

PD-1 blockade induces responses by inhibiting adaptive immune resistance

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Therapies that target the programmed death-1 (PD-1) receptor have shown unprecedented rates of durable clinical responses in patients with various cancer types^{1–5}. One mechanism by which cancer tissues limit the host immune response is via upregulation of PD-1 ligand (PD-L1) and its ligation to PD-1 on antigen-specific CD8⁺ T cells (termed adaptive immune resistance)^{6,7}. Here we show that pre-existing CD8⁺ T cells distinctly located at the invasive tumour margin are associated with expression of the PD-1/PD-L1 immune inhibitory axis and may predict response to therapy. We analysed samples from 46 patients with metastatic melanoma obtained before and during anti-PD-1 therapy (pembrolizumab) using quantitative immunohistochemistry, quantitative multiplex immunofluorescence, and next-generation sequencing for T-cell antigen receptors (TCRs). In serially sampled tumours, patients responding to treatment showed proliferation of intratumoral CD8⁺ T cells that directly correlated with radiographic reduction in tumour size. Pre-treatment samples obtained from responding patients showed higher numbers of CD8⁺, PD-1- and PD-L1-expressing cells at the invasive tumour margin and inside tumours, with close proximity between PD-1 and PD-L1, and a more clonal TCR repertoire. Using multivariate analysis, we established a predictive model based on CD8 expression at the invasive margin and validated the model in an independent cohort of 15 patients. Our findings indicate that tumour regression after therapeutic PD-1 blockade requires pre-existing CD8⁺ T cells that are negatively regulated by PD-1/PD-L1-mediated adaptive immune resistance.

Recently, we reported sustained tumour regression in 38% of patients in a multi-institutional, international, phase 1 expansion study evaluating the safety and clinical activity of pembrolizumab (formerly known as MK-3475 and lambrolizumab), a humanized monoclonal antibody against PD-1, in patients with advanced melanoma (<http://ClinicalTrials.gov> study number NCT01295827)^{3,8}. PD-L1, known to be expressed by cells in the tumour microenvironment, engages PD-1 on T cells and subsequently triggers inhibitory signalling downstream of the TCR, blocking effector functions and reducing T-cell killing capacity⁶. PD-L1 can be constitutively expressed on the surface of cancer cells through poorly characterized oncogenic signalling pathways^{9,10}, or alternatively, expressed in response to the presence of T cells producing immune-stimulating cytokines such as interferons^{7,11,12}. The process of expression of PD-L1 in response to cytokines has been termed adaptive immune resistance⁶, and represents a mechanism by which cancer cells attempt to protect themselves from immune-cell-mediated killing.

We sought to determine whether pre-existing tumour-associated CD8⁺ T cells inhibited by PD-1/PD-L1 engagement represent key factors in determining clinical response to PD-1 blocking therapy. Our study cohort consisted of 46 patients with advanced melanoma treated with single-agent pembrolizumab between December 2011 and October 2013 at UCLA (Institutional Review Board (IRB) study number 11-003066). Patients underwent tumour biopsies before and during treatment. Baseline

biopsy samples from 15 additional patients with advanced melanoma enrolled in the same pembrolizumab phase 1 clinical trial at Gustave Roussy in Villejuif, Paris, France (IRB study number 11-040) were analysed as a validation cohort (Extended Data Table 1).

We first examined the spatiotemporal dynamics of CD8⁺ T cells by performing qualitative and quantitative immunohistochemistry (IHC) analysis for CD8 expression before and during PD-1 blockade in two tumour compartments: the invasive tumour margin (stromal–tumour edge) and inside the tumour parenchyma (tumour centre)^{13,14}. S100 expression was used to define the invasive margin and tumour centre (Extended Data Fig. 1a). Pre-treatment samples obtained from patients who experienced a tumour response (response group; Fig. 1a), showed higher CD8⁺ cell densities at the invasive margin when compared to samples from patients who progressed during therapy (progression group; Fig. 1b). Extended Data Table 2 provides the anatomical location of all tumours serially sampled. Serially sampled tumours during treatment exhibited a parallel increase in CD8⁺ cell density at both the invasive margin and tumour centre in the response group (Spearman's correlation $r = 0.71$, $P < 0.001$; Fig. 1c), but not in the progression group (Fig. 1d). Two patients experienced delayed responses (Fig. 1c, triangles) and showed step-wise accumulation of CD8⁺ cells, with initial increases restricted to the invasive margin, followed by mobilization into the tumour parenchyma (Extended Data Fig. 1b).

Releasing the PD-1 immune checkpoint in pre-existing tumour-antigen-specific T cells should lead to T-cell proliferation, intratumoral infiltration and increased effector function. We found a greater increase in CD8⁺ density from baseline to post-dosing biopsy that significantly correlated with a decrease in radiographic tumour size (Extended Data Fig. 2a; Spearman's correlation $r = -0.75$, $P = 0.0002$). During treatment, we found an increase in cells that were double positive for CD8 and the nuclear proliferation marker Ki67 in samples from patients with a tumour response. We observed all sub-phases of mitosis based on characteristic chromatin patterns (Fig. 2 and Extended Data Fig. 2b, c). The post-dosing increase in CD8/Ki67 double-positive cells was restricted to the tumour parenchyma. We found increased expression of granzyme B, a cytotoxic granule reflective of CD8 effector function, on CD8⁺ cells in post-dosing biopsies in the response group ($P < 0.0001$; Extended Data Fig. 3a, b).

The correlation between T-cell activation/effector function and treatment outcome upon release of the PD-1 immune checkpoint may be driven by the production of interferons by tumour-infiltrating CD8 cells that induce PD-L1 expression on tumour-resident cells^{11,15}. To test this hypothesis, we stained baseline and post-dosing biopsies for phospho-STAT1 (pSTAT1), which is an immediate downstream effector upon interferon- γ binding to its receptor (Extended Data Fig. 3c, d). The response group was associated with significantly higher expression of pSTAT1 at the invasive margin, localized to the area of CD8 infiltrate, before ($P = 0.002$) and during treatment ($P < 0.0001$), when compared to biopsies from the progression group. In serially sampled tumours from

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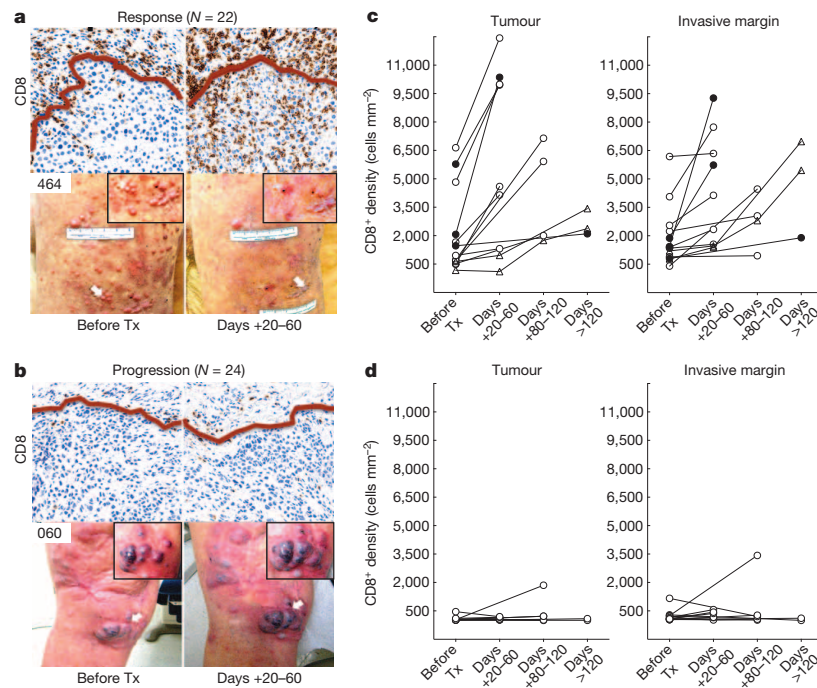


Figure 1 | Immunohistochemical analysis of CD8⁺ T cells in samples obtained before and during pembrolizumab treatment. **a, b,** Examples of CD8 expression in melanoma tumours serially biopsied before PD-1 blocking treatment (Tx) and 20–60 days after treatment began (Days +20–60) from a patient in the response (**a**) and progression (**b**) groups. Red line separates tumour parenchyma (below line) and invasive margin (above line).

Magnification, $\times 20$. **c, d,** CD8⁺-cell density at the tumour parenchyma and invasive margin in samples from all responders (**c**; $n = 13$) and progressors (**d**; $n = 12$) who received a biopsy before and during treatment. Filled circle indicates complete response; open circle indicates partial response; triangle indicates delayed response.

the response group, pSTAT1 expression was also found to be significantly higher during treatment when compared to baseline ($P = 0.007$) (Extended Data Fig. 3e). These findings prompted us to investigate the association between CD8⁺-, CD4⁺-, PD-1⁺- and PD-L1⁺-cell densities in baseline biopsies in regards to response to treatment (Fig. 3a). The response group was associated with significantly higher numbers of CD8⁺-, PD-1⁺ and PD-L1⁺ cells at both the invasive margin and the tumour centre when compared to the progression group (CD8, $P < 0.0001$; PD-1, $P = 0.0002$; PD-L1, $P = 0.006$). However, CD4 expression at baseline was not found to correlate with treatment outcome. No relationship was found between previous treatment history with ipilimumab (anti-CTLA4) and CD8 expression or treatment outcome (Extended Data Table 3).

We next determined the relative proximity of PD-1 and PD-L1 as evidence of a physical interaction between PD-1⁺ and PD-L1⁺ cells, a presumptive requisite for adaptive immune resistance. Figure 3b shows representative examples of chromogenic PD-1 and PD-L1 expression in serially cut tissue sections as well as multiplexed PD-1 $\times$ PD-L1 immunofluorescence in pre-treatment samples according to treatment outcome. Using quantitative multiplexed PD-1 $\times$ PD-L1 immunofluorescence, we found a significant correlation between proximity of PD-1 and PD-L1 and response to therapy (Fig. 3c; $P = 0.005$).

We next investigated the relationship between CD8 and PD-L1 using a Spearman's correlation analysis and found the two markers to correlate in both the tumour (Spearman $r = 0.598$, $P < 0.001$) and the invasive

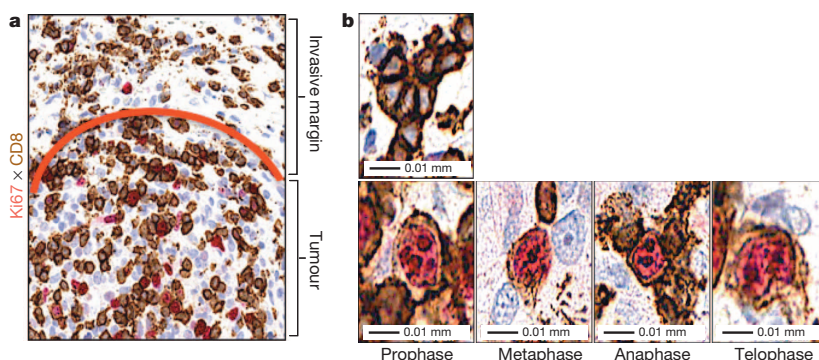


Figure 2 | Regressing tumours during treatment are associated with proliferating CD8⁺ T cells that localize to the tumour. **a,** Representative example of CD8/Ki67 chromogenic double staining from a sample obtained during tumour regression shows double-positive T cells localized to the tumour parenchyma. The red line separates the invasive margin (above line) and

tumour (below line). **b,** Top, representative single-positive quiescent CD8⁺ brown cells (no Ki67 labelling) from the invasive margin. Bottom, representative double-positive cells (red, labelled Ki67 nucleus; brown, labelled CD8 membrane) with characteristic chromatin patterns associated with sub-phases of mitosis. Magnification, $\times 40$.

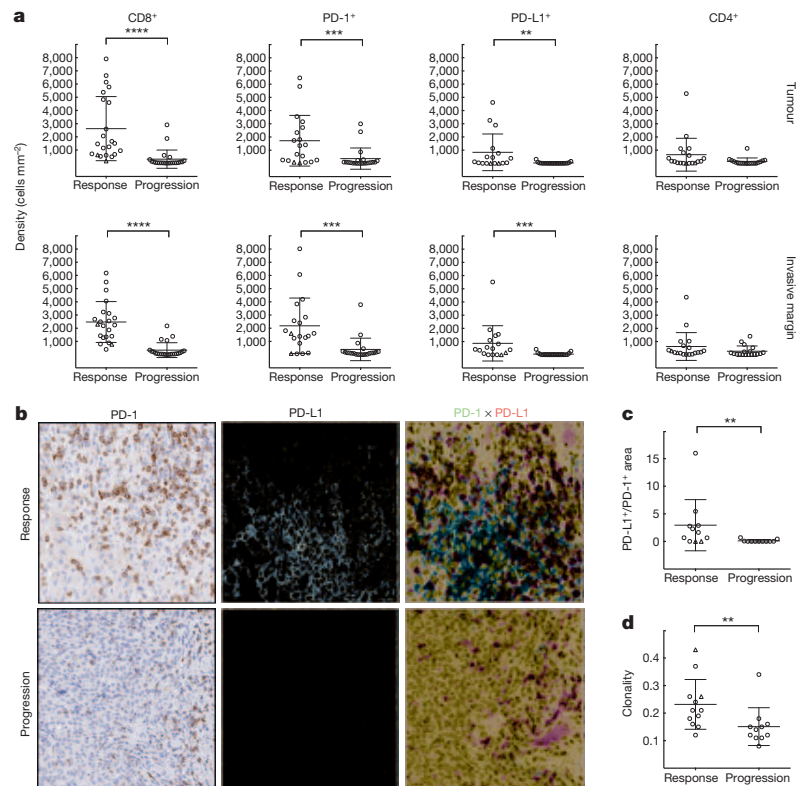


Figure 3 | Baseline density, location and proximity of CD8⁺, PD-1⁺, PD-L1⁺ and CD4⁺ cells, and T-cell repertoire according to treatment outcome. **a**, Melanoma samples collected before treatment with PD-1 blocking therapy were assessed for CD8 (response $n = 22$, progression $n = 24$), PD-1 (response $n = 19$, progression $n = 21$), PD-L1 (response $n = 17$, progression $n = 21$) and CD4 (response $n = 19$, progression $n = 18$) density by quantitative immunohistochemistry in the tumour compartment and at the invasive margin. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **b**, Examples of the relative

proximity of PD-1- and PD-L1-expressing cells in representative baseline samples from a responder and a progressor. **c**, Proximity analysis of PD-1 and PD-L1 based on multiplex quantitative immunofluorescence in baseline tumour samples (response $n = 11$, progression $n = 11$). ** $P = 0.005$. **d**, Results of TCR sequencing performed on 25 whole tumour samples taken at baseline (response $n = 12$, progression $n = 11$). Triangles indicate delayed response. ** $P = 0.004$. Error bars denote the s.d.; horizontal lines denote the mean.

margin (Spearman $r = 0.527$, $P < 0.001$). Furthermore, CD8 and PD-L1 density co-varied with treatment outcome in both the tumour and invasive margin ($P < 0.001$ for both; Extended Data Fig. 4a, b). Immunofluorescence multiplexing for CD8 and PD-1 corroborated our chromogenic IHC findings that CD8⁺ T cells represented the primary cellular source of PD-1 expression (Extended Data Fig. 4c). Using chromogenic double staining for SOX10 and PD-L1, we found PD-L1 expression on melanoma cells and also on cells morphologically consistent with lymphocytes and macrophages in samples obtained during treatment from the response group (Extended Data Fig. 5a). Principal component analysis of samples obtained before treatment showed that CD8, PD-1 and PD-L1 expression in the tumour ($P = 0.001$) and at the invasive margin significantly correlated with treatment outcome ($P < 0.0001$; Extended Data Fig. 5b).

The high density of CD8⁺ cells at the site of the tumour in the response group is suggestive of a specific immune response to tumour antigens. Therefore, we hypothesized that a more restricted TCR sequence usage would reflect a tumour-antigen-specific T-cell accumulation at the tumour site. Using genomic DNA isolated from pre-treatment samples, we performed next-generation sequencing to capture all uniquely rearranged, variable TCR β -chain regions^{16,17}. We found that a more restricted TCR β -chain usage, reflecting a T-cell population that was less diverse in repertoire and more clonal in nature, significantly correlated with clinical response to pembrolizumab treatment ($P = 0.004$; Fig. 3d). The clonality read-out was not found to highly correlate with tumour-infiltrating lymphocyte density ($R^2 = 0.04$; Extended Data Fig. 6a). However, biopsies from patients with a tumour response showed evidence of an enriched

population of T cells with unique specificities. In addition, comparison of the TCR clonality at baseline and post-dosing biopsies showed that in samples from the response group, more than ten times as many clones expanded after anti-PD-1 therapy than in the progression group (Extended Data Fig. 6b, c).

To create the best discriminatory model to assess the probability of clinical response to PD-1 blocking therapies, forward stepwise logistic regression was run on CD8⁺-, CD4⁺-, PD-1⁺- and PD-L1⁺-cell densities within the tumour and the invasive margin. Results of the stepwise procedure, and a logistic regression model, consistently selected the invasive margin CD8⁺ density as the best full predictive parameter (Extended Data Table 4a). The next best predictors were tumour CD8⁺ T-cell density, tumour and invasive margin PD-1⁺ density, and tumour and invasive margin PD-L1⁺ density. Tumour and invasive margin CD4⁺ density were the poorest predictors.

To test this predictive model, we obtained pre-treatment biopsies from 15 patients treated at Gustave Roussy and were blinded to treatment outcome. We quantified CD8⁺ T-cell density in the invasive margin and used our logistic model to calculate a predicted probability of response for each patient in the validation cohort (Extended Data Table 4b). Out of the 15 patients, we accurately predicted 4 out of 5 patients in the true progression group and 9 out of 9 patients in the true response group. There was one false-positive prediction and one patient predicted to respond who remains in stable disease.

Our studies build upon the evidence that response rates to PD-1 or PD-L1 blocking antibodies are higher in patients whose tumours express

PD-L1 (refs 1, 15). Since PD-L1 can be either constitutively expressed or induced upon T-cell recognition and production of interferons^{11,15}, we hypothesized that response to PD-1 blockade would more tightly covariate with the inducible PD-L1 expression in the presence of antigen-specific T cells⁷, termed adaptive immune resistance⁶. Indeed, we found PD-1-positive T cells interfacing with PD-L1-expressing cells within tumours in pre-treatment samples from responders. The clinical relevance of the relative distribution of PD-L1 expression on cancer cells, myeloid-derived cells and activated T cells in tumours, in terms of treatment outcome, remains to be elucidated. Our data suggest that PD-L1 may serve as an indirect marker of adaptive immune resistance in response to tumour-antigen-specific T-cell infiltration rather than as a static constitutive biomarker. Hence, inducing a type-I interferon inflammatory response in combination with PD-L1 blockade merits further clinical investigation¹¹.

T-cell infiltrates have been found to have predictive value with respect to the natural history of primary cancers^{13,14,18}. We build on this and report that the baseline density and location of T cells in metastatic melanomas have predictive value in the treatment outcome of patients receiving therapies that block the PD-1/PD-L1 axis. Releasing the PD-1 immune checkpoint results in clinically relevant anti-tumour activity when there is a greater density of pre-existing tumour-antigen-restricted CD8⁺ T cells that are negatively regulated by PD-1/PD-L1 interactions.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions P.C.T. and A.R. supervised the project and developed the concepts. P.C.T., C.L.H., C.R. and A.R. designed the experiments. P.C.T., C.L.H., M.S., C.R. and A.R. interpreted the data. A.R., P.C.T., C.L.H., C.R., J.H.Y., M.S., I.P.S., E.J.M.T., R.O.E., H.R. and R.H.P. gave conceptual advice and edited the manuscript. P.C.T., J.H.Y., I.P.S., A.N.W. and E.J.M.T. established IHC staining and/or imaging protocols. J.H.Y., I.P.S., E.J.M.T. and R.H.P. provided confirmatory pathology analyses. G.T. worked on IHC samples from the patients from Gustave Roussy. C.L.H., L.R., M.S., G.H., V.C., M.C., C.K. and E.S. provided technical support. P.C.T., T.R.G. and D.A.E. designed and implemented the predictive model and provided statistical support. P.C.T., B.C., A.J.G., C.M., J.A.G., G.C., C.R. and A.R. clinically evaluated patients in the trial.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Readers are welcome to comment on the online version of the paper. The authors declare competing financial interests: details are available in the online version of the paper. Correspondence and requests for materials should be addressed to P.C.T. (ptumeh@mednet.ucla.edu) or A.R. (aribas@mednet.ucla.edu).

METHODS

Tumour samples. Tumour biopsies were obtained from a subset of patients enrolled in a phase 1a clinical trial in which 411 patients were treated¹⁹; patients were selected for this analysis by having adequate tumour biopsy samples and clinical follow-up. All patients in the study and validation cohorts underwent mandatory biopsy of a metastatic tumour within 30 days of starting treatment and one or more optional biopsies at 20–60 days, 60–120 days, or greater than 120 days after starting anti-PD-1. Samples were immediately fixed in formalin followed by paraffin embedding. Biopsy collection and analyses were approved by UCLA IRBs 11-001918 and 11-003066. Tumour samples obtained from the initial cohort of 46 patients from UCLA underwent IHC analysis of CD8 ($n = 46$ patients, 45 samples before and 31 samples during treatment), CD4 ($n = 37$ patients, 37 samples before and 0 samples during treatment), PD-1 (41 = patients, 39 samples before and 26 samples during treatment), PD-L1 ($n = 38$ patients, 38 samples before and 24 samples during treatment), multiplex immunofluorescence for PD-1 and PD-L1 ($n = 26$ patients, 22 samples before treatment, 25 samples during treatment), multiplex chromogenic staining ($n = 13$ patients, 12 samples before and 17 samples during treatment), and TCR (V β) repertoire ($n = 23$ patients, 23 samples before treatment). The validation cohort included baseline biopsies of 15 patients from Gustave Roussy.

Treatment outcome groups. Patients at both sites received single-agent pembrolizumab intravenously in one of three dosing regimens: 2 mg kg⁻¹ every 3 weeks (2Q3W), 10 mg kg⁻¹ every 3 weeks (10Q3W), or 10 mg kg⁻¹ every 2 weeks (10Q2W) within a phase 1 clinical trial¹⁹. Tumour responses to pembrolizumab were evaluated at 12 and 16 weeks after the first infusion, and every 12 weeks thereafter. The response evaluation criteria in solid tumours (RECIST) version 1.1 was used to define objective clinical responses by an independent, central, blinded radiographic review. The protocol allowed us to proceed beyond initial progression at the resting scans at 3 months and have repeated imaging scans to confirm progression or response at least 4 weeks later following the immune-related response criteria (irRC)²⁰. Following this protocol-specified criteria, two patients had evidence of increase in size of target lesions at 12 weeks, but met criteria for objective partial response at 36 weeks and were considered within the response group but denoted as having a delayed response.

IHC staining. Slides were stained with haematoxylin and eosin, S100, CD8, CD4, CD80, Ki67, pSTAT1 and granzyme B at the UCLA Anatomic Pathology IHC Laboratory. Immunostaining was performed on Leica Bond III autostainers using Leica Bond ancillary reagents and REFINE polymer DAB detection system. Antibodies used included rabbit polyclonal S100 (DAKO, 1/1,500 dilution, low pH retrieval), CD8 clone C8/144B (DAKO, 1/100, low pH retrieval), rabbit monoclonal pSTAT1 clone D3B7 (Cell Signaling, 1/300 30 min, low pH retrieval), PD-1 and PD-L1 single-label chromogenic and multiplexed immunofluorescence triple staining were performed at Merck laboratories. All staining was performed on Dako autostainers. Signal in chromogenically labelled slides was visualized with DAB. Signal in immunofluorescently labelled slides was visualized with AlexaFluor 488 and AlexaFluor 594 TSA kits (Invitrogen), and nuclei were visualized with Prolong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). All stained slides were evaluated in a blinded fashion by one dermatopathologist and one investigator trained to identify the features of melanoma. PD-1 and PD-L1 stained slides were independently evaluated by one pathologist at Merck laboratories. Slides were examined for the presence of CD8, CD4, Ki67, PD-1 and PD-L1 within the tumour parenchyma (tumour) and the connective tissue surrounding the tumour (invasive margin).

The anti-PD-L1 antibody clone 22C3 used in the presented IHC studies is a mouse anti-human PD-L1 IgG1 κ generated through murine immunization with a fusion protein containing the human extracellular domain of PD-L1 and subsequent hybridoma formation. Binding was screened initially using a cell-based ELISA employing stably transfected CHO cells expressing human PD-L1 as positive control and parental (non-transfected) CHO cells as negative control. Further screening was performed to assess specificity as an IHC reagent for use in formalin-fixed paraffin-embedded (FFPE) sections through correlation of staining prevalence and intensity on FFPE cell pellets from melanoma cell lines to patterns of expression observed on aliquots of the same cells analysed for PD-L1 expression by flow cytometry, and for messenger RNA expression on serial sections of the same blocks using the Nanostring platform. Appropriateness of signal distribution in FFPE tissue was confirmed by correlation of IHC signal pattern in tissue with PD-L1 *in situ* hybridization studies performed on serial sections of the same blocks. Attribution of IHC signal to binding of complementarity determining regions of the antibody was confirmed by abrogation of signal on pre-adsorption of the antibody with immunogen. Antigen stability in cut slides and within paraffin blocks was evaluated through time course studies to determine changes in dynamic range of the assay on pre-sectioned slides as well as on fresh cut slides from existing paraffin blocks over periods of months.

Sections cut from FFPE blocks were deparaffinized and rehydrated with serial passage through changes of xylene and graded ethanol for PD-1 and PD-L1 IHC. All slides were subjected to heat-induced epitope retrieval in Envision FLEX Target

Retrieval Solution, High pH (catalogue number K8012, Dako). Endogenous peroxidase in tissues was blocked by incubation of slides in 3% hydrogen peroxide solution before incubation with primary antibody (anti-PD-L1 clone 22C3, Merck Research Laboratories, or anti-PD-1 clone NAT105, Cell Marque) for 60 min. Antigen-antibody binding was visualized via application of the FLEX+ polymer system (catalogue number K8012, Dako) and application of 3,3'-diaminobenzidine (DAB) chromogen (K4368, Dako). Stained slides were counterstained with haematoxylin and coverslipped for review.

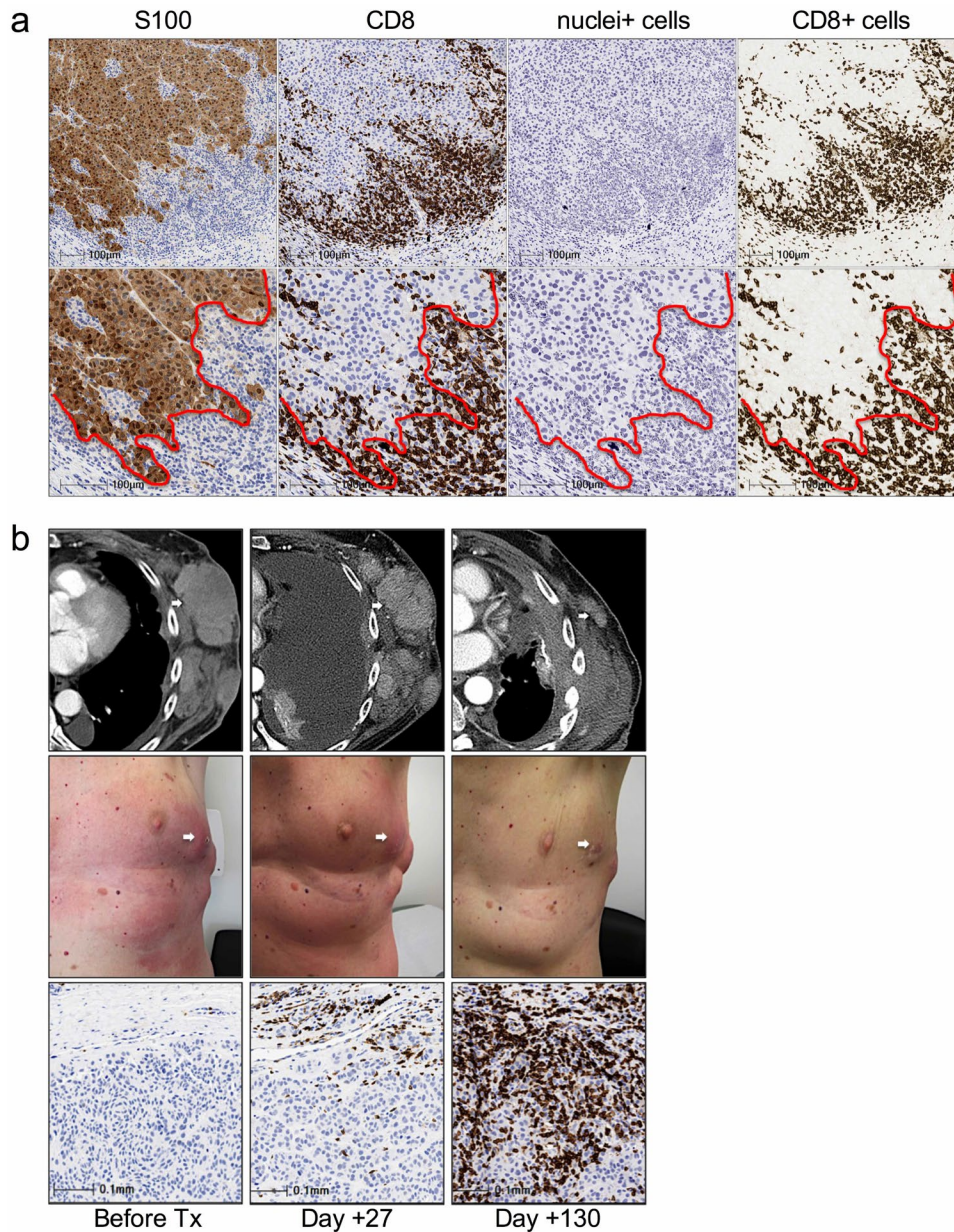
Digital image acquisition and analysis. All slides were scanned at an absolute magnification of $\times 200$ (resolution of 0.5 μ m per pixel). An algorithm was designed based on pattern recognition that quantified immune cells within S100-positive areas (tumour) and S100-negative areas (invasive margin). Image analysis based on RGB (red, green, blue) spectra was used to detect all cells by counterstaining with haematoxylin (blue), and DAB or fast red. The algorithm calculated the density (cells mm⁻²) and percentage cellularity (% positive cells/all nucleated cells) using Indica Labs Halo platform. For the proximity analysis slides fluorescently stained for PD-1 (AlexaFluor 488, green channel) and PD-L1 (AlexaFluor 594, red channel) were scanned to generate digital images in which the entire tumour region was sampled using 10,000 random disks. Disks containing both green and red signal were scored as positive for PD-1/PD-L1 proximity. The total number of positive disks was summed to yield a proximity score for each sample. CD8 expression was determined using two read-outs that were independent of each other to account for tumour heterogeneity: cell density (number of positive cells per mm²) and percentage cellularity (number of positive cells per number of nucleated cells). Cell density and percent cellularity correlated significantly ($R^2 = 0.89$ in tumour and 0.84 in the invasive margin).

Next-generation sequencing for T-cell receptor clonality. TCR sequencing and clonality quantification was performed as previously described^{16,17} from tumour samples preserved using RNAlater (Qiagen) and stored at -80°C . DNA was isolated by mincing followed by extraction using a DNeasy kit (Qiagen). Melanin was then removed from visibly pigmented melanoma samples using a PCR Inhibitor Removal kit (Zymo Research). TCR- β CDR3 regions were amplified and sequenced using the survey ImmunoSeq assay in a multiplexed PCR method using 45 forward primers specific to TCR V β gene segments and 13 reverse primers specific to TCR J β gene segments (Adaptive Biotechnologies). Reads of length 87 bp were obtained using the Illumina HiSeq System. For each sample, Shannon entropy was calculated on the clonal abundance of all productive TCR sequences in the data set. Shannon entropy was normalized to the range by dividing Shannon entropy by the logarithm of the number of unique productive TCR sequences in the data set. This normalized entropy value was then inverted ($1 - \text{normalized entropy}$) to produce our clonality metric.

PCR template abundance estimation. To estimate the average read coverage per input template in our multiplex PCR and sequencing approach, we employed a set of approximately 850 unique types of synthetic TCR analogue, comprising each combination of V β and J β gene segments¹⁷. These molecules were included in each PCR reaction at very low concentration so that most unique types of synthetic template were not observed in the sequencing output. Using the known concentration of the synthetic template pool, we simulated the relationship between the number of observed unique synthetic molecules and the total number of synthetic molecules added to the reaction (this is very nearly one-to-one at the low concentrations we used). These molecules then allowed us to calculate for each PCR reaction the mean number of sequencing reads obtained per molecule of PCR template, and thus to estimate the number of rearranged TCRs per diploid genome (that is, level of T-cell infiltration) in the input material.

Statistical analysis. Demographic, clinical and immunohistochemical variables were compared between responders and progressors using Wilcoxon rank-sum tests for ordinal or quantitative variables and Fisher's exact test for categorical variables. Receiver operating characteristic (ROC) curves for response versus progression were constructed to assess the prognostic ability of CD8, PD-1, CD4 and PD-L1 for both tumour and invasive margin measures. The area under the ROC curve (AUC) was used to measure model performance and the Wilcoxon test was used to assess significance of the AUC results. A logistic regression model was constructed using pre-treatment CD8⁺ density (cells mm⁻²) versus the outcome of clinical response (PR versus PD) using the study cohort. This fixed model was then applied to the CD8⁺ density measurements in the Gustave Roussy validation cohort to compute predicted probabilities of response to treatment. Sensitivity and specificities were calculated. Principal components analysis was used to decompose the variance for the markers (CD8⁺, PD-1⁺, PD-L1⁺ and CD4⁺-cell densities in cells mm⁻²) separately in the tumour and in the invasive margin. The first principal component accounted for the majority of the variability in the four markers (69.6% and 57.1% in the tumour and invasive margin, respectively). Principal component scores for the first principal component were compared between response groups with Wilcoxon rank-sum tests. Analyses were performed using GraphPad Prism, SAS and SPSS. All tests were two-sided and equal variance was not assumed unless otherwise stated. P values < 0.05 were considered statistically significant.

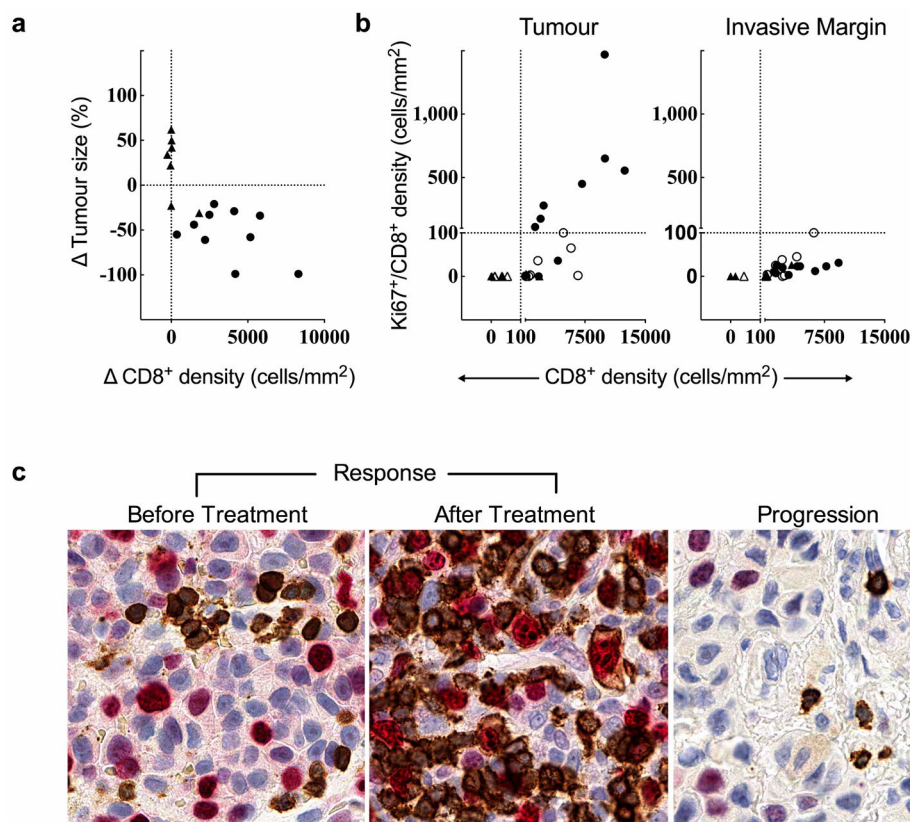
19. Ribas, A. *et al.* Efficacy and safety of the anti-PD-1 monoclonal antibody MK-3475 in 411 patients (pts) with melanoma (MEL). *J. Clin. Oncol.* **32**, 5s (2014).
20. Wolchok, J. D. *et al.* Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin. Cancer Res.* **15**, 7412–7420 (2009).



Extended Data Figure 1 | CD8⁺-cell infiltrates in tumour biopsies.

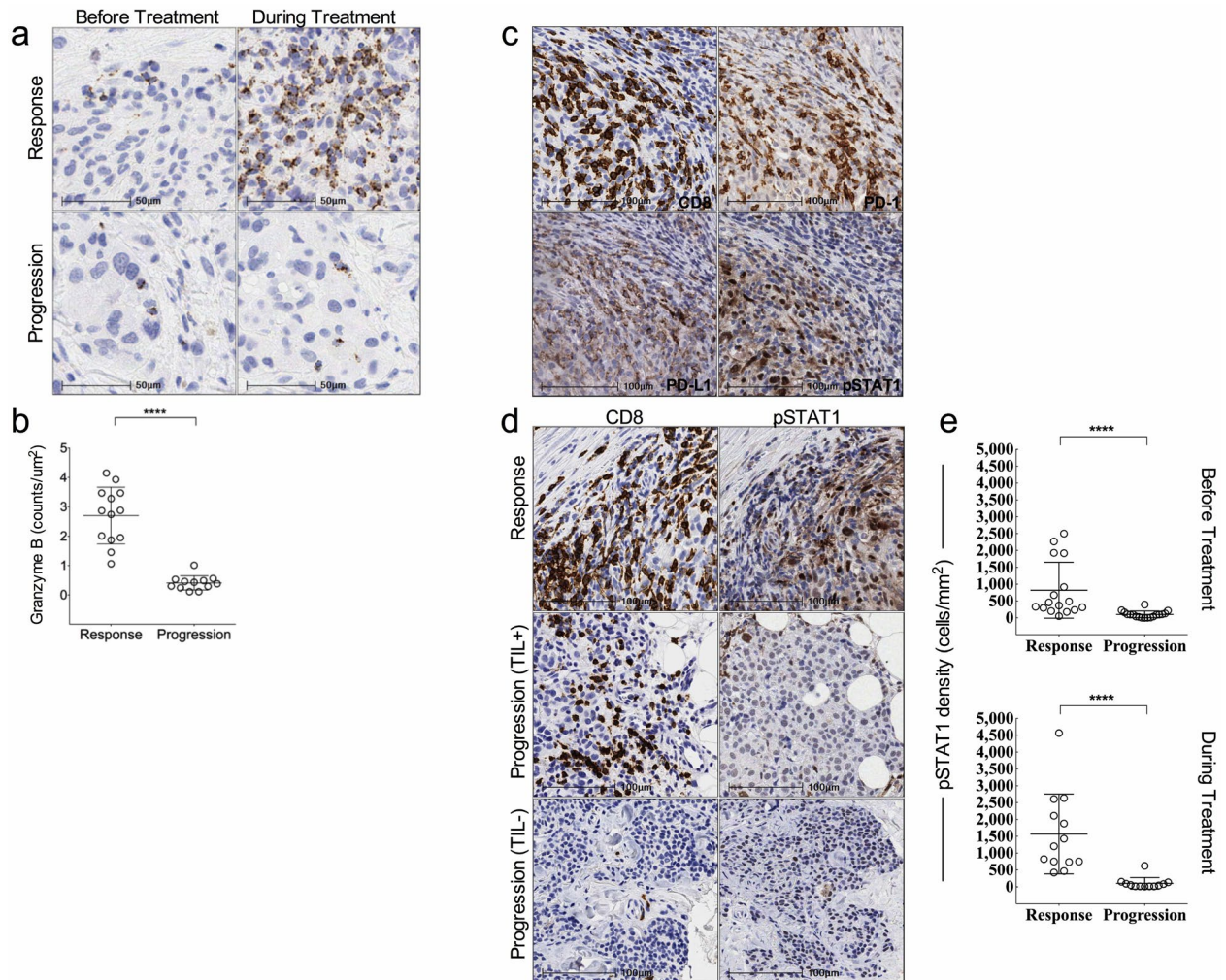
a. Segmentation of the invasive margin and tumour parenchyma using S100 and CD8 chromogenic staining. Low magnification (top row) and high magnification (bottom row) are shown. The red dotted line demarcates S100⁺ tumour (left of red line) from S100⁻ stroma (right of red line). Coordinates of the invasive margin and tumour parenchyma are generated from the S100 stained image (column labelled 'S100') and subsequently imported into the CD8 stained image (column labelled 'CD8'). This is followed by a deconvolution imaging algorithm of the CD8 stained image where first all nuclei (column labelled 'nuclei+ cells') are identified and quantified,

irrespective of what type of cell. This is followed by identifying CD8⁺ membrane (column labelled 'CD8⁺ cells') for cell quantification and analysis. **b.** CD8⁺ T-cell kinetics within the tumour microenvironment in a serially sampled tumour responding to PD-1 blocking therapy. Example of radiographic, clinical, and CD8 IHC in a serially sampled melanoma tumour of the left chest wall that was obtained from a patient with a delayed response. On day +27, clinical and radiographic examinations indicated progressive disease; at a time when CD8⁺ T cells expression increased in density at the invasive margin.



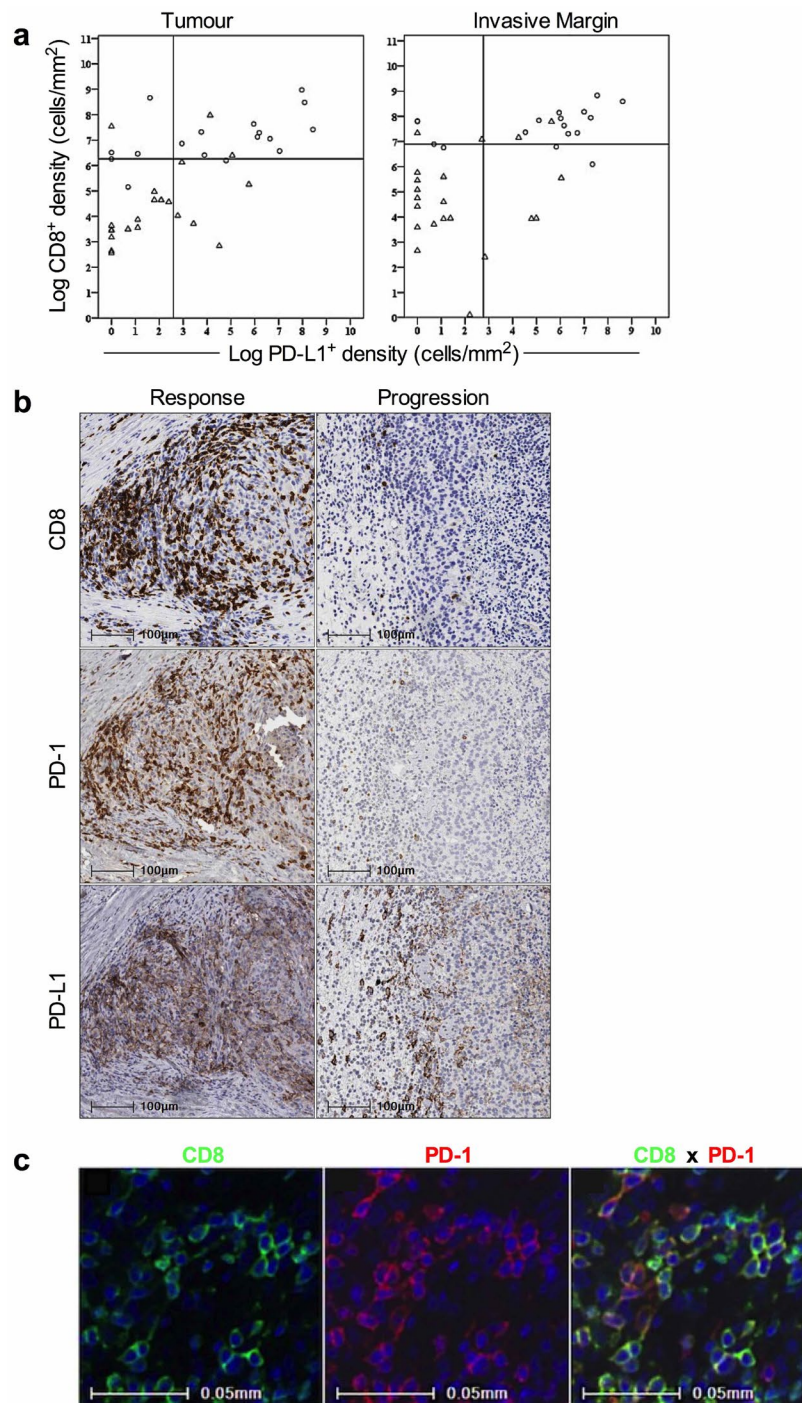
Extended Data Figure 2 | Proliferation of CD8⁺ T cells in regressing tumours. **a**, Relationship of the change in CD8⁺-cell density and best percentage change in tumour size in serially sampled tumours that were assessed using quantitative immunohistochemistry and CT scan measurements ($n = 18$, Spearman $r = -0.75$, $P = 0.0002$). **b**, CD8⁺-cell density and Ki67⁺/CD8⁺-cell density in the response group before treatment ($n = 11$, empty circles) and during treatment ($n = 17$, filled circles) and the progression group before treatment ($n = 9$, empty triangles), and during treatment ($n = 15$,

filled triangles). **c**, Representative examples of CD8/Ki67 chromogenic double staining from a biopsy of a patient with a tumour response and another with progression. Double-positive CD8 cells (red, labelled Ki67 nucleus; brown, CD8 labelled membrane) are not present at baseline, but are present during tumour regression in the biopsy from a patient with a tumour response. The double-positive cells are not in the biopsy of a patient with progression during treatment (no Ki67 labelling in brown CD8 cells). Magnification, $\times 40$.



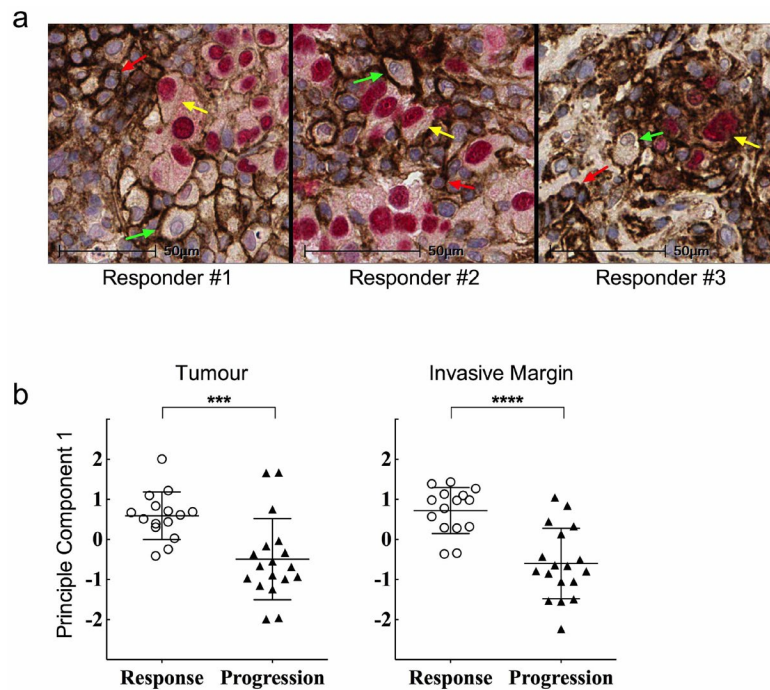
Extended Data Figure 3 | Granzyme B and pSTAT1 expression before and during treatment in terms of clinical response. **a**, Representative examples of granzyme B expression according to clinical response. **b**, Samples collected during PD-1 blocking therapy were evaluated for granzyme B signal (response $n = 13$, progression $n = 12$) using quantitative immunohistochemistry. **** $P < 0.0001$. **c**, Representative example of the proximity between CD8⁺, PD-1⁺, PD-L1⁺ and pSTAT1⁺ cells in the tumour microenvironment in a sample obtained before treatment from the response group. Magnification, $\times 40$. 2 μ M serial cut tissue sections were stained for CD8, PD-1, PD-L1 and pSTAT1. **d**, Localization of CD8⁺ and pSTAT1⁺ cells in samples obtained

before treatment from a biopsy in a patient with response and two patients with progression on anti-PD-1 therapy ($\pm$ a CD8 presence). The biopsy from a patient with disease progression had a moderate presence of CD8 cells that did not show pSTAT1 expression in the area. **e**, Using quantitative IHC analysis, the response group was associated with significantly higher expression of pSTAT1⁺ at the invasive margin before and during treatment (response $n = 16$, progression $n = 18$, $P = 0.002$ for pre-treatment biopsies; and response $n = 13$, progression $n = 12$, $P < 0.0001$ for post treatment biopsies). Within the response group, pSTAT1 expression was significantly higher during treatment when compared to baseline ($P = 0.022$).



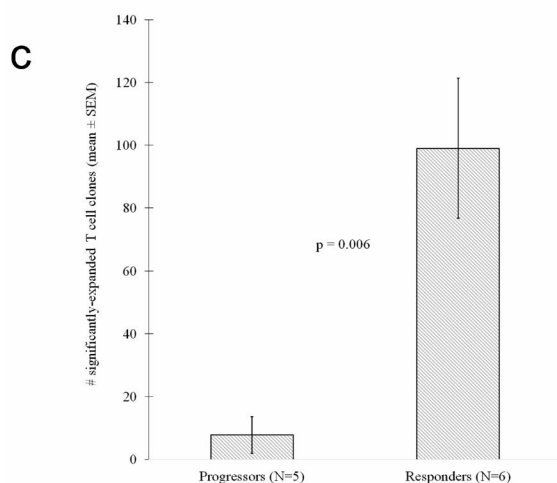
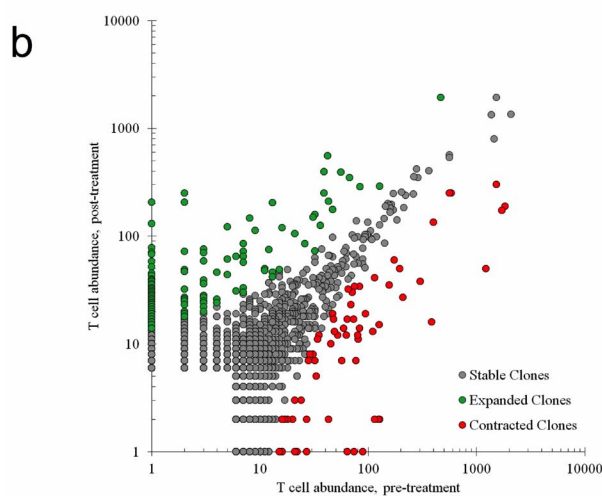
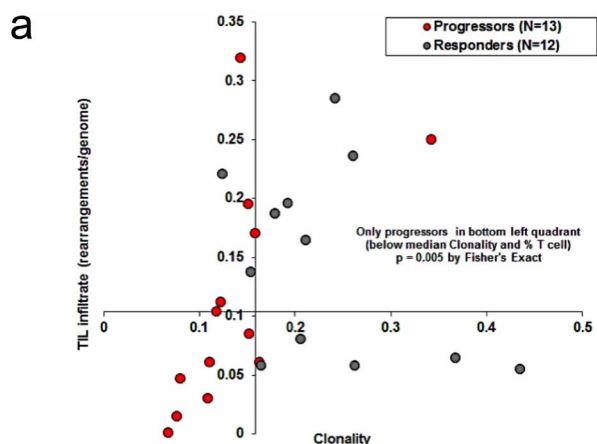
Extended Data Figure 4 | Relationship between CD8 and PD-L1 expression in terms of treatment outcome. **a**, Scatterplots of CD8 and PD-L1 density (cells mm⁻²) using a log($x+1$) scale are shown in samples obtained at baseline stratified by treatment outcome. Reference 'cut-points' for CD8 and PD-L1 densities were based on the median value for each marker across the entire cohort. Samples present in the CD8^{high}PD-L1^{high} quadrant, in both tumour and invasive margin, were predominantly derived from the response group. Samples present in the CD8^{low}PD-L1^{low} quadrants were significantly associated with the progression group (response $n = 17$, progression $n = 21$, $P < 0.001$ at both the invasive margin and tumour). **b**, CD8, PD-1 and PD-L1 expression before treatment in terms of clinical response. Representative

examples of the proximity between CD8⁺, PD-1⁺, PD-L1⁺ cells in the tumour microenvironment in pre-treatment samples obtained from a patient with a tumour response and progression. Magnification, $\times 20$. 2 μ m serial cut tissue sections were stained for CD8, PD-1 and PD-L1. **c**, Multiplexed immunofluorescence staining of CD8 and PD-1 to evaluate the relative co-expression of CD8 and PD-1 on individual cells within the tumour microenvironment. CD8⁺ cells were detected using AlexaFluor 488 staining in the green channel. PD-1⁺ cells were detected using AlexaFluor 594 staining in the red channel. High levels of co-expression of the two antigens were observed when the two channels are combined, yielding yellow signal in areas of co-localization.



Extended Data Figure 5 | PD-L1 expression and relationship with T-cell infiltration. **a**, Multiplexed chromogenic staining of SOX10 (red nucleus) and PD-L1 (brown membrane) to evaluate PD-L1 expression on melanoma cells, lymphocytes and macrophages within the tumour microenvironment. SOX10 is a transcription factor that is melanoma-cell specific. Representative high power fields of double-positive cells (yellow arrows) show melanoma cells expressing PD-L1 and single-positive PD-L1 cells comprising of lymphocytes (high nuclear:cytoplasmic ratio, red arrows) and macrophages (low nuclear:cytoplasmic ratio, green arrows) in three responders from samples

obtained during tumour regression. Magnification, $\times 40$. **b**, Principal component analysis to decompose the variance for the markers ($CD8^{+}$ -, $PD-1^{+}$ -, $PD-L1^{+}$ - and $CD4^{+}$ -cell densities (cells mm^{-2})) separately in the tumour and in the invasive margin. The first principal component accounted for the majority of the variability in the four markers—69.6% and 57.1% in the tumour centre ($***P = 0.001$) and the invasive margin ($****P < 0.0001$), respectively. Principal component scores for the first principal component were compared between response groups with Wilcoxon rank-sum tests.



Extended Data Figure 6 | Clonality of the T-cell repertoire and density of T-cell infiltration in terms of clinical response. **a**, High-throughput quantitative sequencing of the rearranged TCR β genes using the ImmunoSeq assay (response $n = 13$, progression $n = 12$). $***P = 0.005$ by Fisher's exact test. The x-axis represents clonality of the T-cell repertoire ($1 - \text{Pielou's evenness}$) and the y-axis represents the density of tumour-infiltrating T cells (estimated TCR gene rearrangements per diploid genomes, see Methods for further details). The axes cross at the median value for clonality and T-cell infiltration. T-cell infiltration and T-cell infiltration repertoire clonality were found to be independent in this cohort ($R^2 = 0.04$). Progressors were associated with lower levels of T-cell infiltration and lower T-cell infiltration clonality (that is, a more diverse T-cell infiltration repertoire); all patients with below-median clonality and T-cell infiltration progressed. **b**, The uniqueness of the CDR3 TCR sequences enable tracking of clonal expansion or contraction in serially sampled tumours. Representative scatterplot of clones from a responding tumour serially sampled before and during anti-PD-1 treatment. (1 dot = 1 unique clone: green, expanded; grey, stable; red, contracted). The x- and y-axes represent the relative abundance of each clone before and during treatment, respectively. Clones that met a $2\times$ change in frequency from baseline must have also met significance using a Fisher exact test of the clone before and during anti-PD-1 treatment and the full set of clone reads, followed by Storey's Q value for false discovery rate. **c**, Clonal expansion in terms of clinical response (response $n = 6$, progression $n = 5$). $***P = 0.006$.

Extended Data Table 1 | Demographic and clinical characteristics of patients in the study and validation cohorts

Variable	Response	Progression	p-value*
UCLA Patients (N=46)	N=22	N=24	
Male (%)	17 (74%)	19 (79%)	>0.99
Median Age (range)	65 (45-90)	64 (36-86)	0.86†
Median WBC Count (range)	6.9 (3.9-21.3)	7.1 (4.0-24.8)	0.52
Median Pre-TX Tumor Burden in cm (range)‡	8.7 (1.1-32.2)	7.9 (1.1-19.4)	0.9†
Metastatic Status			
M0	3 (13%)	2 (8%)	0.13†
M1a	4 (17%)	2 (8%)	
M1b	7 (30%)	6 (25%)	
M1c	8 (35%)	14 (58%)	
Dosing Regimen			
10Q2W	9 (39%)	5 (21%)	0.2
10Q3W	8 (35%)	8 (33%)	
2Q3W	5 (22%)	11 (46%)	
BRAF Mutation			
Mutant (# not wild type)	7 (30%)	9 (38%)	0.76
Previous Treatment			
chemotherapy	3 (13%)	5 (21%)	0.7
BRAF or MEK inhibitor	3 (13%)	5 (21%)	0.7
immunotherapy			
ipilimumab	8 (35%)	13 (54%)	0.25
other	7 (30%)	9 (38%)	0.76
Pre TX Biopsy Location			
Subcutaneous	14 (61%)	11 (46%)	0.02†
Liver	0	8 (33%)	
Lung	5 (22%)	1 (4%)	
Other	3 (13%)	3 (13%)	
IGR Patients (N=15)	N=10	N=5	
Median Age (range)	55 (26-73)	60 (38-61)	0.77†
Male (%)	4 (40%)	2 (40%)	>0.99
Metastatic Status			
M0	2 (20%)	0 (0%)	0.51†
M1a	3 (30%)	1 (20%)	
M1b	0 (0%)	1 (20%)	
M1c	5 (50%)	3 (60%)	
Dosing Regimen			
10Q2W	5 (50%)	2 (40%)	0.62
10Q3W	3 (30%)	3 (60%)	
2Q3W	2 (20%)	0 (0%)	
BRAF Mutation			
Mutant (# not wild type)	4 (40%)	2 (40%)	>0.99
Previous Treatment			
chemotherapy	2 (20%)	3 (60%)	0.25
BRAF or MEK inhibitor	2 (20%)	0 (0%)	0.52
immunotherapy			
ipilimumab	4 (40%)	2 (40%)	>0.99
other	1 (10%)	1 (20%)	>0.99

* P values calculated using Fisher's exact test.

† P values represent estimations using the non-parametric Kruskal-Wallis test.

‡ Tumour burden represents the sum of the longest measurement of identified target lesions as defined by RECIST 1.1 criteria.

Extended Data Table 2 | Anatomical location of biopsies performed before and during treatment

Patient	Baseline Biopsy	Post-Dosing Biopsy	Anatomical Location	Confirmed Overall Response
1	duodenal lesion	duodenal lesion	Gastrointestinal	Response
2	Segment 4 hepatic mass	Segment 4 hepatic mass	Liver	Progression
3	Segment 5 hepatic mass	Segment 5 hepatic mass	Liver	Progression
4	R. lower lobe lung mass	R. lower lobe lung mass	Lung	Response
5	R. lower lobe lung mass	R. lower lobe lung mass	Lung	Response
6	L. axillary mass	L. axillary mass	Lymph Node (Subcutaneous)	Response
7	L. inguinal mass	L. inguinal mass	Lymph Node (Subcutaneous)	Response
8	R. supraclavicular fossa mass	R. supraclavicular fossa mass	Lymph Node (Subcutaneous)	Response
9	R. cervical neck mass	R. cervical neck mass	Lymph Node (Subcutaneous)	Progression
10	R. axillary mass	R. axillary mass	Lymph Node (Subcutaneous)	Progression
11	R. submandibular mass	R. submandibular mass	Lymph Node (Subcutaneous)	Progression
12	L. medial thigh	L. medial thigh	Subcutaneous/skin	Response
13	L. lower back mass	L. lower back mass	Subcutaneous/skin	Response
14	L. chest wall mass	L. chest wall mass	Subcutaneous/skin	Response
15	Middle back mass	Middle back mass	Subcutaneous/skin	Response
16	L. face mass	L. face mass	Subcutaneous/skin	Response
17	L. scapula mass	L. scapula mass	Subcutaneous/skin	Response
18	L. forearm mass	L. forearm mass	Subcutaneous/skin	Progression
19	L. popliteal mass	L. popliteal mass	Subcutaneous/skin	Progression
20	L. lower abdomen mass	L. lower abdomen mass	Subcutaneous/skin	Progression
21	L. lower abdomen mass	L. lower abdomen mass	Subcutaneous/skin	Progression
22	L. scalp mass	L. scalp mass	Subcutaneous/skin	Progression
23	L. anterolateral leg mass	L. anterolateral leg mass	Subcutaneous/skin	Progression
24	L. upper arm (mets in transit)	L. upper arm (mets in transit)	Subcutaneous/skin (mets in transit)	Response
25	lower leg (mets in transit)	lower leg (mets in transit)	Subcutaneous/skin (mets in transit)	Progression

The anatomical locations that correspond with Fig. 1 are provided (response $n = 13$, progression $n = 12$ tumours biopsied at, before and during aPD-1). The term lymph node/subcutaneous refers to a tumour identified as lymph node on radiographic imaging but with no evidence of lymph node architecture on histological examination. Two out of the 25 patients had metastases in transit which were distinct nodules that were <1 cm apart from each other at the time of biopsy.

Extended Data Table 3 | CD8, PD-1, PD-L1 and CD4 expression and clonality before treatment in terms of clinical response to pembrolizumab and previous treatment with ipilimumab

Variable	Ipilimumab Treatment History		
	Response (Naïve) vs. Response (Treated)	Response (Naïve) vs. Progression (All)	Response (Naïve) vs. Progression (Treated)
	p-value*		
Clonality	0.1604	0.0568	0.4213
Tumor density (cells/mm ²)			
CD8	0.0513	<0.0001	0.0019
PD-1	0.1198	0.0028	0.0573
PD-L1	0.1732	0.0658	0.1645
CD4	0.2453	0.3453	0.3948
Stroma density (cells/mm ²)			
CD8	0.0170	<0.0001	0.0016
PD-1	0.0358	0.0330	0.1872
PD-L1	0.3121	0.0049	0.0123
CD4	0.2051	0.4183	0.4316

*p-values represent estimations using the non-parametric Mann-Whitney test

No significant association was found with previous treatment with ipilimumab (anti-CTLA4) and expression levels of CD8 before receiving anti-PD-1 in terms of treatment outcome.

* P values represent estimations using the non-parametric Mann-Whitney test.

Extended Data Table 4 | Predictive model and validation

a

Variable	AUC (95% CI)*	P-value**
Tumour		
CD8+ Density	.91 (0.81, 1.00)	<0.001
PD-1+ Density	.80 (0.67, 0.94)	0.001
PD-L1+ Density	.71 (0.54, 0.88)	0.026
CD4+ Density	.66 (0.48, 0.84)	0.095
Invasive Margin		
CD8+ Density	.94 (0.88, 1.00)	<0.001
PD-1+ Density	.80 (0.66, 0.94)	0.001
PD-L1+ Density	.79 (0.64, 0.95)	0.002
CD4+ Density	.66 (0.48, 0.84)	0.095

b

Patient ID	CD8+ Density, Before Tx (Invasive Margin)	Predicted Probability of Response (Logistic Model)	Blinded Prediction	True Clinical Response (RECIST 1.1)
IGR - A	58	0.35	Progression	Progression
IGR - B	159	0.37	Progression	Progression
IGR - C	329	0.40	Progression	Progression
IGR - D	341	0.41	Progression	Progression
IGR - E	2120	0.75	Response	Stable
IGR - F	5466	0.98	Response	Progression
IGR - G	2211	0.76	Response	Response
IGR - H	3810	0.92	Response	Response
IGR - I	4294	0.95	Response	Response
IGR - J	4948	0.97	Response	Response
IGR - K	5565	0.98	Response	Response
IGR - L	6004	0.99	Response	Response
IGR - M	5951	0.99	Response	Complete Response
IGR - N	7230	0.99	Response	Complete Response
IGR - O	6320	0.99	Response	Complete Response

a, ROC curve analysis for clinical response based on pre-treatment CD8⁺, PD-1⁺, PD-L1⁺ and CD4⁺ cells. The area under the ROC curve (AUC) was used to measure response prediction performance for pre-treatment CD8⁺, PD-1⁺, PD-L1⁺ and CD4⁺-cell densities (cells mm⁻²). *P* values were computed on the basis of the Wilcoxon rank-sum statistic. **b**, Performance of a model for clinical response using CD8⁺ density (cells mm⁻²). A logistic regression model was constructed using pre-treatment CD8⁺ density (cells mm⁻²) versus the outcome of clinical response (PR versus PD) using the study cohort. This fixed model was then applied to the CD8⁺ density measurements in the validation cohort to compute predicted probabilities of response to treatment.

***P* values were calculated using the Wilcoxon rank-sum test.