Supplementary Figure legends

Supplementary Figure S1: Patient clinical history

A previously healthy woman in her late 40s presented in September 2010 with post coital bleeding and was diagnosed with a localized stage I mucosal melanoma involving the posterior vaginal wall (**Figure S1, Primary biopsy**). Staging scans at the time were negative for regional or distant spread. She initially underwent external beam radiotherapy to the primary, followed by local high dose brachytherapy implant. Given the presence of persistent local disease following radiotherapy, she underwent excision of the primary disease with vaginal repair in May 2011. In March 2012, routine surveillance imaging identified a local recurrence within the vaginal vault. The patient received a single cycle of cytotoxic chemotherapy (dacarbazine) before switching to the CTLA-4 inhibitor ipilimumab due to poor tolerance. Following four cycles of ipilimumab, imaging showed progression of her local disease. She subsequently underwent a pelvic exenteration, with histologic examination revealing two separate foci of melanoma involving the vaginal wall measuring 30 and 25mm. Five months following her surgery, the patient was diagnosed with distant metastatic disease involving a solitary 28mm right lung metastasis. This was resected with a video-assisted thorascopic wedge resection. Recurrent metastatic disease was diagnosed on imaging four months later in March 2013, with a left adrenal nodule, a left lung lesion and a subcutaneous nodule in the left flank. The patient was subsequently enrolled onto a Phase I clinical trial investigating the safety and tolerability of DEDN6526A, an antibody-drug conjugate involving an antibody against Endothelin B receptor and the anti-mitotic agent monomethyl auristatin E (MMAE). Initially, she demonstrated a partial response by RECIST version 1.1, however after five months on treatment the disease progressed with significant enlargement of the adrenal lesion, which was subsequently resected, together with a left flank subcutaneous nodule in

October 2013. The patient's only remaining disease was a left lung lesion, which received stereotactic ablative body radiotherapy. Two months later, the patient developed multiple new subcutaneous nodules and was started on the chemotherapic temozolomide. Since she progressed after two cycles, with enlargement of the subcutaneous metastases and new lymph node involvement, one of the subcutaneous metastases was resected from the central back for symptomatic relief **[Figure S1 Met1]** in April 2014**.** A second subcutaneous metastasis was resected a month later, and she was subsequently started on the PD-1 inhibitor pembrolizumab. The patient's response to pembrolizumab was mixed, with a partial response seen in some subcutaneous nodules while other sites continued to progress, including the development of new metastatic sites. During her immunotherapy treatment, she received several courses of palliative radiation therapy to progressing subcutaneous lesions, both for symptomatic benefit as well as for a possible immune-stimulating effect. After 16 months on treatment, or 24 cycles of pembrolizumab, there was clear progression of her disease involving multiple subcutaneous and liver metastases, as well as a bleeding duodenal metastasis. Pembrolizumab was ceased and a subcutaneous metastasis from her right shoulder was resected for the symptomatic benefit **[Figure S1 Met2]** in November 2015. She underwent radiation therapy to her abdomen in an effort to control the bleeding duodenal metastasis, as she had become transfusion dependent. Unfortunately, her condition deteriorated rapidly over the subsequent weeks and she passed away. Fresh tissue samples as well as formalin fixed paraffin embedded (FFPE) portions from Met1 and Met2 were collected as part of a larger cohort study to study T cell infiltration in metastatic melanoma (1). Peripheral blood mononuclear cells (PBMC) were also banked, and TILs were expanded in vitro from tumour fragments in high dose IL-2 protocol (2).

Supplementary Figure S2: Nanostring data, whole genome RNA sequencing pathway analysis and antigen presenting cells IHC

(A) Heatmaps of Log2 Normalized counts the top 100 upregulated and downregulated genes as identified for fold change >2 and positive DE call. B) Heatmap of the Log2 Normalized counts of tumor inflammation signature (TIS) genes. Samples were hierarchically clustered with Euclidean distance and genes with Pearson correlation. C) Whole transcriptome RNA sequencing and ranked list gene set enrichment analysis of Primary (Pr), Metastasis 1 (Met1) and Metastasis (Met2) samples are depicted in three comparisons (i) Met1 versus Primary, (ii) Met2 versus diagnostic, and (iii) Met2 versus Met1. D) Spatial profiles of antigen presenting cells in tumour samples stained with an OPAL panel (SOX10, CD3, CD19, CD68, CD11c, PDL1). E) Cell density of antigen presenting cell populations in the 3 samples. F-G) Visual representation of the immune GEP of MelTIL 5 vs other primary vaginal melanomas (F) and vaginal melanomas vs other mucosal melanomas and MelTIL 5 samples including primary and metastases 1 and 2 (G)

Supplementary Figure S3: Mass cytometry single markers and $CD4^+$ T cells

A) UMAP of TILs with colorimetric expression of each marker.

B) Violin plots of scaled expression of myeloid markers on myeloid populations in samples Met1 and Met2. C-E) Violin plots showing scaled expression of all markers on $CD4^+$, $CD4^+$ $CD39^-$ and $CD4^+$ Tregs respectively and divided by sample of origin. F) Violin plots of scaled expression of selected markers on NK cell populations and divided by sample of origin. G) CD69 and CD103 profile of CD8+ T cells as determined by mass cytometry and visualised in FlowJo.

Supplementary Figure S4: Cell clusters derived from single-cell RNA expression

(A) UMAP split by sample with cluster colours

(B) Heatmap of Top 10 marker genes for all clusters and samples.

(C) Top 5 marker genes in each cluster.

Supplementary Figure S5: TRM clusters and TCR sharing between tumor samples and PBMC

(A) Proportion of all cells possessing proliferating or activated TRM phenotypes depending on sample sources.

(B) Proportion of TRM cells harbouring proliferating or activated TRM phenotypes in Met1, Met2, and Shared-TRM.

(C) Five-way Venn diagrams of individual clonotype sharing between all four samples for all clusters (left panel) and TRM cells only (right panel). For PBMC clonotypes all cells are used.

Supplementary Figure S6

A) Detection of *in vivo* administered pembrolizumab on tumour samples by staining with anti-human IgG4. (B) PD1 detection on Met1 TILs and expanded TILs via *in vitro* staining with pembrolizumab and detection with anti-human IgG4 (aPD1). ET= expanded TILs. $CD4^+$ T cells are divided in central memory (CM, $CD45Ra^-$ $CCR7^+$), effector memory (EM, $CD45Ra^-$ $CCR7^+$), within EM $CD69^+$ and $CD69^-$ are identified.

(C-F) In vitro overnight stimulation of Met1 or Met2 sample after staining with pembro or Isotype control or addition of different stimuli.

(G-J) In vitro overnight stimulation of expanded TILs from the Met1 or Met2 sample with the addition of FACS-sorted melanoma cells from either the Met1 or the Met2 MEL after staining the TILs with pembrolizumab or Isotype control. Other stimuli are added to the media in the absence of melanoma cells. (NS= non stimulated, CD3= CD3/CD28/CD2 Transactivator,

PMA=PMA/Ionomycin, CEF=CEF Peptivator, aPD1= pembrolizumab). Anti-CD107a was added to the media. For intracellular cytokine production, cells were incubated with Golgi Plug for the duration of stimulation.

Two technical replicates are represented. The experiment was repeated twice for the Met1 sample and expanded TILs and once for the Met2 sample and its expanded TILs.

(K-M) $CD8^+$ T cells subsets and melanoma cells were FACS sorted and cultured overnight at 2:1 ratio. T cells were stained with pembrolizumab or Isotype control before culture.

CD107a was added to the cells during the whole stimulation period. For intracellular cytokine production, cells were incubated with Golgi Plug for the duration of stimulation.

Two technical replicates are represented. The experiment was repeated twice for the Met1 sample and expanded TILs and once for the Met2 sample and its expanded TILs.

(O) IFN-γ, TNF-α and CD107a expression on $CD8^+$ T cells after 3.5 hr *in vitro* re-stimulation of expanded TILs with irradiated autologous lymphoblastoid cell line (LCL) coated with neoantigen peptides. Expanded TILs were previously cultured for 11 days in the presence of the same stimuli used for the restimulation.

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Supplementary Materials and Methods

Multiplex immunohistochemistry

3-µm-thick sections of FFPE tissue were cut onto Trajan Series 3 slides (Trajan Sci Med, Ringwood, VIC, Australia). An automatic serial protocol of staining was performed on BOND RX fully automated research stainer (Leica Biosystems, Mount Waverley, VIC, Australia) at the Centre for Advanced Histology and Microscopy at PeterMac. The immunofluorescent signal was visualised using the OPAL™ 7-color fIHC kit (Perkin Elmer, MA) TSA dyes 520, 540, 570, 620, 650, and 690. Anti-human antibodies, clones and dilutions used were rabbit monoclonal CD4 (Spring Bioscience, clone SP35, 1/100, high pH retrieval), rabbit monoclonal CD3 (Spring Bioscience, clone SP7, 1/1000, low pH retrieval), mouse monoclonal CD8 (Thermofisher, clone 4B11, 1/1000, high pH retrieval), rabbit polyclonal FOXP3 (BioSB, 1/100, high pH retrieval), rabbit monoclonal PD-L1 (Spring Bioscience, clone SP142, 1/1000, high pH retrieval), mouse monoclonal SOX10 (Biocare Medical, clone BC34, 1/200, low pH retrieval), rabbit anti-human CD103 (clone EPR4166(2), 1/2000, Abcam). Slides were imaged on a Vectra® three automated quantitative pathology imaging system (PerkinElmer, Waltham, MA, USA) at 20×. Multispectral images were deconvoluted within inForm® software (PerkinElmer) using multispectral library reference slides of single-colour slides for each of the six OPAL fluorophores and DAPI stain. Multiple individual deconvoluted images were stitched together in the HALO™ image analysis platform (Indica Labs, Albuquerque, NM, USA). Thresholds for individual fluorophore channel were manually determined for each tissue using the High-Plex FL module. Cell phenotyping and spatial plots were calculated within

HALO™ (Indica Labs). Spatial relationships between the cell types were calculated with the software package ISAT (3) . Density and distances were plotted using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). For single colour immunohistochemistry, 3-µm-thick sections of FFPE tissue on Trajan Series 3 slides underwent heat antigen retrieval on the Bond Autostainer. After quenching of endogenous peroxidase activity with 3% H_2O_2 (Merck EMSURE), the slides were stained manually with mouse anti-human MHC I ABC antibody (clone: EMR8-5, ab70328, Abcam), and mouse anti-human HLA-DP, DQ, DR (clone: CR3/43, Dako), and detected with ImmPRESS® HRP-Anti-mouse IgG (MP-7402, Vector Laboratories) followed by Dako Liquid DAB$^+$ Substrate Chromagen system (K3468). Slides were counterstained with haemotoxylin on Jung Autostainer and mounted automatically using the DAKO Coverslipper. The slides are imaged with the Olympus VS120 microscope and viewed with the program OlyVIA (also by Olympus).

CyTOF staining

Cells were incubated with Human TruStain (Biolegend) to bock Fc receptors, at 1/20 dilution in CyTOF staining media (CSM: PBS (Ca^{++}/Mg^{++} free), 2% Fetal Bovine Serum (FBS), 2mM EDTA, 0.05% Sodium Azide). Cells were washed in CSM and resuspended in CXCR6 antibody for 30 min at 37ºC before adding the extracellular staining cocktail for 30 min on ice. After one wash in CSM, cells were washed in PBS and stained for viability with 0.5 uM cisplatin in PBS for 3 min at RT and quenched with CSM.

Cells were permeabilized with eBioscience FoxP3/Transcription Factor Staining Buffer Set (Invitrogen) according to manufacturer's instructions and stained intracellularly with antibodies diluted in Perm Wash for 30 min on ice. After washing, the pellet was resuspended in iridium intercalator (EM Sciences) at 0.1 uM in 2% paraformaldeide in PBS and incubated for max 48 hr in the fridge. Before acquisition on Helios instrument (Fluidigm), cells are washed in CSM and twice in deionized water (MilliQ, Merck).

Targeted gene expression of immune genes

300 ng RNA extracted from FFPE of primary and metastatic lesions were hybridized and gene expression was measured via the nCounter XT CodeSet Gene Expression assay, using the probes contained in the nCounter Human Pan Cancer Immune Profiling Panel with the extension of a Custom Panel Plus of 30 genes (Supplementary table 1). The samples were run on the nCounter PrepStation and measured on the nCounter Digital Analyser (all NanoString Technologies, Inc., Seattle, Washington, USA) as per manufacturer's instructions.

Gene counts were normalized, their fold change and differential expression (DE call) were calculated per each pair comparisons expression using the software nSolver 4.0 (NanoString Technologies) according to manufacturer's instructions. Due to the presence of only one biological replicate, statistical difference between the samples could not be calculated. For this kind of analysis, nSolver performed a differential expression (DE) call based on the likelihood that the expression difference between two samples could be explained by platform technical noise alone. Genes with positive DE call and a fold change of more than 2 between each pair comparison were selected for further analysis because they were more likely to be biologically relevant. Heatmaps were generated in R using the package pheatmap.

To generate ontology pathways, we input the selected genes for each separate pair comparison into the online resource ClueGO plugin (4) in Cytoscape (5). We selected GO-immune pathways and a custom genes reference of the 800 genes that were tested. The false discovery rate adjustment was the Benjamini-Hochberg correction (Supplementary table 2).

Comparison of targeted gene expression with published dataset

Overlapping genes between our custom panel [nCounter Human Pan Cancer Immune Profiling Panel with 30 custom genes (Supplementary table 1)] and that published by Mikkelsen et al. (6) [nCounter Human Pan Cancer 360 IO Panel with 10 custom genes] were used to calculate GSVA scores for each sample against the MSigDB C5 ontology gene set and the C7 immunologic signature gene set (7). The significantly differentially expressed pathways between vaginal mucosa and vaginal mucosal melanoma were calculated and plotted alongside the primary sample with Heatmap. Only pathways with p value <0.05 are represented.

Mutational profile of tumor cells and neoantigen prediction

DNA and RNA from frozen pieces of tumor or PBMCs were extracted using the Qiagen AllPrep Kit (DNA/RNA/Protein). DNA was prepped for exome sequencing using a SureSelect Clinical Research Exome V2 kit (Agilent, U.S.A). RNA was processed for stranded whole-transcriptome sequencing using a NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England Biolabs, U.S.A). Libraries were subsequently sequenced using an Illumina NextSeq platform (2x75 paired end run) in Molecular Genomics Core Facility at Peter MacCallum Cancer Center.

WES analysis and neoantigen prediction

Sequenced reads were quality-checked using FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and detected adapters removed using cutadapt v1.5 (8) with a required minimum length (-m=50) and quality cutoff (-q=15). DNA and RNA reads were aligned against the hg19 version of the human genome using bwa-mem v0.7.10 (9), and hisat2 v2.0.4 (10), resp. Aligned reads were further processed for marking of duplicates with picard v1.119 (http://picard.sourceforge.net/) as well as local insertion/deletion (indel) realignment and base recalibration with GATK v3.2 (11). Reads

marked as duplicates or with a mapping quality < 30 were excluded from further analysis. Somatic point mutations and small indels from DNA were detected using a consensus approach involving multiSNV v2.3 (12), muTect v3.1 (13), varscan v2.3 (14) and Indelocator v1.0.4905 (11). SNVs flagged as 'pass' and 'normal contamination' by multiSNV were kept for further processing. SNVs with an allele fraction >5% in the germline DNA were discarded. Variants (i) with total coverage below 10X in any of the tumor samples or the germline DNA, (ii) overlapping with annotated repetitive sequences by repeatmasker (15), (iii) overlapping with low complexity sequences (16), (iv) present on the Exome Variant Server vESP6500 (http://evs.gs.washington.edu/EVS) or the 1000 genome database (17) and (v) overlapping with artifact-generating and other regions prone to false positives such as CDC27, mucins, ryanodine receptors and olfactory receptors (18, 19) were filtered. Variants detected by at least two callers and passing all filters were kept for downstream analysis. The following steps were applied to optimise the reliability of variant calling in this multi-sample setting. To leverage the sensitivity of muTect in settings of low cellularity, variants absent in one sample based on the procedure above but called only by muTect were included. To leverage the sensitivity of multiSNV as a multi-sample variant caller, the presence of variants detected as above was confirmed in other samples from the same patient via a lenient execution of VarScan (i.e. allowing variant allele frequencies <10%, minimum coverage of 10x and variant present in ≥2 reads). Validation of this pipeline on an independent multisample patient cohort showed an accuracy of 90% (sensitivity 86%, specificity 98%) (20).

Germline mutations were detected from DNA using platypus v0.8.1 (13). Expressed point mutations and small indels were pursued on RNA-Seq using GATK HaplotypeCaller v3.8 (11) and Varscan v2.3 (14). Mutational signatures are obtained using deconstructSigs v1.9 (21) with version 2 of the COSMIC signature set (https://cancer.sanger.ac.uk/cosmic/signatures).

MHC class I and II alleles were predicted using phlat (22) and seq2hla (23). Germline DNA was used to predict the 4-digit types of MHC class I and class II alleles with PHLAT (22) (MHC class I predicted alleles: HLA-A*74:03, HLA-A*03:01, HLA-B*44:03, HLA-B*49:01, HLA-C*07:01, HLA-C*04:01 and MHC class II alleles HLA-DQA1*01:02, HLA-DQA1*02:01, HLADQB1*02:02, HLA-DQB1*06:09, HLA-DRB1*13:05, HLA-DRB1*07:01). Germline DNA and tumor RNA was used with seq2HLA v2.2 (23) to predict HLA-DPA and HLA-DPB alleles (predicted alleles: HLA:DPA1*02:02, HLA-DPB1*05:01, HLA:DPB1*27:01).

Neo-antigenic mutations were called using netMHCpan4.0 (24) and netMHCIIpan3.2 (25) for MHC class I and class II alleles, respectively. Default thresholds (%rank) were used to estimate weak/strong binding affinity of 8-11mers (for class I) and 14-17mers (for class II) containing the mutated aminoacid.

Estimates of allele-specific copy number were obtained using Sequenza v3.0 (26) with a median normalization method and with appropriate gender setting for the patient.

Whole transcriptome analysis

SmartSeq adapter trimming was performed with *cutadaptPE* v2.9 pipeline, reads were subsequently aligned using *Hisat2* v2.0.4, with gene counts quantified using *Htseq* in samtools v1.8 and normalisation performed with *limma-voom*. Normalisation $\log_2$ counts were subsequently used for downstream analysis.

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