lines show upper and lower quantile in I-J, median by solid line.

354



355 **Figure 3 Immunofluorescence analyses of PD-L1 in infected and uninfected cells**

356 Dual IHC-FISH using an *Amastin* probe was performed on pre-treatment sections of patients
 357 enrolled in the validation cohort. (A) A 400x confocal image showing infection of PD-
 358 L1⁺CD68⁺ (arrows) and PD-L1⁺CD68⁺ (arrowhead) cells. Scale bar, 50 pixels (B)
 359 Relationship between PD-L1 expression and parasite burden (*Amastin* dot count).
 360 Scattergram from a representative patient (P24 at presentation) showing *Amastin*⁺ low (cyan),
 361 medium (red) and high (green) PD-L1 expressing cells with respect to parasite abundance.
 362 (C) Fluorescence intensity distributions of infected and uninfected PD-L1 cells (D) Mean
 363 fluorescent intensity of PD-L1 expression on *Amastin*⁻ cells compared to *Amastin*⁺ cells from
 364 a representative patient P24. The upper and lower whisker represents highest and lowest
 365 value that is within 1.5 * interquartile range. N=9159 parasite positive cells and N=41520 for
 366 parasite negative cells. Significance score was generated using Wilcoxon signed rank test.
 367 (E) PD-L1 expression on *Amastin*⁺PD-L1⁺ cells vs. *Amastin*⁻PD-L1⁺ cells (n=7 patients).
 368 Significance score was generated using Students two-tailed paired t-test after testing for
 369 normality using Shapiro Wilk and Kolkogorov-Smirnov tests.



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Figure 4 Clinical correlates of PDL1 reduction on treatment in CL patients

(A) Patients (validation cohort; n=23) were stratified based on high (>geomean value; n=11) and low (< geomean value; n=12) pre-: on-treatment expression ratio. (B) Kaplan-Meier curve based on pre-:on-treatment ratio of PD-L1 expression (high vs low). (C) Patients stratified based on on-treatment expression of PD-L1 (> geomean value; n=11 vs < geomean value; n=12). (D) Multivariate Cox Proportional Hazards model plotted as a forest plot. p-values for each covariate represent Wald statistic value and overall statistical significance is also indicated. (E) Patients stratified by *LITS1* PCR status (n= 9 PCR⁺ vs n=14 PCR⁻ or ^{+/-} (equivocal)) on treatment. (F) PD-L1 expression in *LITS1* PCR⁺ vs. PCR⁻ individuals on treatment. Dotted lines show upper and lower quantile, solid line shows median. P-value generated using two-tailed Mann-Whitney test. Vertical line drawn in B, C, E on the X axis shows time when on-treatment biopsies were collected. Curves in B, C, E were compared using Log-rank (Mantel-Cox) test. Blue and red shaded area show 95% CI of the two groups.