Acquired resistance to anti-MAPK targeted therapy confers an immune-evasive tumor microenvironment and cross-resistance to immunotherapy in melanoma

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How targeted therapies and immunotherapies shape tumors, and thereby influence subsequent therapeutic responses, is poorly understood. In the present study, we show, in melanoma patients and mouse models, that when tumors relapse after targeted therapy with MAPK pathway inhibitors, they are cross-resistant to immunotherapies, despite the different modes of action of these therapies. We find that cross-resistance is mediated by a cancer cell-instructed, immunosuppressive tumor microenvironment that lacks functional CD103+ dendritic cells, precluding an effective T cell response. Restoring the numbers and functionality of CD103+ dendritic cells can re-sensitize cross-resistant tumors to immunotherapy. Cross-resistance does not arise from selective pressure of an immune response during evolution of resistance, but from the MAPK pathway, which not only is reactivated, but also exhibits an increased transcriptional output that drives immune evasion. Our work provides mechanistic evidence for cross-resistance between two unrelated therapies, and a scientific rationale for treating patients with immunotherapy before they acquire resistance to targeted therapy.

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In contrast, immunotherapy with checkpoint blockade antibodies directed against programmed cell-death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) results in durable responses in a subset of melanoma patients, but shows lower response rates. Therefore, the American Society of Clinical Oncology and European Society for Medical Oncology guidelines recommend both targeted therapies and immunotherapies as first-line treatment for metastatic melanoma. However, there is little mechanistic understanding of which choice of first-line therapy is better. Many centers treat patients first with targeted therapy (TT) until the tumors acquire resistance, and then switch patients to immunotherapy. It is interesting that there is some clinical evidence to suggest that patients who have relapsed on TT have a lower overall response rate to immunotherapy compared with patients who are naïve to TT. TT-resistant tumors lose CD8 T cells, as determined by immunohistochemistry, and their gene expression profiles show an overlap with tumors resistant to checkpoint inhibitors. Moreover, a few factors have been implicated in both,

1Targeted therapies, inhibiting oncogenic signaling in cancer cells, and immunotherapies, stimulating the immune system to eliminate cancer cells, have revolutionized the treatment of patients with metastatic cancer and led to durable tumor control in a subset of patients. However, low response rates and acquired resistance remain daunting problems. Based on the different modes of action of targeted therapies and immunotherapies, and the expectation that resistance mechanisms do not overlap, targeted therapies and immunotherapies are often administered sequentially. Yet how these therapies change the tumor and its microenvironment, and thereby influence subsequent therapeutic responses, remains poorly understood.

BRAF-mutated, metastatic melanoma exemplifies the sequential treatment approach, because it responds well to both classes of therapies. Targeted therapy with mitogen-activated protein kinase (MAPK) pathway inhibitors (RAfi, MEKi) leads to prompt responses in most patients; however, responses are often not durable with a median duration of response of approximately 1 year.

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diminishing responsiveness to TT and immune evasion. Yet whether acquired resistance to TT could jeopardize a treatment response to immunotherapy, and how this could be achieved mechanistically, are unknown.

In the present study, we provide decisive experimental evidence for the evolution of cross-resistance between targeted MAPK pathway inhibition and immunotherapy in matched, TT-naive and -resistant mouse models and patients. We found that cross-resistance was mediated via an immune-evasive tumor microenvironment (TME), characterized by low abundance and impaired maturation of CD103+ dendritic cells (DCs). The immune-evasive TME is directly instructed by the TT-resistant cancer cells. Using our lineage tracing method CRISPRa Tracing of Clones in Heterogeneous cell populations (CaTCH)29, which allows the retrospective isolation of founding clones before evolutionary selection, we demonstrate that cross-resistance is acquired during treatment with MAPK inhibitors (MAPKi). We found that cross-resistance is not a consequence of a selective bottleneck imposed by the immune system during the evolution of TT resistance, but that it arises from the enhanced transcriptional output of the MAPK pathway in TT-resistant cancer cells. We identified two strategies to overcome immunotherapy resistance: (1) direct modulation of the TME via CD103+ DC maturation and expansion, and (2) inhibition of the MAPK pathway that instructs the TME. Our work strongly suggests that immunotherapy should be administered before patients develop resistance to targeted MAPK pathway inhibitors, and underscores the need to understand how tumors evolve during treatment response and resistance to identify the most potent therapeutic strategies for durable tumor control.

**Results**

**Resistance to MAPKi confers cross-resistance to immunotherapy.**

In our melanoma patients and public datasets30-32, we and others have observed that patients have a lower response rate to immunotherapy and shorter progression-free survival (PFS) when they acquired resistance to TT (RTT) with RAF inhibitors (RAFi, for example, vemurafenib and dabrafenib) alone or in combination with MEK inhibitors (MEKi, for example, cobimetinib and trametinib), compared with patients who are naive to TT (NTT) (Fig. 1a,b, Extended Data Fig. 1a,b and Supplementary Table 1). Moreover, in RTT tumors, inhibitors (MEKi, for example, cobimetinib and trametinib), compared with patients who are naive to TT (NTT) (Fig. 1a,b, Extended Data Fig. 1a,b and Supplementary Table 1). Moreover, in RTT tumors, cytotoxic CD8+CD8+ T cells and tumor-reactive CD39+CD103+ T cells, which mediate anti-tumor immune responses, were reduced compared with biopsies taken from the same patient before TT, which is in line with observations in other datasets33-35 (Fig. 1c, Extended Data Fig. 1c-e and Supplementary Table 2). These clinical correlations could suggest that RTT tumors do not just overcome growth restraints of oncogenic inhibition by TT, but simultaneously acquire immune-evasive traits.

To assess whether acquired resistance to TT could indeed impair a treatment response to immunotherapy, we modeled the evolution of TT resistance in the presence of an intact immune system in mice. We utilized two murine melanoma cell lines, one that is driven by BrafV600E/W10/Cdkn2a-/- (YUMM3.3 melanoma) and one by BrafV600E/W10/Cdkn2a-/-Pten-/- (YUMM17 melanoma). From these treatment-naive cell lines, we established NTT tumors in immunocompetent mice and treated them with RAFi or a RAFi/MEKi combination. Mirroring the clinical course seen in patients,1 tumors initially regressed but eventually relapsed and grew into RTT tumors (Fig. 1d,e). To generate matched, transplantable tumor models, we derived cell lines from NTT and RTT tumors and confirmed their sensitivity and resistance to RAFi or RAFi/MEKi (Extended Data Fig. 2a-e).

We investigated the response to immunotherapies used in the clinic, by establishing tumors from NTT and RTT cell lines of the BRAF melanoma model in immunocompetent mice and treating them with the immune checkpoint inhibitor anti-PD-1/CTLA-4. All NTT tumors responded to anti-PD-1/CTLA-4 in a T cell–dependent manner and regressed (Fig. 1f and Extended Data Fig. 2g). However, the RTT tumors grew unperturbed, independently of whether they were resistant to RAFi or RAFi/MEKi (Fig. 1f and Extended Data Fig. 2h). In line with these results, anti-PD-1/CTLA-4 treatment led to a threefold increase of CD8+ T cells in NTT, but not in RTT, tumors (Fig. 1g,h). Importantly, these mice were never treated with a RAFi or RAFi/MEKi to exclude direct drug effects, for example, on immune cells. To exclude potential drug withdrawal effects on tumor cells in this experimental setup, we also exposed RTT tumors continuously to a RAFi and also observed cross-resistance (Extended Data Fig. 2i). In summary, cross-resistance is a heritable trait of RTT cancer cells, which upon transplantation into mice establish immunotherapy-resistant tumors.

Immunotherapies typically act by promoting T cell responses. To dissect the T cell response in a well-controlled setting with a single antigen and in the presence of an equal number of antigen-specific T cells, we took advantage of the OT-1 T cell receptor/ovalbumin antigen system. We engineered the BrafV600E/Pten melanoma model, which shows primary resistance to checkpoint inhibitors (Extended Data Fig. 3a), to present equal levels of the ovalbumin (OVA) antigen on major histocompatibility complex (MHC)-I, making it susceptible to killing by OVA-specific OT-1 T Cells (Extended Data Fig. 3b). We established NTT-OVA and RTT-OVA tumors (RAFi or RAFi/MEKi resistant) in Rag2−/− mice lacking endogenous T cells, and performed adoptive T cell transfer (ACT) of luciferase-expressing effector OT-1Luc T cells, which are traceable by bioluminescence imaging (BLI) (Fig. 2a). NTT-OVA tumors were rapidly infiltrated by OT-1Luc T cells and tumors regressed; in contrast, RTT-OVA tumors showed lower T cell infiltration and grew unperturbed (Fig. 2b,c and Extended Data Fig. 3c,d). In addition, RTT tumors continuously exposed to RAFi were also cross-resistant to ACT, excluding drug withdrawal effects on tumor cells (Extended Data Fig. 3e-g). In conclusion, RTT tumors are cross-resistant to immunotherapy, even in the presence of a potent antigen and an excess of antigen-specific T cells.

Cross-resistance is mediated via an immune-evasive TME. Cancer cells can become resistant to immunity via cell-autonomous mechanisms (for example, loss of antigen presentation, defective interferon (IFN)-γ response) or non-cell-autonomous mechanisms (for example, by instructing an immune-evasive TME)36. We found a conserved IFN-γ response in NTT and RTT cells, and comparable MHC-I levels and OVA peptide presentation in NTT and RTT cells, excluding the most common cell-autonomous resistance mechanisms (Fig. 2d,e, Extended Data Fig. 3h-k and Supplementary Table 3). Importantly, we observed that NTT-OVA and RTT-OVA cells were killed equally well in an in vitro co-culture assay by the same effector OT-1Luc T cells that failed to control RTT-OVA tumor growth in vivo (Fig. 2f and Extended Data Fig. 3f). To explore non-cell-autonomous mechanisms, we probed the role of the TME in mediating cross-resistance. We experimentally exchanged the TME surrounding a minority (0.05%) of luciferase-positive, target NTT-OVA-Luc cells by mixing them with a majority (99.95%), thus, TME-instructing, luciferase-negative RTT-OVA cells (Fig. 2g). After we transferred OT-1 T cells, we tracked the survival of luciferase-positive target cells by bioluminescence imaging (BLI), and found that the NTT-OVA-Luc cells were shielded from OT-1-mediated killing and grew unperturbed, consistent with an immune-evasive TME established by RTT-OVA cells. Conversely, a minority of RTT-OVA-Luc cells within NTT-OVA tumors were eradicated by OT-1 T cells (Fig. 2h and Extended Data Fig. 3m). This was not a bystander effect due to widespread, unselective T cell killing, because 0.05% OVA-negative cells spiked into 99.95% NTT-OVA cells were not eradicated by OT-1 T cells (Extended Data Fig. 3n). These data indicate that NTT tumors establish an immune-permissive TME, whereas RTT tumors establish an...
Fig. 1 | TT resistance induces cross-resistance to immunotherapy. a,b, Clinical response rate (a) and PFS (b) of patients with metastatic melanoma in the Lausanne patient cohort (Supplementary Table 1) receiving immunotherapy (IT) with checkpoint inhibitors (n = 54 patients). NTT, TT-naive patients (n = 38); RTT, TT (RAFi or RAFi/MEKi)-resistant patients (n = 16). c, Quantification of T cells in tumors of NTT and RTT patients with melanoma receiving MAPKi (RAFi or RAFi/MEKi combination), assessed by IF staining of matched patient biopsies (n = 10 patients; Supplementary Table 2). d, Scheme (1) outlining generation of NTT and RTT cell lines, before therapy or after tumor relapse on treatment, and (2) testing immunotherapy response in tumors derived from NTT and RTT cell lines. e, Treatment response of subcutaneously injected Braf melanoma (Braf\textsuperscript{V600E/WT},Cdkn2a\textsuperscript{−/−}) tumors continuously treated with indicated TT. The arrow indicates the start of therapy (Ctrl, n = 6 tumors; other groups, n = 8). The experiment was performed three times and a representative example is shown. f, Treatment response in mice bearing NTT and RTT Braf melanoma. The arrows indicate immunotherapy administration (all groups, n = 8 tumors). No TT was administered. The experiment was performed nine times and a representative example is shown. P values: \(*P = 0.011, \,**P < 0.001, \,**\,**P < 0.0001; \) NS, nonsignificant. Data in e, f and h are displayed as mean ± s.e.m. Data analysis was done by one-sided \(\chi^2\) test (a), two-sided log-rank (Mantel–Cox) test (b), two-way ANOVA (f) and one-way ANOVA (h). \(*P < 0.05, \,**P < 0.01, \,**\,**P < 0.001, \) NS, nonsignificant.

To identify the mediators of the immune-evasive TME in R\textsuperscript{T}T tumors, we investigated the immune cell composition of the tumors, focusing first on the T cell compartment. In the Braf melanoma model, we identified reduced T cell influx in R\textsuperscript{T}T tumors (Extended Data Fig. 4a,b) and reduced T cell expansion on checkpoint therapy (Fig. 1g,h). In the Braf\textsuperscript{Pten\<sup>\textsuperscript{flx}\> model, T cell numbers post-ACT were substantially reduced in the R\textsuperscript{T}T compared with N\textsuperscript{T}T tumors (Fig. 3a,b). Low-input RNA-sequencing (Smart-seq) revealed reduced expression of activation markers and effector molecules, such as IFN-\(\gamma\), granzyme A and B, perforin 1 and CCL5 in T cells.
isolated from RTT tumors, also indicating a functional impairment of T cells, characteristic for a tumor with an immunosuppressive TME\(^{26,27}\) (Fig. 3c,d and Extended Data Fig. 4c,d).

We found that suppressive myeloid cells, which can inhibit T cell function and have been implicated in RAFi resistance\(^{15}\), were increased in RTT tumors of the Braf/Pten\(^{\text{V600E/WT}}\) melanoma model (Fig. 4g and Supplementary Table 3). CD103\(^{+}\) DCs have been implicated in T cell activation and recruitment, and their stimulation can enhance the acute response to TT and immunotherapy in melanoma\(^{19,28–30}\). Importantly, immunofluorescence (IF) staining for CLEC9a, a marker that is largely, although not exclusively, specific for this DC population in humans\(^{31}\), revealed a reduction in CLEC9a\(^{+}\) cells on TT resistance in biopsies taken from the same patient before and after TT (Fig. 3g and Supplementary Table 2).
**Fig. 3** | The TME of R\textsuperscript{TT} tumors shows reduced and dysfunctional CD103\textsuperscript{+} DCs. a, T cell influx into N\textsuperscript{TT} and R\textsuperscript{TT} Braf/Pten\textsuperscript{OVA} melanoma 5 d post-ACT assessed by IF. Scale bars, 100 \(\mu\)m and 20 \(\mu\)m. The experiment was performed twice and a representative image is shown. b, T cell influx into N\textsuperscript{TT} and R\textsuperscript{TT} Braf/Pten\textsuperscript{OVA} melanoma 5 d post-ACT quantified separately at the tumor margin and center (\(n=2\) tumors per condition; all groups \(n=10\) ROIs, except R\textsuperscript{TT} center, \(n=11\) ROIs). \(P\) values: ****\(P=1.08 \times 10^{-5} , 5.7 \times 10^{-6}\). c, Gene set enrichment analysis of IFN-\(\gamma\) response in T cells sorted from R\textsuperscript{TT} versus N\textsuperscript{TT} Braf/Pten\textsuperscript{OVA} melanoma (N\textsuperscript{TT}, R\textsuperscript{TT}, \(n=4\) tumors per condition). NES, normalized enrichment score. d, Heatmap displaying T cell effector genes in T cells sorted from N\textsuperscript{TT} and R\textsuperscript{TT} tumors (N\textsuperscript{TT}, R\textsuperscript{TT}, \(n=4\) tumors per condition). e, Suppressive myeloid cells (left) and CD103\textsuperscript{+} DCs (right) in N\textsuperscript{TT} and R\textsuperscript{TT} tumors of Braf/Pten\textsuperscript{OVA} melanoma, assessed by flow cytometry (\(n=9\) tumors each). The experiment was performed five times and a pool of two representative experiments is displayed. \(P\) values: ****\(P=8 \times 10^{-4} , 9.4 \times 10^{-4}\). f, CD103\textsuperscript{+} MHCII\textsuperscript{+} DCs in N\textsuperscript{TT} and R\textsuperscript{TT} Braf/Pten\textsuperscript{OVA} melanoma, assessed by IF. Scale bars, 100 \(\mu\)m and 20 \(\mu\)m. The experiment was performed twice and a representative image is shown. g, Quantification of CLEC9\textalpha\textsuperscript{+} cells in tumors of N\textsuperscript{TT} and R\textsuperscript{TT} melanoma patients receiving MAPK pathway inhibitors (RAFi or RAFi/MEKi combination), assessed by IF staining of matched patient biopsies (\(n=10\) patients; Supplementary Table 2). h, Gene expression changes in sorted CD103\textsuperscript{+} DCs from N\textsuperscript{TT} and R\textsuperscript{TT} Braf/Pten\textsuperscript{OVA} melanoma assessed by Smart-seq (N\textsuperscript{TT}, \(n=4\); R\textsuperscript{TT}, \(n=5\) sorted tumors; in technical triplicates; Supplementary Table 5). FC, fold-change. i, Scheme outlining co-culture of CD103\textsuperscript{+} DCs isolated from N\textsuperscript{TT} and R\textsuperscript{TT} Braf/Pten\textsuperscript{OVA} melanoma using magnetic-activated cell sorting (MACS) with CFSE-labeled naive OT-1 T cells to assess T cell activation potential (left) and representative histogram illustrating CFSE signal in T cells (right). The experiment was performed six times, and a representative example is shown. TCR, T cell receptor. Data in b and e are displayed as mean \pm s.e.m. and analyzed using two-tailed, unpaired Student’s \(t\)-test with Welch’s correction for unequal variance or the Mann–Whitney \(U\)-test if not normally distributed. ****\(P<0.0001\).

In ingenuity pathway analysis (IPA) of bulk tumor transcriptomes, we found that the term ‘DC maturation’ scored as the top downregulated pathway in R\textsuperscript{TT} tumors (Extended Data Fig. 3a and Supplementary Table 4). To directly assess the maturation state of CD103\textsuperscript{+} DCs, we isolated them from tumors and found a decreased expression of maturation markers, antigen-presentation machinery, IFN-\(\alpha\) response and T cell stimulation, and recruitment factors (for example, interleukin (IL)-12b, CCL5, CXCL9, CXCL10)\textsuperscript{10} and an...
increased expression of immune-suppressive factors (for example, cyclooxygenase 1/2 (COX or PTGS1/2), transforming growth factor β (TGF-β), ARG1/2) in CD103+ DCs from RTT compared with NTT tumors31-35 (Fig. 3b, Extended Data Fig. 5b–d and Supplementary Table 5). Given that the maturation state of DCs is imperative for their immunostimulatory function36,37, we assessed the ability of CD103+ DCs from NTT and RTT tumors to induce antigen-specific proliferation of naïve OT-1 T cells. Indeed, CD103+ DCs isolated from RTT tumors failed to activate T cells and stimulate their proliferation in vitro (Fig. 3i and Extended Data Fig. 5e). In conclusion, the abundance and functionality of CD103+ DCs were impaired in RTT tumors, which, together with the increase in suppressive myeloid cells, could preclude a functional T cell response.

Modulation of myeloid cells restores sensitivity to immunotherapy. To determine whether therapeutic modulation of the myeloid compartment could restore the immunotherapy response in RTT tumors, we depleted suppressive myeloid cells, but observed tumor control only in 7 of 15 mice, probably due to rapid repopulation and high plasticity of myeloid cells (Extended Data Fig. 5f–i). To overcome the quantitative and qualitative defects of CD103+ DCs and assess their contribution to cross-resistance, we induced DC expansion by expressing FMS-like tyrosine kinase 3 ligand (FLT3L) in RTT cells and induced DC maturation by injecting poly(I:C) into the tumors (Fig. 4a and Extended Data Fig. 5j). FLT3L-mediated DC expansion alone led to a 15-fold increase of CD103+ DCs in RTT-GVA tumors, but did not enhance T cell infiltration or induce tumor control (Fig. 4a–d and Extended Data Fig. 5k). Poly(I:C)-mediated DC maturation enhanced T cell infiltration by inducing T cell recruitment factors such as CCL5, CXCL9 and CXCL10, and was sufficient to restore tumor control (Fig. 4b–e and Extended Data Fig. 5l). CD103+ DCs isolated from poly(I:C)-treated RTT tumors regained their capacity to activate T cells and stimulate their proliferation in vitro (Fig. 4f,g). A combination of FLT3L and poly(I:C) further increased T cell infiltration and tumor control and, notably, T cell memory protected mice from tumor formation on reinjection of RTT- OVA cells (Fig. 4h,i). In the second melanoma model (Braf melanoma), poly(I:C)-mediated DC maturation alone was also sufficient to re-sensitize RTT tumors to checkpoint inhibition in wild-type mice. Importantly, in BATF3−/− mice, which lack functional CD103+ DCs48, tumors did not regress, demonstrating that the poly(I:C)-mediated re-sensitization of cross-resistant tumors is dependent on DCs (Extended Data Fig. 5m).

To investigate cross-resistance in a nonmelanoma model, we used the KrasG12D-driven murine colon carcinoma model CT26, which is responsive to MAPK pathway inhibition with MEKi. We generated matched NTT and MEKI-resistant R TT cell lines and also identified cross-resistance to anti-PD-1 in R TT tumors (Extended Data Fig. 6a–d). We confirmed the lack of common cell-autonomous immunotherapy resistance mechanisms and the immune–evasive TME with reduced T cell infiltration, increased suppressive myeloid cells and reduced and immature CD103+ DCs (Extended Data Fig. 6e–n). To investigate whether local, intratumoral DC maturation and expansion induce regressions of distant secondary tumors, we established subcutaneous R TT tumors at the opposite flanks. Poly(I:C) -mediated DC maturation, in combination with FLT3 and anti-PD-1, led to complete control of the injected and the contralateral tumor (Extended Data Fig. 7a,b). This response was preceded by antigen-specific expansion of T cells in both tumors and abrogated by T cell depletion (Extended Data Fig. 7c–e). As an alternative to poly(I:C), focal radiotherapy has been described as an activator of DCs49,50. Indeed, combining FLT3L with radiation acted synergistically with anti-PD-1, and may represent a clinically relevant intervention strategy (Extended Data Fig. 7f,g). In summary, inducing a functional CD103+ DC compartment can overcome the immune-evasive TME of R TT tumors and may represent a treatment strategy for cross-resistant patients.

Cross-resistance is acquired and linked to the R TT signaling program. Understanding the mechanistic basis of cross-resistance could help to prevent or revert it. The immunosuppressive TME in R TT tumors is established only on resistance to TT, whereas we noticed a remodeling of the TME51 and increased T cell infiltration on short-term MAPK pathway inhibition in NTT tumors (3–7 d), consistent with previous reports52,53,54 (Extended Data Fig. 8a,b). Thus, we investigated whether this immune cell influx could drive the evolution of cross-resistance by selecting for immune-evasive clones. However, R TT cell lines generated in vitro (R TT-vivo) and in immunocompromised, non-obese diabetic (NOD)/severe combined immunodeficient (SCID)-gamma mice (R TT-NSG) also displayed cross-resistance to immunotherapy, phenocopying R TT cells generated in immunocompetent mice (Fig. 5a–d and Extended Data Fig. 8c). Next, we investigated whether cross-resistance is a general consequence of sequential therapies and generated cells resistant to dacarbazine, but these chemotherapy-resistant cells did not show cross-resistance to immunotherapy (Fig. 5e and Extended Data Fig. 8d). In summary, these data indicate that cross-resistance is mediated via a cancer cell–intrinsic program that is directly linked to MAPKi resistance and is not the consequence of selection by the immune system.

To examine whether cross-resistance is pre-existent in treatment-naïve tumors or arises during inhibition of the MAPK pathway, we used our lineage tracing tool CaTCH55. CaTCH allows, via diverse barcodes, the identification of R TT clones and can retrospectively isolate their founding NTT clones before evolutionary selection using a Cas9–VPR-inducible green fluorescent protein (GFP) reporter linked to the barcode (Fig. 5f). We isolated such a clonal pair and found that tumors established by the R TT clone showed low OT-1 T cell infiltration and grew unperturbed on ACT, but tumors from its matched NTT founder clone showed high T cell infiltration and tumor regression, indicating that cross-resistance is acquired during MAPKi treatment (Fig. 5g and Extended Data Fig. 8e,f).

The R TT signaling program predicts immunotherapy response. To characterize the cancer cell–intrinsic program mediating cross-resistance, we performed transcriptomic profiling of the Braf and Braf/Pten bulk tumors, as well as FACS-purified Braf/Pten melanoma cells. We identified the shared, differentially expressed tumor-specific gene signatures, altogether suggesting that R TT cells instruct a signaling program that is associated with an immune-evasive TME and poor response to immunotherapy (Fig. 6c, Extended Data Fig. 8m and Supplementary Tables 8 and 9).

Cross-resistance is mediated via the MAPK pathway in R TT tumors. To identify the regulator governing the ccIES, we performed a computational upstream regulator analysis (IPA) and identified MAPK signaling as the top-scoring signaling pathway (Fig. 6f). Motif analysis of the upregulated genes in the ccIES and transcriptome data from bulk tumors also showed an enrichment of key transcription factors (TFs) of MAPK signaling, such as members of the AP-1 complex (Fig. 6g and Extended Data Fig. 9a).
Fig. 4 | Restoration of a functional CD103⁺ DC compartment restores immunotherapy response. a, Scheme outlining experiments to assess the impact of DC maturation (mat.; intratumoral poly(I:C)) and DC expansion (exp.; FLT3L overexpression from tumor cells) on ACT using effector T cells. b, c, Tumor infiltration of OT-I transgenic T cells in NTT and RTT Braf/PtenOVA melanoma measured by BLI 24 h post-ACT: representative image (b) and quantified 24 h post-ACT (c; n = 4, 4, 3, 4, and 4 mice from left to right). The experiment was performed twice and a representative example is shown. P values: ***P = 0.0004, *P = 0.0376, *P = 0.0351. d, Spider plot indicating response to ACT ± poly(I:C) ± FLT3L in RTT Braf/PtenOVA tumors (RTT Ctrl, RTT Ctrl + FLT3L, RTT + ACT, RTT FLT3L + ACT, n = 3; RTT + ACT + poly(I:C), RTT FLT3L + ACT + poly(I:C), n = 4 mice). The experiment was performed twice and a representative example is shown. e, Gene expression changes in CD103⁺ DCs sorted from Braf/PtenOVA NTT, RTT or RTT tumors 24 h post-poly(I:C) injection, assessed by Smart-seq and normalized to NTT (NTT, RTT + poly(I:C), n = 4; RTT, n = 5 sorted tumors in technical triplicates). f, Representative histogram illustrating CFSE signal in T cells co-cultured with CD103⁺ DCs isolated from NTT, RTT and RTT Braf/PtenOVA melanoma treated with poly(I:C). g, Quantification of T cell proliferation based on CFSE dilution in DC co-culture assays displayed in f (n = 3 tumors per condition). The experiment was performed once. P values: ****P = 0.0003. h, Scheme illustrating experiments to address immunological memory in mice that were bearing RTT tumors and had complete regression in response to poly(I:C) + FLT3L + ACT. i, Tumor growth curve in mice that previously had complete response to ACT, poly(I:C) and FLT3L reinjected with RTT Braf/PtenOVA melanoma compared with mice (naive, n = 5; reinjected, n = 8 mice). The experiment was performed twice and a representative example is shown. Data in c and g are displayed as mean ± s.e.m. and analyzed using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, nonsignificant.

The MAPK pathway has been implicated in immune evasion. However, intriguingly, the MAPK pathway is already active in NTT melanomas, yet they are largely sensitive to immunotherapy, suggesting that, when the MAPK pathway is reactivated in RTT melanomas, it drives a different immune phenotype that confers immunotherapy resistance.

To investigate whether the MAPK pathway in RTT cells gains access to different gene regulatory regions, we assessed genome-wide alterations in chromatin accessibility in NTT and RTT cells using an assay for transposase-accessible chromatin using sequencing (ATAC-seq). The top TF motifs enriched in accessible chromatin of RTT cells were effectors of the MAPK pathway (Fig. 6). We found that this enrichment stems from: (1) chromatin regions that are open in NTT cells but are more accessible with increased activity of MAPK effectors in RTT cells (shared peaks) and (2) regions that are accessible only in RTT cells (unique peaks) and also enriched for motifs of central MAPK effectors (Fig. 6). Notably, shared peaks containing no MAPK motifs displayed similar levels of accessible chromatin, indicating that the enhanced chromatin accessibility is specific for MAPK effectors (Extended Data Fig. 9).
**Fig. 5 | Cross-resistance is acquired during evolution of MAPKi resistance and is directly linked to a cell-intrinsic signaling program.**

**a.** Scheme outlining experiments to test ACT or anti-PD-1/CTLA-4 response in mice bearing R\textsuperscript{TT-vitro} tumors, of cell lines made resistant in vitro. IT, immunotherapy.

**b.** Tumor infiltration of OT-1\textsuperscript{Luc} T cells into R\textsuperscript{TT-vitro} tumors, measured by BLI at indicated days (N\textsuperscript{TT}, n = 3; R\textsuperscript{TT-vitro}, n = 3 mice). The experiment was performed twice and a representative example is shown. P values: *P* = 0.0382, **P* = 0.0340, ***P* = 0.0074.

**c.** Tumor infiltration of OT-1\textsuperscript{Luc} T cells into RTT-vitro tumors, measured by BLI at indicated days (N\textsuperscript{TT}, R\textsuperscript{TT-NRG}, n = 4 mice). The experiment was performed twice and a representative example is shown. P values: *P* = 0.0168, **P* = 0.0058, NS = 0.2687.

**d.** Cross-resistance is acquired during evolution of MAPKi resistance and is directly linked to a cell-intrinsic signaling program. Experimental steps: (1) Injection of barcoded N\textsuperscript{TT} cell line into mice; (2) resistance generation using RAFi/MEKi; (3) isolation of RTT cell line; (4) next-generation sequencing of barcodes in RTT cell line (NTT-vitro, R\textsuperscript{TT-vitro}, N\textsuperscript{TT}, R\textsuperscript{TT-NRG}, n = 6 tumors; N\textsuperscript{TT}, R\textsuperscript{TT-Dec} anti-PD-1/CTLA-4, n = 10 tumors). The experiment was performed once. P values: ****P = 7.5 × 10\textsuperscript{-5}, ***P = 0.0008.

**e.** Scheme illustrating the use of the lineage tracing system CaTCH employing a barcode-guided inducible GFP reporter to isolate the treatment-naive (N\textsuperscript{TT}) founding clone of a TT-resistant (R\textsuperscript{TT}) clone. Experimental steps: (1) Infection of barcoded N\textsuperscript{TT} cell line into mice; (2) resistance generation using RAFi/MEKi; (3) isolation of R\textsuperscript{TT} cell line; (4) next-generation sequencing of barcodes in R\textsuperscript{TT} cell line and isolation of matching N\textsuperscript{TT} and R\textsuperscript{TT} clones; (5) OVA engineering; and (6) ACT experiment. P values: NS = 0.9367, *P* = 0.0298, **P* = 0.0133.

**f.** Infiltration of OT-1\textsuperscript{Luc} T cells into matched CaTCH-isolated N\textsuperscript{TT} and R\textsuperscript{TT} Braff/Plen\textsuperscript{OVA} tumors (all groups, n = 5 mice). The experiment was performed twice and a representative example is shown. P values: NS = 0.9367, *P* = 0.0298, **P* = 0.0133.

**g.** Infiltration of OT-1\textsuperscript{Luc} T cells into matched CaTCH-isolated N\textsuperscript{TT} and R\textsuperscript{TT} Braff/Plen\textsuperscript{OVA} tumors (all groups, n = 5 mice). The experiment was performed twice and a representative example is shown. P values: NS = 0.9367, *P* = 0.0298, **P* = 0.0133.
The RTT signaling program predicts immunotherapy response in patients and is controlled by MAPK signaling. a, Scheme outlining the generation of the ccIES (106 genes, Supplementary Table 7) by overlapping bulk transcriptomic data from both melanoma models (immune-evasion signature, 279 genes; Supplementary Table 6) with genes deregulated in sorted Braf/Pten tumor cells. b, Expression of genes comprising the ccIES in NTT and RTT cancer cells sorted from Braf/Pten−/− tumors derived from Rag2−/− mice (RTT tumors in this experiment were continuously exposed to 10 mg kg−1 of RAFi; n = 3 mice). c, PFS of patients receiving anti-PD-1 monotherapy (n = 41 patients) stratified based on ccIES expression43. PCC, Pearson correlation coefficient. d, Clinical response in patients receiving anti-PD-1 (n = 41 patients) stratified based on ccIES expression43. e, Correlation of ccIES with CD103 score and T cell score (Supplementary Tables 8 and 9) in patients receiving anti-PD-1 (n = 41 patients) stratified based on the z-score. f, HOMER motif enrichment analysis of upregulated ccIES genes, comparing RTT versus NTT. g, Volcano plot of differential TF activity (weighted mean difference) in ATAC-seq data comparing RTT versus NTT Braf/Pten−/− melanoma cells sorted from tumors (n = 3 tumors per condition). TFs with a TF activity ≥2 (0.25) are highlighted in black. The corresponding significance is computed using the analytical approach and adjusted for multiple testing using the Benjamini–Hochberg procedure (y axis). i, Line plots highlighting the ATAC-seq profile for indicated TF motifs in NTT and RTT Braf/Pten−/− cell lines. j, Top: heatmap of normalized (reads per genomic content (RPGC)) gene accessibility tracks (ATAC-seq). Accessibility profiles for peaks uniquely identified in NTT and RTT cell lines are depicted. Profile plots and heatmaps represent accessibility around the unique peak center (±1kb). Heatmaps are sorted by descending accessibility. Bottom: HOMER motif enrichment analysis of peaks uniquely identified in NTT and RTT cell lines. The top three predicted motifs for each condition are displayed. The P value in e is derived from Cox’s proportional hazards model using the gene score as a continuous variable and analysis in f is by two-sided Pearson’s correlation.

Next, we used SLAM-seq, a metabolic RNA-labeling method for time-resolved measurement of newly transcribed (nascent) RNA, to understand whether the altered chromatin landscape changes the immediate transcriptional output of the MAPK pathway37,48 (Extended Data Fig. 9c). We inhibited the MAPK pathway for 2 h using MEKi in NTT and RAFi-resistant RTT cells, which remain
responsive to MEKi, and found that 62 genes uniquely changed in
N\textsuperscript{T\text{+}}R T\text{+}, 204 in R T\text{+}, and 222 in both R T\text{+} and N T\text{+} cells (N T\text{+}-specific, 'R T\text{+}-specific' and 'common' targets, respectively) (Extended Data
Fig. 9d–f and Supplementary Table 10). Notably, the common and
R T\text{+}-specific targets were also transcribed at higher rates, altogether
regulation of mediators of immune evasion and an upregulation of
ccIES genes in response to 72 h of MEKi in R T\text{+} Braf/Pten\text{DVA} melanomas treated with MEKi (0.5 mg kg\textsuperscript{-1} for three doses) with
CFSE-labeled, naive OT-1 T cells displayed as a representative histogram of CFSE signal in T cells. 

To address whether inhibition of the enhanced MAPK pathway output is sufficient to restore responsiveness to immunotherapy, we inhibited the MAPK pathway in RAFi-resistant R T\text{+} tumors using MEKi (Fig. 7a). Inhibition of the MAPK pathway in vivo reverted
the expression of 80% of the ccIES genes in RAFi-resistant R T\text{+}
tumors, but only 12% of these genes were altered in N T\text{+} tumors (Fig. 7b,c). MAPK pathway inhibition in R T\text{+} cells led to a down-
regulation of mediators of immune evasion and an upregulation of genes associated with an active immune response, such as genes in the type I IFN pathway (Extended Data Fig. 9h,i). Within the TME, we observed an increase in CD103\textsuperscript{+} DCs and a reduction in sup-
pressive myeloid cells (Fig. 7d). The CD103\textsuperscript{+} DCs isolated from MEKiktreated R T\text{+} tumors showed a regained ability to activate
T cells, and T cells infiltrated R T\text{+} tumors upon inhibition of the
MAPK pathway (Fig. 7e,f and Extended Data Fig. 10a). We excluded that TME remodeling resulted from general cell death by activating

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**Fig. 7** Inhibition of the reactivated MAPK pathway restores sensitivity to immunotherapy in R T\text{+} tumors. a, Scheme illustrating the rationale and
experiment to inhibit MAPK signaling in R T\text{+} cells and profile the contribution of MAPK signaling to cross-resistance. b, Gene expression changes in ccIES genes in response to 72 h of MEKi in R T\text{+} Braf/Pten\text{DVA} melanomas sorted from Rag2\textsuperscript{-/-} mice (R T\text{+}, n = 5; R T\text{+}+ MEKi n = 6 tumors). c, Gene expression changes analyzed via Smart-seq in ccIES genes (divided into up- and downregulated genes) in N T\text{+} and R T\text{+} Braf/Pten\text{DVA} melanomas sorted from tumors after 72 h of MEKi treatment (n = 3 tumors per condition, in technical triplicates). The black line highlights the mean. d, Suppressive myeloid cells and
CD103\textsuperscript{+} DC levels in N T\text{+} and R T\text{+} Braf/Pten\text{DVA} tumors 3 d post-MEKi (N T\text{+}, R T\text{+} n = 9 tumors; R T\text{+}+ MEKi, n = 10 tumors). The experiment was performed twice and the data represent the pool of both experiments. P values: CD103\textsuperscript{+}−, "P = 0.0087, "P = 0.0159, f, Co-culture of CD103\textsuperscript{+} DCs isolated from N T\text{+}, R T\text{+} and R T\text{+} Braf/Pten\text{DVA} melanoma treated with MEKi (0.5 mg kg\textsuperscript{-1} for three doses) with CFSE-labeled, naive OT-1 T cells displayed as a representative histogram of CFSE signal in T cells. 

g, Spider plot indicating tumor volume in R T\text{+} Braf/Pten\text{DVA} tumor-bearing mice treated with the indicated therapies (all groups n = 4; except MEKi+ACT, n = 5 mice). The experiment was performed twice and a representative example is shown. h, Summary scheme highlighting the core findings. IT, immunotherapy; MCs, myeloid cells. Data in d and e are displayed as mean ± s.e.m. Data analysis in d is by one-way ANOVA, and in e by two-tailed, unpaired Student’s t-test with Welch’s correction for unequal variance or the Mann-Whitney U-test if not normally distributed. *P < 0.05, **P < 0.01, ***P < 0.001.
the suicide gene thymidine kinase in \textsuperscript{RT} cancer cells, which led to tumor regression but not to TME remodeling (Extended Data Fig. 10b,c). Next, we combined MEKi treatment with ACT of OT-1\textsuperscript{Luc} T cells and, whereas single treatment with ACT or MEKi had no or minor effects on overall survival, the combination was strongly synergistic and led to durable tumor control in all mice (Fig. 7g and Extended Data Fig. 10d). The combination of MEKi with anti-PD-1/CTLA-4 in the RAFi-resistant \textit{Braf} melanoma model also extended survival (Extended Data Fig. 10e).

These data indicate that \textit{RT} cancer cells instruct an immune-evasive TME through an enhanced MAPK pathway output, mediating cross-resistance to immunotherapy (Fig. 7h). Moreover, in our ACT model a brief treatment with targeted therapy did not interfere with subsequent T cell infiltration or tumor cell killing (Extended Data Fig. 10f–h). Although this experimental setting is highly controlled and does not test immunotherapy administration directly, taken together our results support the notion that TT should be limited to a short period and patients should be switched to immunotherapy before resistance develops. Cross-resistance can be overcome by inhibiting the MAPK pathway or—in tumors that are resistant to MAPK pathway inhibition—by directly modulating the DC compartment to increase the efficacy of immunotherapy (Fig. 7h).

Discussion

Tumor development relies on oncogenic signaling to initiate and maintain tumor growth and the tumor’s ability to evade elimination by the immune system. Growing evidence suggests that oncogenic signaling pathways may also impair anti-tumor immune responses\(^9\). Consistent with this idea, the MAPK pathway in TT-naive melanomas has been implicated in immune evasion\(^10\). However, despite their hyperactive MAPK pathway, most TT-naive melanomas are responsive to immunotherapies\(^1\), suggesting that oncogenic hyperactivation of the MAPK pathway does not suppress anti-tumor immunity sufficiently to facilitate immunotherapy resistance.

In the present study, we show that, when melanomas acquire resistance to TT, they also become resistant to immunotherapy. Cross-resistance is driven by the MAPK pathway, which is reactivated and drives resistance in most of the TT-resistant melanomas. We find that the reactivated MAPK pathway has an enhanced transcriptional output, indicated by canonical and new gene regulatory regions that are more accessible to the critical MAPK effectors of the AP-1 TF complex. This enhanced transcriptional output drives immune evasion and leads to an entirely different, impaired response to immunotherapy in TT-resistant tumors compared with TT-naive melanomas. We find that cross-resistance is acquired during MAPKi treatment, challenging the notion that resistant cells generally pre-exist before therapy (see, for example, ref. \(^50\)). Our findings highlight that tumors can acquire a strongly immune-evasive state by modulating key oncogenic signaling pathways that initially drive tumor initiation, without the need to engage additional pathways.

The immunosuppressive TME of cross-resistant tumors is potent enough to protect cancer cells from T cell–mediated killing, even in the presence of a strong antigen underscoring the central role of the TME in determining therapy responses. We show that CD103\(^+\) DCs are reduced and functionally impaired in the TME of \textit{RT} tumors in patients and mice, and that restoration of DC functionality via poly(l:C)-induced maturation, focal irradiation or inhibition of the enhanced MAPK output is sufficient to restore immunotherapy responsiveness. CD103\(^+\) DCs transport tumor antigens to draining lymph nodes and prime naive CD8\(^+\) T cells\(^29\). Moreover, they produce CXCL9/10, recruiting activated T cells to the tumor and IL-12, which boosts anti-tumor activity of T cells\(^30\). Notably, with several strategies to generate a functional DC compartment currently in clinical trials\(^31,32\), the direct perturbation of the immune-evasive TME represents an exciting opportunity to sensitize patients to immunotherapy.

Prior studies in patients have provided correlative data suggesting cross-resistance between TT and immunotherapy. TT-resistant tumors show reduced T cell infiltration, an increase in M2 macrophages and a shared gene expression program with immunotherapy-resistant patients\(^33\). Moreover, retrospective analysis of clinical studies has suggested that melanoma patients who develop resistance to TTs respond worse to subsequent immunotherapies\(^34\). However, patients with acquired resistance, including patients in our own dataset, tend to have more widespread metastases, including brain metastases, and have higher lactate dehydrogenase levels, complicating the biological interpretation of these correlative observations. Our study provides experimental evidence and mechanistic insights into the molecular basis of cross-resistance between TTs and immunotherapies, making a leap in our understanding of this clinically relevant biology. A key strength of our model systems is, for example, that TT-resistant tumors can be established by implanting resistant cell lines in mice with continuous exposure to TT or in mice that have never been exposed to TTs. This allowed us to show that cross-resistance is instructed by the cancer cells and does not stem from a direct, inhibitory effect of TT on immune cells. Moreover, the models allowed us to test the hypothesis whether the influx of T cells into the tumor, observed in the acute response to TTs, drives the emergence of cross-resistance, for example, via immune editing. We established tumors from TT-resistant cell lines that were generated in immunocompromised mice and in vitro, where immune cells are absent, and also observed cross-resistance, ruling out immune response-induced selective pressure during resistance development as a driver of cross-resistance. Ultimately, both insights from patients and interventional experiments in mouse models are needed to reach firm conclusions about relevant disease mechanisms.

Our work has important implications for the treatment of patients, and it will influence future studies. With a large repertoire of mechanism-based therapies to hand, the future of cancer treatment lies in rational therapy combinations and the sequential administration of different treatment regimens for durable tumor control in patients. However, not even in melanoma, where both TTs and immunotherapies have been approved for years, is there a consensus on the right sequence of TT and immunotherapy\(^35\). Our study, together with other work\(^36,37,38,39\), provides a strong scientific rationale for using immunotherapy as a first-line treatment in \textit{BRAF}-mutant melanoma patients. In advanced patients, where the prompt and reliable responses of TT can be desired, patients should be switched to immunotherapy before resistance develops. Moreover, the concept of TT–immunotherapy cross-resistance that we have discovered in \textit{BRAF}-mutant melanoma may also extend to other tumor types and therapies, for example, \textit{KRAS}-mutant tumors. KRAS inhibitors represent a promising new treatment option and are currently in clinical trials in large patient populations with lung and colon carcinomas, which are at least in part also responsive to immunotherapies\(^40\). To identify effective therapeutic strategies, our work shows that it will be necessary to understand how cancer cells and their TMEs evolve through all phases of treatment, including the phase of active response and resistance to therapies.

Methods

Ethical regulations. The research performed in the present study complies with all ethical regulations. The retrospective analysis of \textit{BRAF} V600 mutant melanoma patients from Lausanne University Hospital, Switzerland was conducted in accordance with the Declaration of Helsinki, the Swiss legal requirements and the principles of good clinical practice. Patients signed the Lausanne University Hospital general consent and accepted the use of their data for research purposes, or did not explicitly refuse the use of personal data (following Art. 34 HRA). Patients did not receive compensation. The protocol was approved by the Research Ethics Committee of Canton de Vaud, Switzerland (protocol 2019-00448). All experiments...
using animals were performed in accordance with our protocol approved by the Austrian Ministry (BMBWF-66.015/0009-V/3b/2019 or GZ: 340118/2017/25). The procedures involving irradiated animals were approved by the Animal Ethics Committee at the Institute of Cancer Research, in accordance with National Home Office Regulations under the Animals (Scientific Procedures) Act 1986.

Clinical data. The retrospective study includes 54 BRAF V600-mutated patients treated with immunotherapy between 1 January 2011 and 29 February 2019 at the Lausanne University Hospital, Switzerland. This cohort is divided into two groups: the TT-naive (NTT) group refers to patients (n = 38) who received immunotherapy as a first-line treatment (nivolumab, pembrolizumab or ipilimumab–nivolumab) and may have received it as a second-line treatment BRAFi (dabrafenib or vemurafenib) ± MEKi (trametinib or cobimetinib) on progression. The TT-resistant (RTT) group refers to patients (n = 16) who received a BRAFi as a first-line treatment (dabrafenib or vemurafenib) ± MEKi (trametinib or cobimetinib) and received immunotherapy as a second-line treatment (nivolumab, pembrolizumab or ipilimumab–nivolumab). The assignment of patients to the responder or nonresponder groups was defined by the radiological reports of scans by position emission tomography/computed tomography and magnetic resonance imaging. Responders were defined as patients with complete response (CR), partial response (PR) or stable disease (SD) of ≥3 months. Nonresponders were defined as patients with progressive disease (PD) or SD for ≤3 months before disease progression. All patients were V600 BRAFi-mutation positive. A detailed list of clinical characteristics and inclusion criteria is summarized in Supplementary Table 1. Survival analysis was performed using the Kaplan–Meier method and a two-sided log-rank test was used to test statistical significance. The association between the response and the group was investigated using a one-sided z test.

Cell culture. BRAF/PTEN melanoma (BrafV600E/PTEN−/−/Cdkn2a−/−) and Braf melanoma (BrafV600E/PTEN−/−/Cdkn2a−/−) cells were cultured in Dulbecco’s modified eagle’s medium (DMEM), containing 2 mM l-glutamine and 100 IU ml−1 of penicillin–streptomycin. Primary T cells were grown in RPMI-1640 containing 10% FBS, 2 mM l-glutamine, 100 mM l-glutamine and 100 IU ml−1 of penicillin–streptomycin, 1x nonessential amino acids, 1x sodium pyruvate, 20 mM 4-((2-hydroxyethyl)-1-piperazine-ethanesulfonic acid and 0.05 mM β-mercaptoethanol. All cells were grown at 37°C with 5% CO2 and regularly tested negative for Mycoplasma contamination. Recombinant INF-γ (BioLegend) was administered to the cells at 10 ng ml−1 for 24 h. RTT cells isolated from mice were continuously cultured on TT, 100 nM RAFl (dabrafenib, Selleckchem) or 10 nM MEKi (trametinib, Selleckchem) for BRAF/PTEN and Braf melanoma or 10 nM MEKi for CT26 colon carcinoma. To generate RAFl- or RAFl/MEKi-resistant melanoma or MEKi-resistant CT26 lines in vitro, cells were seeded at a low density and exposed to 100 nM–3μM RAFl or 10–300 nM MEKi. After 6–8 weeks of continuous drug exposure, resistant cell lines were derived and cultured continuously on 100 nM RAFl, 100 nM RAFl/30 nM MEKi or 10 nM MEKi. RAFl-resistant melanoma cell lines and MEKi-resistant colon carcinoma cell lines are referred to as RT2. RAFl/MEKi-resistant double-resistant cell lines are highlighted as RAFl/MEKi RT2. To generate dacebazine-resistant cell lines, Braf melanoma cells were seeded in 50 μl ml−1 of dacebazine (Sigma-Aldrich) for 3–4 weeks, and then the concentration was increased to 100 μg ml−1. All RT2 cell lines were continuously cultured on treatment and resistance was confirmed using proliferation assays.

Isolation of NTT and RTT cell lines from in vivo tumors. To generate cell lines from tumors, tumors were excised, cut into small pieces and dissociated for 1.5 h at 37°C using collagenase A (1 mg ml−1, Roche) in phosphate-buffered saline (PBS). Single-cell suspensions were stained with a 7-μm nylon mesh, washed twice in PBS and plated in complete medium containing 100 nM RAFl, 100 nM RAFl/30 nM MEKi or 10 nM MEKi for resistant cell lines. Cells were allowed to adhere overnight, followed by medium exchange after 16 h. NTT and RTT cell lines were subjected to whole-exome sequencing to identify mutations in cancer-associated genes that could mediate therapy resistance (Supplementary Tables 11–13).

Plasmids and viruses generation. For lentivirus production, LentIX 293T cells were transfected with 4 μg of the plasmid of interest, 2 μg of Pax2 and 1 μg of VSVG plasmid using polyethyleneimine. The virus was collected 48 h, 64 h and 72 h post-transfection in DMEM containing 1% FBS, and used for infection in complete medium with polybrene at a concentration of 8μg ml−1. Then, pLOBI (pRLL-SVFF-LUC2-P2a-OVA-mPGK-GLAST-IRES-IRP720), pOBI (pRLL-SVFF-OVA-mPGK-GLAST-IRES-IRP), pFLTN (pRLL-SVFF-FLT3Lg; P2A-NEO), pGP (pRLL-hu6-Luc2-GFP-PURO) and pB7 (pRLL-GLAST-IRES-IRP720) were used. The virus production and retroviral gene transfer of the triple-modality reporter gene TGL has been described previously (pSFG-NES-HSV1-TK/GFP/Luc)17.

Proliferation assay. Cells, 1000, were plated on a 96-well plate in 100 μl of medium containing vehicle control (dimethylsulfoxide for RAFl and MEKi; 1 M HCl for dacebazine), RAFl, MEKi or dacebazine at increasing concentrations. At seeding (day 0) and after 72 h, the cell number was determined using a Cell-Titer Glo Assay (Promega), according to the manufacturer’s instructions. Luminescence readout was performed using a Synergy H1 Plate Reader (Biotek). The fold-change in proliferation was calculated relative to the day-3 vehicle control for the respective condition.

Mouse models. For injections of BRAFi and BRAF/PTEN melanoma, 6- to 12-week-old male/female immunocompetent B6(Cg)-Tyr−/− or male/female immunocompetent B6(Cg)-Prkdc−/−Il2rgtm1張/Natl2afl mice were used. As recipients for ACT 6- to 12-week-old, male/female B6(Cg)-Rag2−/−G12D/G12D ly5.2 mice were used. CT26 colon carcinoma cells were injected into 6- to 12-week-old male/female BALB/c mice. These mice were received from the in-house breeding facility, and bred and housed under standard pathogen-free conditions at a housing temperature of 22 ± 1°C, 55 ± 5% humidity and a photoperiod of 14 h light:10 h dark. T cell isolation was performed from 8- to 20-week-old male/female OT-1 Rag2−/− mice, or 8- to 20-week-old male/female OT-1 Ly5.2 mice. B6.129S(S)-Bat3tm1aHpl/1 mice were purchased from Jackson Laboratories and used for injection of the Braf melanoma model at age 6–12 weeks.

Tumor cell injections and in vivo treatment studies. For subcutaneous tumor cell injections, mice were anesthetized using ketamine hydrochloride (100 mg kg−1), xylazine (10 mg kg−1) and acepromazine (3 mg kg−1), or isoflurane and tumor cell suspensions were subcutaneously injected into the shaved flank in 50 μl of Matrigel:PBS (1:1 (v/v)). For the in vivo resistance generation, mice were subcutaneously injected with 106 cells on each flank to increase the change of tumor formation and daily treatment by oral gavage was initiated at 25–100 mg tumor volume using vehicle (PBS), 30 mg kg−1 of RAFl (dabrafenib, Tafinlar, Novartis), 1 mg kg−1 of MEKi (trametinib, Mekinist, Novartis), or combinations of 15 mg kg−1 of RAFi and 0.5 mg kg−1 of MEKi. For checkpoint inhibition, 0.5–0.7×106 CT26 cells colon carcinoma were transplanted into NOSG mice using poly(I:C) at a concentration of 150 mg kg−1, followed by checkpoint inhibition on days 6, 9 and 12. Ganciclovir (Cymevene), for depletion of myeloid cells, was injected i.p. at a concentration of 15 mg kg−1, followed by poly(I:C) at a concentration of 50 mg kg−1 i.p. and a photoperiod of 14 h light:10 h dark. T cell isolation was performed from 8- to 20-week-old male/female OT-1 Rag2−/− mice, or 8- to 20-week-old male/female OT-1 Ly5.2 mice. B6.129S(S)-Bat3tm1aHpl/1 mice were purchased from Jackson Laboratories and used for injection of the Braf melanoma model at age 6–12 weeks.
to the long and short tumor diameters, respectively. For survival curves, a defined tumor volume (according to the animal protocol, 1,000 mm³ or tumor volume at first appearance of necrosis) was used as a cut-off criterion across all mice in the respective experiment.

**Spinee-in experiments to probe the TME contribution.** For ‘spike-in’ experiments N² and R² BlaG/Plenex cells line cells were counted, mixed at indicated ratios (0.05% (500 cells) Luc® minority population and 99.95% (999,500 cells) Luc-negative, TME-instructing majority population) and subcutaneously injected into Rag2−/− mice. No MAPKi was applied in these experiments, to avoid drug effects on the TME or N² cells. On the day of ACT, BLI was performed to have a reference value of the spiked-in populations, mice were assigned into Ctrl and ACT groups and ACT of 4×10⁶ pre-activated effector OT-1 T cells was performed on day 7.

**Focal radiation therapy.** CT26 RT² cells, 3×10⁶, were injected subcutaneously into the right flank of female 5–8-week-old Balb/c mice (Charles River). Animals were irradiated under anesthesia with Hypnorm/Hynovel (fentanyl–fluanisone/midazolam), administered i.p. Irradiation was performed on day 14 using an AGO 250-kV X-ray machine. Radiation dose (9 Gy) was measured using an AGO 250-kV X-ray machine. Pre-activated OT-1 Luc T cells was performed on day 7 post-injection, as described in ‘Adoptive T cell transfer’ above.

**CD8 T cell isolation and activation.** T cell isolation was performed using naïve CD8 T cell isolation kit (Miltenyi Biotec). Splenic and mesenteric lymph nodes were isolated from donor mice and processed according to the manufacturer’s protocol. Naïve CD8 T cells were then either used directly or activated with anti-CD3 (2 ng ml⁻¹) and anti-CD28 (1 μg ml⁻¹) and anti-CD28 (1 μg ml⁻¹) (both eBioscience) for 16 and expanded for 6 days in IL-2 (15 ng ml⁻¹, in-house produced).

**In vitro T cell killing assay.** OVA+ cancer cells, 2×10⁵, were seeded into 24-well plates and allowed to attach overnight. Pre-activated effector OT-1 T cells were then seeded on to the cancer cells in the indicated effector:target ratio (accounting for one doubling overnight) in complete T cell medium containing IL-2 (15 ng ml⁻¹). After 24 h of co-culture, the remaining cells were stained with CD8α and DAPI as a live–dead marker and analyzed by flow cytometry. OVA- cancer cells were used as a specificity control for killing.

**In vitro DC co-culture assay.** For CD103+ DC isolation, tumors (derived from BlaG/Plenex melanoma-bearing Rag2−/− mice) were dissected, cut into small pieces and dispersed for 1 h at 37 °C using collagenase A (1 mg ml⁻¹) and trypsin (0.25% v/v) in PBS. In the case of experiments profiling the effect of MEKi on the DC compartment, mice received three doses of MEKi (0.5 mg kg⁻¹) before DC isolation. In the case of experiments profiling the effect of polyclonal (PC) on the DC compartment, mice received two doses of polyclonal (PC) before DC isolation (24 h after the second dose). Single-cell suspensions were strained through a 70-μm nylon mesh, washed in FACS buffer (0.5% BSA, 2 mM ethylenediaminetetraacetic acid (EDTA)) and incubated for 10 min on 4°C with anti-mouse Fc-Bloc CD16/CD32 antibody. Cells were stained with biotinylated anti-CD103 (clone REA789, Miltenyi Biotec) and isolation of labeled CD103+ cells was performed with anti-biotin microbeads (Miltenyi Biotec), according to the manufacturer’s protocol; purity was confirmed using flow cytometry (MACS II, Miltenyi Biotec). For co-culture 150,000 naïve OT-1 CFSE-labeled (0.25 μM, Thermo Fisher Scientific) T cells were co-cultured with 50,000 freshly isolated CD103+ DCs in a 96-well plate in complete T cell medium. After 24 h, the cell suspension was stained with a live–dead marker and CD3ε/BV605. The proliferation of OT-1 T cells was determined based on carboxyfluorescein succinimidyl ester (CFSE) dilution. As a positive control naive OT-1 CFSE-labeled T cells were activated with anti-CD3 (2 μg ml⁻¹) and anti-CD28 (1 μg ml⁻¹) (both eBioscience) for 16 h and expanded for 3 d in IL-2 (15 ng ml⁻¹, in-house produced).

**Flow cytometry.** For flow cytometry of whole tumors, cells were dissected, cut into small pieces and dissociated for 1:5 h on 37°C using collagenase A (1 mg ml⁻¹). Tumor suspensions were strained through a 70-μm nylon mesh, washed in FACS buffer (0.5% BSA, 2 mM EDTA) and incubated for 10 min at 4°C with anti-mouse Fc-Bloc CD16/CD32 antibody. Cells were subsequently stained with antibodies detecting immune cells in FACS buffer for 30 min at 4°C. Cells were washed twice in PBS and live–dead exclusion was performed using DAPI staining or fixable viability dye eFluor780 (eBioscience, 1:1,000).

CD103+ DCs were defined as CD103+CD11c+ out of alive, singlet CD45+ cells. CD103+CD11c+ DCs were also gated as CD103+CDHILC+ out of alive, singlet CD45+ cells. Flow cytometry of lineage-negative (CD11b~Gr1−, NK1.1−, B220−, CD11c+) CD103+CD11c+ TME or NTT cells. On the day of ACT, BLI was performed to have a reference value of the spiked-in populations, mice were assigned into Ctrl and ACT groups and ACT of 4×10⁶ pre-activated effector OT-1 T cells was performed on day 7.

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**Western blotting.** Radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (CST, Thermo Fisher Scientific) was used according to the manufacturer’s instructions. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce). Proteins were separated using a 4–12% gradient Bis–Tris polyacrylamide gel in the MOPS buffer system (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad) using standard protocols. Proteins were blocked in 5% milk and incubated with antibodies in 5% milk overnight on 4°C (for antibody information see Reporting Summary). After primary antibody incubation and washing, membranes were probed with horseradish peroxidase (HRP)-coupled secondary antibodies (1:5,000 in 5% milk) and developed using the ECL system (GE Healthcare).

**IF staining murine tumors.** Tissues for IF staining of tumors were obtained after fixation in 4% paraformaldehyde at 4 °C, and two subsequent dehydration steps from 15% sucrose to 30% sucrose. Tissues were then sliced using a sledge microtome and slices (30 μm) were blocked in 10% goat serum, 2% BSA and 0.25% Triton X-100 in PBS for 2 h at room temperature (RT). Primary antibodies (Reporting Summary) were incubated overnight in the blocking solution at 4°C and on the next day for 30 min at RT. After five washes in PBS/0.25% Triton, secondary antibodies were added for 1 h at RT at a concentration of 1:1,000 followed by five additional washes. Then, nuclei were stained with Hoechst solution (Thermo Fisher Scientific), tumor slices were washed twice with PBS, transferred on to glass slides and mounted with ProLong Gold Antifade Reagent (Thermo Fisher Scientific). For quantification of IF staining, five regions of interest (ROIs) were acquired per tumor and counted manually using ImageJ. The margin was defined as 300 μm from the tumor border and the center was defined as ~1 mm from the tumor margin onward. For each condition two or more tumors each with ≥2 ROIs were counted.

**IF staining melanoma patient biopsies.** Patient biopsies from a cohort of 10 patients before (NTT) and after progression on MAPK pathway inhibitors (BRAFi or BRAFi+MEKi) combination therapy, R² were processed for multiplex IF staining on formalin-fixed paraffin-embedded (FFPE) tissue sections. Melanoma (5 mm² fixed, paraffin-embedded) melanoma biopsies were cut into 3-μm sections and mounted on Superfrost Plus slides (Thermo Fisher Scientific). FFPE slides were heated in the oven at 65 °C for 30 min, deparaffinized in xylene (5 min in 2x xylene) and rehydrated in ethanol (5 min in 2x 100% ethanol, 5 min in 1x 95% ethanol, 5 min in 70% ethanol). Antigen retrieval was performed in AR9 buffer (Akoya; catalog no. AR900) at 110 °C for 10 min in a pressurized decloaking chamber (Biocare Medical). Slides were cooled in a flowing water bath for 5 min before commencing staining using an automated slide stainer (intelliPATH FLX, Biocare Medical). Tissue sections were blocked with 3% hydrogen peroxide in Tris-buffered saline–Tween 20 (TBS-T) for 5 min. Primary antibodies (Reporting Summary) were incubated for 30 min. Positive controls for antibodies were detected in the presence of 10 μM poly-L-lysine (further washed with TBS-T) for 10 min and visualized using Tyramide Signal Amplification with 10-min incubations (Olap 7-Colour HSC, Akoya; CD103 Opal 520, CD103 Opal 570, CD3 Opal 620, CD3 Opal 650, SOX10 Opal 690). Between subsequent staining runs, the sections were deboxed in AR buffer, pH 9 in the deboxing chamber at 110°C for 10 min to strip the antibody–HRP complex from the sections. Samples were counterstained with DAPI (2 drops in 1 ml) for nuclei visualization, and subsequently coverslipped using ProLong Diamond Antifade Mountant (Invitrogen).

For CLEC9a staining on patient biopsies, staining was performed using the Alexa Fluor-488 Tyramide SuperBoost Kit (Thermo Fisher Scientific, catalog no. B40922). In brief, biopsies were dewaxed, antigen retrieval was performed in EDTA (pH 9.5), slides were blocked in 3% hydrogen peroxide for 10 min in the dark, followed by three washing steps in TBS-T and blockade in 10% goat serum for 30 min. Slides were stained with primary antibody (Reporting Summary) for 1 h at RT, followed by three washes in TBS-T and incubation with secondary antibody (goat-anti-rabbit polyclonal (HRP), Thermo Fisher Scientific, catalog no. B40922) for 1 h at RT. Slides were washed three times in TBS-T and incubated with Tyramide 488
SLAM-seq sample preparation. Braf/Ftenova N'T cell line and R'T cell line were seeded and allowed to adhere overnight, and the medium was replaced with a complete medium (10% FBS to activate signaling and induce a transcriptional response mimicking that allowed to adhere overnight, and the medium was replaced with complete medium (10% FBS to activate signaling and induce a transcriptional response mimicking that allowed to adhere overnight, and the medium was replaced with complete medium (10% FBS to activate signaling and induce a transcriptional response mimicking that allowed to adhere overnight, and the medium was replaced with complete medium (10% FBS to activate signaling and induce a transcriptional response mimicking that allowed to adhere overnight, and the medium was replaced with complete medium (10% FBS to activate signaling and induce a transcriptional response mimicking that allowed to adhere overnight, and the medium was replaced with complete medium (10% FBS to activate signaling and induce a transcriptional response mimicking.
4. Larkin, J. et al. Five-year survival with combined nivolumab and ipilimumab in advanced melanoma. N. Engl. J. Med. 381, 1355–1364 (2019).
5. Seth, R. et al. Systemic therapy for melanoma: ASCO guideline. J. Clin. Oncol. 38, 3947–3970 (2020).
6. Michielin, O. et al. Cutaneous melanoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann. Oncol. 30, 1884–1901 (2019).
7. Ackerman, A. et al. Outcomes of patients with metastatic melanoma treated with immunotherapy prior to or after BRAF inhibitors. Cancer 120, 2665–2671 (2014).
8. Johnson, D. R. & Sullivan, R. J. Sequencing treatment in BRAFV600E mutant melanoma: anti-PD-1 before and after BRAF inhibition. J. Immunother. 40 (2017).
9. Téno, P. et al. Benefit of the nivolumab and ipilimumab combination in pretreated advanced melanoma. Eur. J. Cancer 93, 147–149 (2018).
10. Mason, R. et al. Combined ipilimumab and nivolumab first-line and after BRAF-targeted therapy in advanced melanoma. Pigment Cell Melanoma Res. 33, 358–365 (2019).
11. Frederick, D. T. et al. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. Clin. Cancer Res. 19, 1225–1231 (2013).
12. Cooper, Z. A. et al. Distinct clinical patterns and immune infiltrates are observed at time of progression on targeted therapy versus immune checkpoint blockade for melanoma. OncolImmunology 5, e1136044 (2016).
13. Hugo, W. et al. Non-genomic and Immune evolution of melanoma acquiring MAPK4 resistance. Cell 162, 1271–1285 (2015).
14. Hugo, W. et al. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. Cell 165, 35–44 (2016).
15. Steinberg, S. M. et al. Myeloid cells that impair immunotherapy are restored in melanomas with acquired resistance to BRAF inhibitors. Cancer Res. 77, 1599–1610 (2017).
16. Ergaz, J. I. et al. Myosin II reactivation and cytoskeletal remodeling as a hallmark and a vulnerability in melanoma therapy resistance. Cancer Cell 37, 85–103.e9 (2020).
17. Walsh, S. J., Rizos, H., Scolyer, R. A. & Long, G. V. Resistance to combination PD1 blockade with in vivo vaccination. Nat. Med. 25, 814–824 (2019).
18. McLaughlin, M. et al. Inflammatory microenvironment remodelling by tumour cells after radiotherapy. Nat. Rev. Cancer 20, 203–217 (2020).
19. Oberaun, A. C. et al. Therapy-induced tumour secretomes promote resistance and tumour progression. Nature 520, 368–372 (2015).
20. Liu, C. et al. BRAF Inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. Clin. Cancer Res. 19, 393–403 (2013).
21. Koya, R. C. et al. BRAF inhibitor vemurafinib improves the antitumor activity of adoptive cell immunotherapy. Cancer Res. 72, 3928–3937 (2012).
22. Gide, T. N. et al. Distinct immune cell populations define response to anti-PD1 monotherapy and anti-PD1/anti-CTLA4 combined therapy. Cancer Cell 35, 238–255.e6 (2019).
23. Liu, D. et al. Integrative molecular and clinical modeling of clinical outcomes to PD1 blockade in patients with metastatic melanoma. Nat. Med. 25, 1916–1927 (2019).
24. Zhang, P. A. & Bhardwaj, N. Impact of MAPK pathway activation in BRAF(V600) melanoma on T cell and dendritic cell function. Front. Immunol. 4, 346 (2013).
25. Shi, H. et al. Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. Cancer Discov. 4, 80–93 (2014).
26. Mühler, M. et al. SLAM-seq defines direct gene-regulatory functions of the BRD4–MYC axis. Science 360, 800–805 (2018).
27. Herranz, V. A. et al. Thiolo-linked alkylation of RNA to assess expression dynamics. Nat. Methods 14, 1198–1204 (2017).
28. Spranger, S. & Gajewski, T. Impact of oncogenic pathways on evasion of antitumour immune responses. Nat. Rev. Cancer 18, 139–147 (2018).
29. Diaz, L. A. et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 486, 537–540 (2012).
30. Gajewski, T. F. & Higgs, E. F. Immunotherapy with a sting. Science 369, 921–922 (2020).
31. Wculek, S. K. et al. Dendritic cells in cancer immunology and immunotherapy. Nat. Rev. Immunol. 20, 7–24 (2020).
32. Long, J. E. et al. Therapeutic resistance and susceptibility is shaped by cooperative multi-compartment tumor adaptation. Cell Death Differ. 41, 2416–2429 (2019).
33. Bar-Sagi, D., Knebel, E. H. & Sequist, L. V. A bright future for KRAS inhibitors. Nat. Cancer 1, 25–27 (2020).
34. Meeth, K., Wang, X. J., Micevic, G., Damsky, W. & Bosenberg, M. W. The YUMM lines: a series of congeneric mouse melanoma cell lines with defined genetic alterations. Pigment Cell Melanoma Res. 29, 590–597 (2016).
35. Castle, J. C. et al. Immunogenic, genomic and transcriptomic characterization of CT26 colorectal carcinoma. BMC Genom. 15, 190 (2014).
36. Minn, A. J. et al. Distinct organ-specific metastatic potential of individual mouse melanoma cell lines and primary tumours. J. Clin. Invest. 115, 44–55 (2005).
37. Ochly, L. J. & Moon, J. J. Whole-animal imaging and flow cytometric techniques for analysis of antigen-specific CD8+ T cell responses after nanoparticle vaccination. J. Vis. Exp. 98, e52771 (2015).
38. Picelli, S. et al. Full-length RNA-seq from single cells using Smart-seq2. Nat. Protoc. 9, 171–181 (2014).
39. Corces, M. R. et al. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat. Methods 14, 959–962 (2017).

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Author contributions
L.H. and A.C.O. conceived the study, designed the experiments and interpreted the results. A.C.O. supervised the study. L.H. developed experimental tools, performed in vitro experiments, in vivo treatment studies, flow-cytometry analysis, gene expression profiling and parts of the computational analysis, and analyzed the data. A.E. validated in vivo treatment studies in independent experiments, performed the in vitro co-culture in Figs. 3i, 4f and 7f, and performed the ATAC-seq library prep. C.U. performed large parts of the SLAM-seq experiment (Extended Data Fig. 9d–h) and generated matched CaTCH clones (Fig. 5f and Extended Data Fig. 8e). I.K. performed western blotting and IF staining (including quantifications) and helped with mouse colony maintenance. M.P. and H.W. performed experiments involving focal radiation. M.K. helped with flow-cytometry studies and in vivo studies (including CT26 flow-cytometry characterization displayed in Extended Data Fig. 6k,m). D.H. contributed to experimental design, computational analysis, in vivo studies and data interpretation. C.L.G. collected the patient information for the retrospective analysis in Fig. 1a,b, M.A.C and O.V. analyzed and interpreted the collected patient data. J.S.W., G.V.L. and R.A.S. analyzed and interpreted VECTRA image analysis data of melanoma biopsies and related clinical data of patients treated with MAPKi therapies, and provided matched patient biopsies for the CLEC9a staining for Fig. 3g. L.L., M.N. and T.N. analyzed gene expression data, SLAM-seq data and whole-exome sequencing data. L.L. analyzed ATAC-seq data and contributed to experimental design and data interpretation. J.Z. provided conceptual input for experiment design and data interpretation of SLAM-seq. S.V. analyzed RNA-seq data, generated the ccIES and probed it in the RNA-seq data of TCGA and published melanoma patient datasets. S.C. and K.J.H. provided conceptual input to the experiment design and data interpretation. T.W. provided clinical expertise and input to experiment design, interpretation and presentation. L.H., T.W. and A.C.O. wrote the manuscript, and all authors read and approved it.

Competing interests
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Extended Data Fig. 1 | Targeted therapy resistant patients display reduced T cell infiltrate and cross-resistance to immunotherapy. **a.** Overall survival (OS) of metastatic melanoma patients in the Lausanne Patient Cohort (Supplementary Table 1) receiving immunotherapy with checkpoint inhibitors \((n=54\) patients). N\textsuperscript{TT}, targeted therapy (TT) naïve patients \((n=38\); R\textsuperscript{TT}, TT (RAFi or RAFi/MEKi) resistant patients \((n=16\). **b.** Summary of responses to immunotherapy in N\textsuperscript{TT} and R\textsuperscript{TT} patients in published patient cohorts (ORR = overall response rate, PFS = progression-free survival). **c.** CD3\textsuperscript{+}CD8\textsuperscript{+} T cells in patient-matched N\textsuperscript{TT} and R\textsuperscript{TT} melanoma biopsies [scale bar pre-treatment: 2 mm (left), 200 \(\mu\)m (right), 50 \(\mu\)m (zoom-in); scale bar post progression: 5 mm (left), 200 \(\mu\)m (right), 50 \(\mu\)m (zoom-in)]. Experiment performed once on 10 matched biopsies. **d.** CD8\textsuperscript{+}CD39\textsuperscript{+}CD103\textsuperscript{+} T cells in patient-matched N\textsuperscript{TT} and R\textsuperscript{TT} melanoma biopsies [scale bar: 200 \(\mu\)m, 50 \(\mu\)m (zoom-in)]. **e.** Quantification of tumour reactive (CD8\textsuperscript{+}CD39\textsuperscript{+}CD103\textsuperscript{+}) T cells in patient-matched N\textsuperscript{TT} and R\textsuperscript{TT} melanoma biopsies, assessed by IF staining \((n=10\) patients, Supplementary Table 2). Data analysis (a) two-sided log-rank (Mantel-Cox) test.
Extended Data Fig. 2 | The Braf melanoma model responds to checkpoint inhibition in the NTT state, but is resistant in the RTT state. a, Proliferation fold change (FC) of Braf melanoma cells after 72 h at indicated drug conditions. Line indicating FC in proliferation of NTT cells on lowest drug condition (n = RAFi: technical triplicates; RAFi/MEKi technical duplicates), (drug concentrations: RAFi: DMSO CTRL, 100 nM, 300 nM, 1 µM, 3 µM; RAFi/MEKi: DMSO CTRL, 10 nM/3 nM, 30 nM/10 nM, 100 nM/30 nM, 300 nM/100 nM). b, pERK status in NTT and RTT Braf melanoma cells, 1-hour post drug exposure. Experiment performed twice; representative example shown. c, Treatment response of subcutaneously injected Braf/Pten melanoma (CTRL, n = 4 tumours; other groups, n = 6 tumours) continuously treated with TT; arrow indicating start of therapy. Experiment repeated 5 times; representative example shown. d, Proliferation FC of Braf/Pten melanoma cells after 72 h at indicated drug conditions. Line indicating FC in proliferation of NTT cells on lowest drug condition (n = RAFi: technical triplicates; RAFi/MEKi technical duplicates), (drug concentrations: RAFi: DMSO CTRL, 100 nM, 300 nM, 1 µM, 3 µM; RAFi/MEKi: DMSO CTRL, 10 nM/3 nM, 30 nM/10 nM, 100 nM/30 nM, 300 nM/100 nM). e, pERK status in NTT and RTT Braf/Pten melanoma cells, 1-hour post drug exposure. Experiment performed twice; representative example shown. f, Gating strategy highlighting successful CD8 T cell depletion in blood of mice treated with anti-CD8 versus CTRL antibody. g, Treatment response to anti-PD-1/CTLA-4 in combination with CD8 depletion in Braf melanoma. (CTRL, n = 6; all other groups, n = 10 tumours). Black arrows indicate anti-PD-1/CTLA-4 administration and blue arrows administration of CD8 depletion antibody. Experiment performed once. P-value: ** 0.0012, ns 0.9970. h, Spider plots indicating individual tumour growth curves of NTT and RTT Braf melanoma receiving checkpoint blockade (CTRL n = 6 tumours; anti-PD-1/CTLA-4, n = 8 tumours). Experiment repeated 9 times; representative example shown. i, Treatment response to anti-PD-1/CTLA-4 of NTT and RTT Braf melanoma (CTRL, RAFi n = 6 tumours; anti-PD-1/CTLA-4 ± RAFi, n = 10 tumours); arrows indicate therapy administration. RTT mice continuously treated with RAFi (5 mg/kg). Experiment performed once. P-value: **** 8.4e-6, ns 0.9924. Data in (a, c, d, g, i) displayed as mean ± SEM. Data analysis (g, i) two-way ANOVA. ** P < 0.01, **** P < 0.0001, ns = non-significant.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Cross-resistance is mediated via the tumour microenvironment. a, Treatment response to immunotherapy in NTT Braf/Pten melanoma tumour bearing mice, black arrows indicate therapy administration (NTT CTRL, n = 2; NTT anti-PD-1/CTLA-4, n = 4 mice). Experiment performed once. b, Generation of OVA antigen-expressing NTT and RTT Braf/Pten cell lines using indicated expression vector (top) and quantification of processed MHC-I loaded ovalbumin peptide (SIINFEKL) by flow-cytometry (bottom). c, Spider Plots indicating individual tumour growth curves of NTT and RTT Braf/PtenOVA tumours receiving ACT (CTRL n = 3; ACT, n = 5 tumours). Experiment performed 7 times; representative example shown. d, Treatment response to ACT in RAFi/MEKi RTT Braf/PtenOVA tumours; arrow indicating day of ACT (NTT, RAFi/MEKi RTT CTRL, n = 3 mice; NTT, RAFi/MEKi RTT ACT, n = 5 mice. P-value: **** 3.9E-11, ns 0.6) (left) and infiltration of OT-1Luc T cells measured by bioluminescence imaging (BLI) at indicated days (all groups, n = 5 tumours P-value: * 0.021, ** 0.0079, ns 0.0952) (right). Experiment performed twice with two independent clones; representative example shown. e, f, Treatment response to ACT in Braf/PtenOVA tumours, RTT tumours assessed (e) off RAFi for the entire experiment [P-value: **** 1.7E-6, ns 0.33] and (f) under continuous exposure to RAFi (10 mg/kg) [P-value: ****5.8E-8, ns 0.35]; arrow indicating day of ACT (NTT, RTT CTRL, n = 3 mice NTT; RTT ACT, n = 5 mice). g, Tumour infiltration of OT-1Luc T cells into NTT Braf/PtenOVA tumours and RTT Braf/PtenOVA tumours ± RAFi (10 mg/kg), (all groups, n = 3 mice). Experiment performed once. P-value: **** 0.0004, *** 0.0003. h, i Principal Component Analysis (PCA) plots displaying top 500 most variable genes for (h) Braf/Pten and (i) Braf melanoma treated with IFN-γ. j, MHC-I surface expression of NTT and RTT Braf melanoma cell lines (baseline and 24 h post 10 ng/ml IFN-γ exposure). Experiment performed 3 times; representative example shown. k, Gene expression changes in NTT and RTT Braf melanoma cell lines treated with IFN-γ. Correlation between genes deregulated in NTT (x-Axis) and RTT (y-Axis) cell lines (P < 0.05), dots display individual genes. P-value: <1E-15. Supplementary Table 3. l, BrafOVA melanoma cell viability after 24 h of co-culture in in vitro killing assay using pre-activated OT-1 T cells at indicated effector:target ratios (all groups, n = 2 replicates). Experiment performed twice; representative example shown. m, Treatment response to ACT in tumours consisting of NTT and RTT Braf/PtenOVA cell lines at indicated ratios; arrow indicating day of ACT (0.05% NTT/RTT CTRL, n = 4; ACT, n = 5; 0.05% RTT/NTT CTRL, n = 4; ACT, n = 4 tumours). Experiment performed twice; representative example shown. n, Scheme outlining experiments to test antigen-specificity of T cell killing in vivo (left) and BLI signal at day 6 post ACT for tumours containing 0.05% OVA+Luc+ or 0.05% OVA+Luc+ CTRL cells (N1/ OVA+ n = 4; N1/ OVA- CTRL n = 5 tumours) (right). Data in (a, d-g, m, n) displayed as mean ± SEM. Data analysis (d-f) two-way ANOVA (d) two-tailed unpaired t-test (g) one-way ANOVA (k) two-sided Pearson correlation. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = non-significant.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | The tumour microenvironment of RTT tumours is strongly remodelled. a, T cell influx into NTT and RTT Braf melanoma, assessed by IF (scale bar 100 µm and 20 µm). Experiment performed twice; representative image shown. b, T cell quantified separately at tumour margin and centre (n = 3 tumours per condition; NTT n = 15, RTT n = 16 ROI). P-value: * 0.0204, ns 0.0756. c, PCA plot displaying top 500 most variable genes for T cells sorted from NTT and RTT Braf/PtenOVA tumours. d, e Gating strategy highlighting identification of CD3+ CD8+ T cells, CD103+ CD11c+ DCs and CD11b+ GR-1+ suppressive myeloid cells. f, Suppressive myeloid cells in Braf melanoma, assessed by flow cytometry (n = 7 tumours per condition). P-value: * 0.023. Experiment performed 3 times; data represents pool of 2 experiments. g, CD103+ DCs in Braf melanoma, assessed by flow cytometry (n = 8 tumours per condition). P-value: *** 0.0005. Experiment performed 3 times; data represents pool of 2 experiments. h, Gating strategy highlighting the identification of CD103 DCs in an alternative gating strategy (Lineage negative (CD11b−;Gr-1, NK1.1, CD3−, B220−, F480−) MHCII+CD103+ cells). i, Quantification of CD103+ MHCII+ DCs with alternative gating strategy. (Braf/PtenOVA melanoma both groups, n = 4 tumours; Braf/Pten melanoma NTT = 5 tumours, RTT = 8 tumours). Experiment performed once. P-value: * 0.0185, ** 0.0083. j, k CD103+ MHCII+ DCs in NTT and RTT Braf melanoma, assessed by IF staining in (j) displayed as a representative picture (scale bar 100 µm and 20 µm) (Experiment performed twice) and (k) quantified separately at tumour margin and centre (n = 3 tumours per condition, all groups 15 ROIs). P-value: *** 0.0002, **** 0.0001. l, CD103+ MHCII+ DCs in NTT and RTT Braf/PtenOVA melanoma quantified separately at tumour margin and centre (n = 2 tumours per condition, all groups 10 ROI, except NTT margin n = 11). P-value: **** 5.7E-6, 1.1E-5. Data in (b, f, g, i, k, l) displayed as mean ± SEM and analysed by two-tailed unpaired t-test with Welch correction for unequal variance or with Mann-Whitney-U-test if not normal distributed. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = non-significant.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Modulation of the myeloid cell compartment restores immunotherapy response. a, DC maturation score comparing gene expression profiles of RTT vs. NTT tumours (Braf/Pten NTT; Braf NTT, BRAF RTT n = 3; Braf/Pten RTT n = 4 tumours). b, Mean fluorescence intensity (MFI) of maturation markers on CD103+ DCs from NTT and RTT Braf/Pten melanoma (N TT, n = 5; R TT, n = 6 tumours), assessed by flow cytometry. Experiment performed twice with independent cell lines; representative example shown. P-value: **** 6.9E-5, ** 0.0043, * 0.0149. c, PCA plot displaying top 500 most variable genes for CD103+ DCs sorted from NTT and R TT ± Poly I:C Braf/PtenOVA tumours. d, GSEA of IFN-alpha response in CD103+ DCs sorted from R TT vs. N TT Braf/PtenOVA melanoma. e, Quantification of T cell proliferation based on CFSE dilution in DC co-culture assays displayed in Fig. 3i (n = 4 tumours per condition). P-value: * 0.029. f, Scheme outlining experiment to assess impact of depleting suppressive myeloid cells on ACT in Braf/PtenOVA tumours. g, Depletion of Ly6C+CD11b+ and Ly6G+CD11b+ cells in blood 3 days post anti-GR-1 administration. h, Tumour infiltration of effector OT-1Luc T cells measured by BLI at 24 h post ACT in Braf/PtenOVA tumour bearing mice treated with Isotype CTRL or anti-GR-1 antibody (n = 9, 9, 8, 8 mice from left to right). Experiment performed 3 times; representative example shown. P-value: ns 0.53, * 0.0128. i, Treatment response of Braf/PtenOVA tumours treated with ACT or anti-GR-1 plus ACT (N TT ACT + ISO, n = 6; R TT ACT + ISO, n = 4; N TT ACT + anti-GR-1, n = 6; R TT ACT + anti-GR-1, n = 4 mice). j, DC maturation in Poly I:C injected Braf/PtenOVA tumours assessed by CD40 expression using flow cytometry. k, CD103+ DC influx in R TT tumours overexpressing FLT3L, assessed by flow cytometry (n = 3 tumours). P-value: * 0.0118. l, Survival in response to ACT ± Poly I:C ± FLT3L in R TT Braf/PtenOVA tumours. (R TT CTRL, R TT + ACT, R TT FLT3L + ACT, n = 3; R TT + ACT + Poly I:C, R TT FLT3L + ACT + Poly I:C, n = 4 mice). Experiment performed twice; representative example shown. P-value: ** 0.0100. m, Treatment response of R TT Braf melanoma in WT mice (left) and BATF3−/− mice (right) treated with indicated therapies; black arrows indicate anti-PD-1/CTLA-4 administration, red arrows indicate Poly I:C injection (CTRL, anti-PD-1/CTLA-4, Poly I:C, n = 6 tumours; Poly I:C + anti-PD-1/CTLA-4, n = 4 tumours). Experiment performed twice; representative example shown. P-value: ** 0.01, ns 0.1931. Data in (b, e, h, k, m) is displayed as mean ± SEM. Data analysis (b, h, k) two-tailed unpaired t-test with Welch correction for unequal variance or with Mann-Whitney-U-test if not normal distributed l two-sided log-rank test (Mantel-Cox) test (m) two-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = non-significant.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | The CT26 colon carcinoma model displays cross-resistance and an immune-evasive TME. a, Treatment response of subcutaneously injected CT26 colon carcinoma (CTRL or MEKi, n = 8 tumours) continuously treated with TT; arrow indicating start of therapy. Experiment performed twice; representative example shown. b, Proliferation FC in CT26 colon carcinoma cell lines after 72 h at indicated drug conditions. Line indicating FC in proliferation of NTT cells on lowest drug condition (n = technical triplicates) (drug concentration: DMSO CTRL, 10 nM, 30 nM, 100 nM, 300 nM MEKi). c, pERK status in NTT and RTT CT26 colon carcinoma cell lines, 1-hour post drug exposure. Experiment performed twice; representative example shown. d, Treatment response in mice bearing NTT and RTT CT26 (Kras<sup>G12D/G12D Cdkn2a<sup>-/-</sup></sup>) tumours (NTT and RTT CTRL, n = 6; NTT and RTT anti-PD-1, n = 14 tumours) treated with anti-PD-1, arrows indicate therapy administration. Experiment performed 5 times; representative example shown. P-value: **** 1.5E-8, ns 0.2838.  

Extended Data Fig. 6 continued e, MHC-I surface expression of NTT and RTT cell lines (baseline and 24 h post 10 ng/ml IFN-γ exposure). Experiment performed 3 times; representative example shown. f, Gene expression changes in NTT and RTT CT26 colon carcinoma cell lines treated with IFN-γ. Correlation between genes deregulated in NTT (x-Axis) and RTT (y-Axis) cell lines (P < 0.05), dots display individual genes. P-value: <1E-15. g, PCA plot displaying top 500 most variable genes for CT26 colon carcinoma cell lines treated with IFN-γ. h, i T cells in untreated NTT and RTT CT26 colon carcinoma tumours assessed by IF staining and (h) quantified separately at tumour margin and centre (n = 3 tumours per condition; all 15 ROI, except RTT centre n = 16) and (i) displayed as a representative picture (scale bar 100 µm and 20 µm). Experiment performed twice. P-value: ** 0.0017, **** 3E-5. j, CD103<sup>+</sup> DCs in untreated NTT and RTT CT26 colon carcinoma tumours assessed by IF staining and quantified separately at tumour margin and centre (n = 3 tumours per condition, all 15 ROI). P-value: **** 0.0002, * 0.046. k, CD103<sup>+</sup> DC infiltration NTT and RTT tumours of CT26 colon carcinoma, assessed by flow cytometry (n = 16, 14 tumours each). Data represents pool of 2 independent experiments. P-value: * 0.016. l, CD103<sup>+</sup> DC infiltration NTT and RTT tumours of CT26 colon carcinoma, alternative gating strategy displayed in Extended Data Fig. 4h (n = 5, 8 tumours). P-value: ** 0.0081. m, Suppressive myeloid cell infiltration in NTT and RTT tumours of CT26 colon carcinoma, assessed by flow cytometry (n = 16, 14 tumours each). Data represents pool of 2 independent experiments. P-value: **** 7E-6. n, MFI of indicated maturation markers on CD103<sup>+</sup> DCs from NTT and RTT CT26 colon carcinoma (NTT, n = 6; RTT, n = 8 tumours) assessed by flow cytometry. P-value: * 0.0426, ns 0.1419, * 0.0293. Experiment performed once. Data in (b, d, h, j-n) displayed as mean ± SEM. Data analysis (d) two-way ANOVA (f) two-sided Pearson correlation (h, j-n) two-tailed unpaired t-test with Welch correction for unequal variance or with Mann-Whitney-U-test if not normal distributed. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = non-significant.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Modulation of the CD103+ DC compartment restores immunotherapy response in R1+CT26 colon carcinoma.  

**a**, Scheme outlining experiment to assess impact of maturation (intratumoural Poly I:C) and expansion (FLT3L overexpression from tumour cells) of DCs on anti-PD-1 treatment in mice bearing R1+CT26 colon carcinoma (left) and survival curve of mice (right, CTRL, n = 3; all other groups, n = 5 mice). Experiment performed twice; representative example shown. P-value: ** 0.0067.  

**b**, Treatment response of R1+CT26 colon carcinoma to anti-PD-1 (day 6, 9, 12) in combination with intratumoural Poly I:C injection (day 5, 9, 12) for intratumoural (left) or contralateral tumour. (CTRL, n = 8; anti-PD-1 n = 10; contralateral and intratumoural, n = 5 tumours each).  

**c**, Influx of H2-Ld MuLV gp70 specific T cells into N1 and R1+CT26 colon carcinoma treated with anti-PD-1 and Poly I:C (injected and contralateral tumour displayed separately) (N1 CTRL, n = 4; R1+CT26, n = 3; R1+anti-PD-1, n = 4; anti-PD-1 + Poly I:C intratumoural, anti-PD-1 + Poly I:C contralateral, n = 5 tumours). Experiment performed once. P-value: all ns.  

**d**, Gating strategy highlighting the identification of gp70 Tetramer positive T cells.  

**e**, Treatment response to anti-PD-1 (day 6, 9, 12) in combination with intratumoural Poly I:C injection (day 5, 9, 12) ± CD8 depleton (day 3, 5, 10, 14), injected and contralateral tumour displayed separately. (CTRL, anti-PD-1, anti-PD-1 + Poly I:C + CD8 depletion n = 6; anti-PD-1 + Poly I:C, n = 7 mice). Experiment performed once. P-value: **** 1.9E-6, **** 2.5E-5, ns 0.9989.  

**f**, Scheme outlining experiment to assess impact of focal radiation ± anti-PD-1 ± FLT3L in mice bearing R1+CT26 colon carcinoma (left) and survival curve of mice treated with indicated therapies (CTRL, n = 9; anti-PD-1, n = 8; XRT + anti-PD-1, n = 8; XRT + anti-PD-1 + FLT3L, n = 8 mice). Experiment performed once. P-value: **** 0.0006, ** 0.0389.  

**g**, Treatment response of RT26 colon carcinoma to anti-PD-1 (day 15, 18, 21, 24) in combination with focal radiation (9 Gy, day 14) and FLT3L administration (10 consecutive doses, initiated on day 7). Number of responding mice indicated in graph. Data in (b, c, e) displayed as mean ± SEM. Data analysis (a, f) two-sided log rank (Mantel-Cox) test (e) two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = non-significant.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Cross-resistance to immunotherapy is cell intrinsic, acquired during resistance formation and specific to MAPK pathway inhibition. a, Active response to RAFi in N10 Braf/Pten tumours (7 doses) and resistance formation upon 27 doses (CTRL, n = 6; 7 doses n = 8, 27 doses n = 10 tumours). b, Characterization of suppressive myeloid cells, T cells and CD103+DCs in Braf/Pten tumours actively responding to RAFi (7 doses) and in relapsing tumours, fully resistant to RAFi (27 doses) (n = 8 tumours per group; except CD3+ 7 doses, n = 7; CD3+ 27 doses, n = 9; CD11b+ Gr-1, CD103+ 27 doses, n = 10). Experiment performed twice; representative example shown. P-value top row: ** 0.0011, ** 0.0085, ** 0.0014; bottom row: ns 0.08, ** 0.0018, *** 0.0003. c, Proliferation FC in Braf/Pten and Braf melanoma cell lines (made resistant to TT in vitro) after 72 h at indicated drug concentrations. Line indicating FC in proliferation of N10 cells on lowest drug condition (n = technical triplicates), (drug concentration: DMSO CTRL, 100 nM, 300 nM, 1 µM, 3 µM RAFi). d, Proliferation FC of Braf melanoma cell lines after 72 h in indicated drug conditions of NTT and NTT-Dacarbazine cell lines (n = technical duplicates), (drug concentration: CTRL, 10 µg, 50 µg, 100 µg, 500 µg Dacarbazine). e, pERK status in CaTCH-isolated N10 and R1 Braf/Pten cell lines, 1-hour post drug exposure. Experiment performed twice; representative example shown. f, Treatment response to ACT in matched CaTCH isolated N10 and R1 Braf/Pten cells (CTRL, n = 3 mice, ACT, n = 5 mice). Experiment performed twice; representative example shown. P-value: **** 3E-5, ns 0.987. g, h PCA plot displaying top 500 most variable genes for (g) Braf/Pten and (h) Braf melanoma tumours. i, Expression of genes comprising the ccIES in sorted N10 and R1 Braf/Pten cells (all groups n = 3 tumours) and in sorted RAFi/MEKi R1 melanoma cells (all groups n = 3 tumours) (right). j, Overall survival stratified based on ccIES expression in TCGA melanoma patients (n = 469 patients). k, I Progression-free survival stratified based on ccIES expression in patients receiving (k) anti-PD-1/CTLA-4 combination therapy (n = 32 patients) or (l) anti-PD-1 monotherapy (n = 121 patients). m, Correlation of ccIES with CD103 score and T cell score in TCGA melanoma patients (n = 469 patients). Data in (a, b, c, d, f) displayed as mean ± SEM. Data analysis (b) two-tailed unpaired t-test with Welch correction for unequal variance or with Mann-Whitney-U-test if not normal distributed (f) two-way ANOVA. P-value in (j-l) derived from a Cox proportional hazards model using gene score as a continuous variable and analysis in (m) two-sided Pearson Correlation coefficient (PCC). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = non-significant.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | The reactivated MAPK pathway in RTT tumours has a qualitatively and quantitatively different output. a, HOMER motif enrichment analysis of upregulated genes comparing RTT vs. NTT tumours of indicated models. b, Heatmap of normalized (RPGC) gene accessibility tracks. Depicted are accessibility profiles for peaks containing motifs of MAPK effectors (left, containing any of the following motifs: AP-1, Fosl1, Fra1, Fra2, Jun-AP-1, c-Jun-CRE, JunB, JunD, ATF2, ATF3) or peaks without MAPK motifs (right). c, Scheme illustrating workflow of SLAM-seq experiment in NTT and RTT (RAFi resistant) Braf/PtenOVA melanoma. d, pERK status in NTT and RTT Braf/PtenOVA melanoma, 1-hour post exposure to MEKi. Experiment performed twice; representative example shown. e, PCA Plot highlighting the Top 500 most variable genes (based on reads containing TC conversions) in SLAM-seq dataset. f, Changes in abundance of newly synthesized mRNA (detected in SLAM-seq based on T > C conversions) in NTT (left) or RTT (right) Braf/PtenOVA melanoma treated with MEKi for 2 hours. Significant targets genes identified in SLAM-seq in NTT cells (black), RTT cells (red) or both (blue) are labelled. Only genes with >2RPMu in CTRL or MEKi conditions displayed. g, Expression of newly synthesized mRNA (RPMu) of 488 target genes identified with SLAM-seq (log2FC < -1, >1, padj<0.1, >2 RPMu) in NTT and RTT Braf/PtenOVA melanoma ± MEKi. Target genes are grouped according to their expression change upon MEKi in both cell lines. (NTT: genes that change expression upon MEKi only in NTT cell line (RTT FC < 1.5), RTT: genes that change expression upon MEKi only in RTT cell line (RTT FC < 1.5), Common: gene expression FC upon MEKi exceeds ±1.5 in both cell lines). h, Expression of selected immune-related genes in NTT, RTT and RTT + MEKi (72 h) sorted Braf/PtenOVA melanoma cells from Rag2-/- mice (NTT, n = 3 R11, n = 8; R11 + MEKi n = 6 tumours). i, PCA plot displaying top 500 most variable genes for Braf/PtenOVA melanoma cells sorted from NTT and RTT tumours after 72 h of MEKi or CTRL treatment.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Inhibition of the reactivated MAPK pathway in RAfi resistant RTT tumours restores immunotherapy response. a, Quantification of T cell proliferation based on CFSE dilution in DC co-culture assays displayed in Fig. 7f (n = 3 tumours per condition). Experiment performed once. P-value: ns 0.21, **** 3E-5. b, Scheme illustrating the use of the ‘thymidine kinase’ (HSV-TK) suicide gene (activated by ganciclovir [GCV]) to induce apoptosis in the R16 Braf/PtenOVA cancer cell line (left) and BLI image and quantification of TGL+ R16 Braf/PtenOVA cancer cells at day 0 and 3 post GCV/MEKi administration (n = 5 mice) (right). Experiment performed twice; representative example shown. P-value: * 0.0188, * 0.0154. c, CD103+ DCs (left) and suppressive myeloid cells (right) in R16 Braf/PtenOVA tumours in response to GCV or MEKi administration (all groups, n = 5 tumours), assessed by flow cytometry. Experiment performed twice; representative example shown. P-value: ns 0.3766, * 0.0303, ns; ns 0.9350, **** <E-15. d, Survival curve illustrating treatment response in R16 Braf/PtenOVA tumour bearing mice treated with indicated therapies (all groups, n = 5 mice). Experiment performed twice; representative example shown. P-value: ** 0.0029. e, Survival curve and corresponding spider plot illustrating treatment response in R16 Braf tumour bearing mice treated with indicated therapies (n = 6 mice per group, 2 tumours each). Black arrows indicate immunotherapy administration, continuous MEKi was initiated on Day 5. Experiment performed twice; representative example shown. P-Value: *** 0.0005. f, Scheme illustrating experiments where mice bearing established NTT Braf/PtenOVA tumours were treated with a short run-in phase (4 doses) of RAfi or RAfi/MEKi and subsequently switched to ACT. g, Tumour infiltration of effector OT-1Luc T cells measured by BLI at 48 h post ACT in Braf/PtenOVA tumour bearing mice (n = 5 mice per group). Experiment performed once with two independent clones. Data represents one representative clone. P-value: ns 0.07, 0.071. h, Treatment response of Braf/PtenOVA tumours to ACT (all groups: CTRL n = 3 mice, ACT n = 5 mice per group). Data in (a - c, g, h) displayed as mean ± SEM. Data analysis (a) two-tailed unpaired t-test (b, c) one-way ANOVA (d, e) two-sided log-rank (Mantel-Cox) test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = non-significant.
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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Software and code

Our web collection on statistics for biologist contains articles on many of the points above.

Data collection

- The melanoma TCGA data (normalized RSEM counts) were downloaded from the cbio portal using the R package ctgsdr v1.3.0 (cbio portal June 2019).

Data analysis

- Gene expression analysis:
  - Trimming was performed for Quanti-seq 3' mRNA-seq experiments (LH5, LH6, LH7, LH8, LH10, LH11, LH12) using BBDuk v36.92 [ref=polyA.fa.gz, truseq.fa.gz k=13 ktrim=r forclrulef=11 useshorthkmer=r minlen=5 ktrim=t trimq=10 minlength=30], and standard RNA-seq experiments (LH1, LH2, LH4) using fastx-toolkit v0.0.13 (fastx_trimmer-f 13). Reads mapping to mouse rRNA transcripts (refseq) were removed using bwa v0.7.12 (DOI: 10.1093/bioinformatics/btp324) alignment and samtools v1.3.1. The remaining reads were aligned to mouse genome mm10 using TopHat v2.1.1 (GTF annotation file mm10, RefSeq from UCSC, 2015/01) (DOI: 10.1186/gb-2013-14-4-r36), and reads in genes were counted with htseq-count v 0.6.1 (DOI: 10.1093/bioinformatics/btu638). The parameters for both tools were set dependent on the strandness of the experiments. For Quanti-seq 3' mRNA-seq experiments tophat was run with the parameters –library-type fr-firststrand and htseq-count with -s reverse. For Smart-seq (LH9, LH13, LH14) libraries tophat was run with the parameters –library-type fr-unstranded and htseq-count with -s no. DESeq2 v1.16.1 in R v3.4.1 was used to perform differential expression analysis, data normalization by variance stabilizing transformation, and to generate PCA plots based on principle component analyses of the normalized expression data. Motif discovery was performed using HOMER v4.11 (DOI: 10.1016/j.jolc.2010.05.004) using the program findMotifs.pl with default settings. Heatmap visualization was performed using the ggrepel package of R or Heatmapper (http://www.heatmap.ca/expressio/). Differentially expressed gene sets were analysed using the gene-set enrichment analysis tool (GSEA, Broad Institute) GSEA Pre-ranked v3.0 using DESeq2 test statistic for ranking and gene signatures from MSigDB v7.0. Ingenuity pathway analysis (IPA, version May 2019) was used to identify deregulated pathways and upstream mediators. CD103 DC score was determined as the geometric mean of the expression of BAF73, IRF8, THBD, CLC9A, CXCL9 and CXCL10, Supplementary Table S8 (adapted from DOI: 10.1016/j.cell.2017.07.003). T cell score was based on TEM-T cell signature, derived from Jerby-Aron et al., Supplementary Table S9 (DOI: 10.1016/j.cell.2018.09.006). The melanoma TCGA data (normalized RSEM counts) were downloaded from the cbio portal using the R package ctgsdr, and expression data for the anti-PD-1 treated cohort was processed in house for h38 using the approach described above for mouse RNA-seq data. To probe IES and ccIES in clinical data, human orthologues were assigned using ENSEMBL and DIOPr.
SLAM-Seq data analysis:
Gene and 3’TUT annotations were obtained from the UCSC table browser (https://genome.ucsc.edu/cgi-bin/hgTables, June 2016). 3’TUT annotations were assigned to Entrez Gene IDs and collapsed on a per-gene basis with the use of bedtools’ (DOI: 10.1093/ bioinformatics/btfq033) merge command. For genes lacking an annotated 3’TUT, Ensembl v84 3’TUTs were added (if available), resulting in a total of 33,163 annotated 3’TUT intervals for 22,552 genes. Adapter trimmed reads were extracted from raw reads using cutadapt through the trim_galore (v0.3.7) wrapper tool with adapter overlaps set to 3bp for trimming. Trimmed reads were further processed using SLAMDunkv0.4.2 (http://github.com/t-neumann/slamdunk) (DOI: 10.1186/s12859-018-2849-7), running the full analysis procedure [slamdunk all] and aligning against the mouse genome (GRCm38), trimming 12bp from the 5’ end, reporting up to 100 alignments for multi-mappers and activating the multi-mapper retention strategy, filtering for variants with a variant fraction of 0.2 and filtering for base-quality cutoff of 22. Remaining parameters were left to their defaults. For gene-level analysis, raw reads mapped to different UTR annotations of the same gene were summed up by Entrez Gene ID. SLAM-seq experiments were performed in replicates and analysed as follows: Differential gene expression calling was performed on raw read counts with ≥2 T-C conversions using DESeq2 (version 1.14.1) (DOI: 10.1186/s13059-014-0650-4) with default settings, and with size factors estimated on corresponding total mRNA reads for global normalization. SLAM-seq target genes were defined as genes with an adj-P-value ≤ 0.1 and a log2FC > 1 or < -1, as well ≥ 2 RPPM in either CTRL condition or Meki treated condition. After definition of these initial set of 488 genes [159 genes identified in NTT, 383 in RTT, 54 shared among both], these genes were further divided into three groups: NTT only targets, common targets and RTT only targets. To avoid calling false- positive “NTT or RTT specific targets” more stringent selection criteria were introduced for this stratification: genes were classified as “common” if Meki affected their expression with a FC > 1.5 or < -1.5 in both cell lines, independent of significance, direction of regulation or expression levels; all other genes were defined as specific targets.

ATAC-seq data analysis:
ATAC-seq data from RTT and NTT Braf/Fn1ova melanoma cells sorted from tumors (n = 3 biological replicates per condition) was analysed using the nf-core/ATACseq pipeline (https://nf-co.org/atacseq) aligning against the mouse genome mm10 [–genome mm10] and calling peaks with MACS2 (v2.2.7.1) in narrow peak calling mode [–narrow_peak]. To infer differential transcription factor activity from genome-wide chromatin accessibility data, we employed diffTF v1.7.1 on peaks called in narrow-peak mode with MACS2. Statistical significance for differentially active TFs was assessed using the “analytical approach” with the following parameters: TFs = all, nPermutations = 0, bcGSim = 10, minOverlap = 2, RNASeqInegration = false (DOI: 10.1016/j.celrep.2019.10.106). Lineplots of individual transcription factor motif occupancy were generated using the footprinting, motifanalysis, and differential functions of the HINT-ATAC (DOI: 10.1186/s13059-019-1642-2) package with the following parameters: –organism mm10, –paired-end. BWA aligned reads of the individual replicates were merged using samtools v1.9 (DOI: 10.1093/bioinformatics/bts324), and RPBC-normalized tracks were calculated using the bamCoverage tool of the deepTools v3.3.1 package (lbs=1, –normalizeUsingRPBC –effectiveGenomeSize 2308125349). Peak-based motif analysis was performed using Homer [4.3.1] (DOI: 10.1016/j.mccl.2010.05.004) with the findMotifs.pl script and default settings. Peaks with MAPK transcription factor binding sites (AP-1, Fos2, Fra1, Fra2, Jun-A, –c-Jun, CRE, Jun-B, JunD, ATF2, ATF3) were identified using the annotatePeaks.pl tool with the mm10 genome with default parameters. Peaks were defined as MAPK-pathway dependent when using one peak MAPK TF binding site was identified within a peak. Conversely, peaks without any MAPK TF binding site were called as MAPK-pathway independent. Composite density plots for peaks unique for or shared between conditions were computed with the computeMatrix function (–referencePoint center -a 1000 -b 1000 –missingDataAsZero -b 10 –sortRegions “descend”) and visualized with the plotProfile and plotHeatmap functions with default parameters (DOI: 10.1093/nar/gkw257).

Whole exome sequencing analysis:
Genomic DNA was isolated from frozen cell pellets with the use of DNeasy Blood & Tissue kit (Qiagen). Library preparation and sequencing was performed by Macrogen, Inc. using the Agilent SureSelect Human All Exon Library and NovaSeq 6000 with 2 x 150 bp and at least 50X on-target coverage. Analysis of exome sequencing data was performed using the bcftools tool (https://databases/doi.org/10.5281/zenodo.3564938) with use of the following tools: alignment was performed with bwa v0.7.17 [mm10], duplicates were marked using biobambam v2.0.87, paired parental-clone SNV variant calling was performed with Strelka2 (DOI: 10.1038/s41592-018-0051-x) and parental cell line calling with mutect2 v4.1.7 [DOI: 10.1101/861054]. Variants were annotated using VEP [GRCm38] (DOI: 10.1186/s13059-016-0974-9) selecting one consequence per gene and annotation variants in coding regions only. The final annotated variant set was analysed and visualized with mutTools (DOI: 10.1101/ijr.239244.118). WES results are shown in Supplementary Tables 11-13.

Flow cytometry data:
Data was acquired using a BD LSR Fortessa and analysed using FlowJo 10.7.1.

Statistical analysis:
GraphPad Prism v7 and v8.3.1 was used for data entry, graph construction and data analysis.

For manuscripts utilizing custom algorithms or software that are not central to the research but yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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Gene expression data (RNA-seq, Quant-seq, SMART-seq and SLAM-seq) and ATAC-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession codes GSE132443. The human melanoma transcriptomic data was derived from the TCGA Network. Gene expression data for patients receiving checkpoint blockade is available from Gide et al. (PRESB3709) and Liu et al. (dbGaP phs000452.v3.p1).
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No sample size calculations were performed. Sample size was determined either 1) based on preliminary experiments that defined the adequate number of samples to consistently identify differences between groups or 2) in means comparable to Obenauf et al., Nature 2015 or Umkehrer et al. Nature Biotech 2020. Sample sizes are indicated in figure legends.

Data exclusions

No data-points were excluded, except: 1) In mouse experiments we excluded mice that had to be euthanized due to necrotic tumors, 2) In the Smart-seq experiment we excluded 2 samples (for CD103 DCs) and 3 samples (for T cells) with poor library quality from the analysis and 3) for image quantification one tumour of the Braf melanoma model was excluded due to necrosis.

Replication

On the graphs individual dots represent individual samples/mice used. For all experiments, the replication experiments were successful and showed comparable results. The number of replications for each experiment is listed in the figure legends. Phenotype defining experiments (e.g. cross-resistance) were performed >5 times, all other experiments were performed at least twice, with a few exceptions listed in the figure legends (e.g. stainings on matched patient biopsy cohorts, Co-culture of CD103 DCs from MEK and Poly I:C treated tumors, Radiotherapy).

Randomization

Mouse experiments: Mice were randomized based on tumor burden to achieve equal tumor volume between treatment groups. Mice used were litter-mates and sex-matched whenever possible. Other experiments: Sample allocation was performed randomly.

Blinding

Mouse experiments: Investigators were not blinded to mouse treatment groups. Mice were assigned into therapy groups based on tumour burden and therapy administration was then done based on ear tag numbers. After multiple doses of therapy administration, numbers and linked therapies are known to the investigators, making complete blinding difficult. However, experiments were performed by independent investigators. In addition tumors were frequently weighed upon excision or pictures of the tumor bearing animals were taken, providing an additional, unbiased read-out.

For experiments not involving mice e.g. flow cytometry the authors were blinded to the treatment group.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |
| ☒ | Clinical data |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | ChiP seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Antibodies

Antibodies used

Flow cytometry:
CD45 PE-Cy7 (clone 3D-F11, BD PharMingen, Cat: 552848, 1:500)
CD11b APC (clone M1/70, eBioscience, Cat: 17-0112-81, 1:200)
Gr-1 PerCpCy5.5 (clone RB6-8C5, eBioscience, Cat: 45-5931-80, 1:100)
CD103 PE (clone 2E7, Biolegend, Cat:121406, 1:100)
CD11c FITC (clone N418, Biolegend, Cat: 117306, 1:200)
CD3e FITC (clone 17A2, Biolegend, Cat: 100210, 1:300)
CD3e-BV605 (clone 17A2, BD Bioscience,Cat: 564005, 1:100)
CD8a AF594 (clone 53-6.7, Biolegend, Cat: 100758, 1:300)
CD40 PE [clone 3/23, BD Bioscience, Cat: 553565, 1:100]
Ly6C BV785 [clone HK1.4, Biolegend, Cat: 128041, 1:100]
Ly6G BV510 [clone IA8, Biolegend, Cat: 127633, 1:100]
MHC (H2Kb) PE [clone AF6-88.5/5.3, ebioscience, Cat: 12-5958-821, 1:300]
MHC (H2Kd) FITC [clone SF1-1.1, BD, Cat: 562003, 1:100]
H2Kb S1NFEKEL PE [clone 25-D1.16, ebioscience, Cat: 12-5743-81, 1:50]
CD80 APC [clone 16-10A1, Biolegend, Cat: 104713, 1:100]
CD86 BV510 [clone Gl-1, Biolegend, Cat: 105039, 1:100]
MHCII eR450 [M5/114.15.2, ebioscience, Cat: 48-5321-82, 1:100]
CD40 FITC [clone 3/23, Biolegend, Cat: 124607, 1:100]
NK1.1 BV711 [clone PKH6, Biolegend, Cat: 108745, 1:100]
B220 BV570 [clone RA3-6B2, Biolegend, Cat: 1032371, 1:100]
F4/80 BV510 [clone BM8, Biolegend, Cat: 123135, 1:100]
CD8 FITC [clone KT15, MBL, Cat: #D271-4, 1:100]
H2-Ld MuLV gp70 APC [MBL Cat: #7B-M521-2, 10uL per test]
Fc-block CD16/CD32 antibody [clone 2.4G2, BD Pharmingen, Cat: 5531411, 1:100]

Westerns:
pERK [clone D13.14.4E, CST, Cat: 4370S, 1:2000]
pERK [clone L34F12, CST, 46965, 1:2000]
Vinculin [clone hVIN-1, Sigma, Cat: V9131, 1:1000]

IF staining mouse tissue:
CD3e FITC [clone 17A2, Biolegend, Cat: 100210, 1:100]
CD8a [clone EPR21769, ABCAM, ab217344, 1:100]
CD103 [clone AF1990, R&D systems, Cat: AF1990, 1:200]
MHCII [M5/114.15.2, ebioscience, Cat: 14-5321-82, 1:200]

IF staining human tissue:
CD103 (clone EPR41666, ABCAM, ab129202, 1:800)
CD3 (Cell Marque, 103-R9S, 1:2000)
CD8 (Cell Marque, 108-R15, 1:1000)
CD39 (clone EPR20627, ABCAM, ab223842, 1:2000)
SOX10 (clone BC34, Biocare, AC0399C, 1:200)
CLEC9a (clone EPR22324, ABCAM, ab245121, 1:500)

T cell activation:
Anti Mo CD3e [Clone 145-2C11, ebioscience, Cat: 16-0031-85, 2ug/ml for plate coating]
Anti Mo CD28 [Clone 37.51, ebioscience, Cat: 16-0281-85, 1ug/ml in suspension]

Mouse treatments:
Anti PD-1 [Clone RPM1-14, BioXCell, Cat: BE0146, 100ug per dose]
Anti CTLA-4 [Clone 909, BioXCell, Cat: BE0164, 100ug per dose]
Anti Gr-1 [Clone RB6-8C5, BioXCell, Cat: BE0075, 6ug/ml per dose]
Anti CD-8 [Clone 2.43, in-house produced, 50ug per dose]
isotype CTRL Rat IgG2b [Clone 11F-2, BioXCell, Cat: BE0090, 100ug per dose]
isotype CTRL Rat IgG2a [Clone 2A3, BioXCell, Cat: BE0089, 100ug per dose]

Validation

The antibodies used are commercially available and validated throughout experiments.

Flow cytometry:
http://www.bdbiosciences.com/us/applications/research/cell-cell-research/cancer-research/mouse/pe-cy7-rat-anti-mouse-cd45-30-111/p/561868
https://www.thermofisher.com/antibody/product/CD11b-Antibody-clone-M1-70-Monoclonal/17-0112-82
http://www.thermofisher.com/antibody/product/Ly-6G-Ly-6C-Antibody-clone-R6-C5-Monoclonal/45-5931-80
https://www.biologie.com/be-anti-mouse-cd103-antibody-3574.html
https://www.biologie.com/en-us-products/ftc-anti-mouse-cd11c-antibody-1815
https://www.biologie.com/en-us-products/ftc-anti-mouse-cd3-antibody-45
https://www.biologie.com/en-us-products/alexafluor-594-anti-mouse-cd8a-antibody-9608
https://www.bdbiosciences.com/us/applications/research/b-cell-research/surface-markers/mouse/pe-rat-anti-mouse-cd40-323/p/561846
https://www.biologie.com/fr-ch/search-results/brilliant-violet-785-anti-mouse-ly-6c-antibody-11982
https://www.biologie.com/en-ie/products/brilliant-violet-510-anti-mouse-ly-6g-antibody-9121
https://www.thermofisher.com/antibody/product/MHC-Class-i-H-2Kb-Antibody-clone-AF6-88-5-5-3-Monoclonal/12-5958-82
https://www.thermofisher.com/antibody/product/OWA257-264-SIFKEK peptide-bound-to-H-2Kb-Antibody-clone-eBla25-01-16-D1-01-16-Monoclonal/12-5743-82
https://www.biologie.com/en-us/products/brilliant-violet-510-anti-mouse-cd86-antibody-8745
https://www.biologie.com/fr-ch/products/apc-anti-mouse-cd80-antibody-2340
http://www.ebioscience.com/mouse-mhc-class-ii-antibody-efluor-450-m5-114152.htm
https://www.biologie.com/en-us/products/ftc-anti-mouse-cd40-antibody-4982
https://www.biologie.com/en-us/search-results/brilliant-violet-711-anti-mouse-human-cdf45r-2220-antibody-7378
https://www.biologie.com/en-us/products/brilliant-violet-510-anti-mouse-f4-80-antibody-8934
**Eukaryotic cell lines**

**Policy information about cell lines**

**Cell line source(s)**

| Cell line | Source |
|-----------|--------|
| Braf/Pten melanoma (BRAFV600E/WT, Pten-/-, CDKN2a-/-) | Parental NTT cell line - Markus Bosenberg Laboratory |
| NTT parental OVA clones: 3A6, 2A8 |
| NTT passaged cell lines (bulk): #387 OVA clone: 2B3 |
| RTT resistant derivatives: |
| in vivo B6 resistant lines: |
| RAFI/MEKI: RTT #447L, #447R, #450 - clonal derivatives of these: 2B12, 1A2, 1G8, 1C8 |
| in vivo NSG resistant lines: #401, #403, #409 |

| Braf (BRAFV600E/WT, CDKN2a-/-) | Parental NTT cell line - Markus Bosenberg Laboratory |
| NTT passaged in vivo derivatives: |
| YUML3.3 #235L |
| RTT derivatives: |
| in vivo B6 resistant lines: |
| RAFI resistant: RTT #26L, #29 |
| RAFI/MEKI: RTT #27R |
| in vivo NSG resistant lines: #161, #166, #168 |
| in vitro resistant lines: #235L in vitro resistant, parental in vitro resistant |

CT26 colon carcinoma: ATCC
| RTT derivatives: |
| in vivo BALB/c resistant lines: |
| #140L, #310L |
| in vivo NSG resistant lines: |
| #303 |
Animals and other organisms

| Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research |
|---|
| Laboratory animals | Laboratory animals – Mus Musculus  
6-12 week-old female/male B6(Cg) Tyr-C 2J/ (albino BL6)  
6-24-week-old female/male NOD.Cg-Prkdcscid I2rgtm1Wjl /Szl [NSG]  
6-12-week-old female/male BALB/cJ  
6-12-week-old female/male B6(Cg)-Rag2tm1.1Cgn/J Ly5.2 [Rag2/-]  
2-5 months-old female/male OT-1 Luc Thy1.1  
2-5 months-old female/male OT-1 B6(Cg)-Rag2tm1.1Cgn/J Ly5.2  
6-12-week-old female/male B6.129S(C)-Batf3tm1Kenny/J mice [Batf3/-] |
| Wild animals | Did not involve wild animals. |
| Field-collected samples | Did not involved field-collected samples. |
| Ethics oversight | All experiments using animals were performed in accordance with our protocol approved by the Austrian Ministry (BMBWF-66.015/0009-V/3b/2019 or 6Z: 340118/2017/25). Experiments involving focal radiation were performed at the ICR London and approved by the Animal Ethics Committee at the Institute of Cancer Research in accordance with National Home Office Regulations under the Animals (Scientific Procedures) Act 1986. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

| Policy information about studies involving human research participants |
|---|
| Population characteristics | The retrospective study includes 54 BRAFV600 mutated patients treated with immunotherapy between 01.01.2011 and 29.02.2019 at the Lausanne University Hospital, Switzerland. This cohort is divided in two groups: The targeted therapy naive (NTT) group refers to patients (n = 38) who received as a second line treatment BRAF inhibitor (BRAFI), dabrafenib or vemurafenib ± MEK inhibitor (MEKI), trametinib or cobimetinib) upon progression. The targeted therapy resistant (RTT) group refers to patients (n = 16) who received as a first-line treatment a BRAF inhibitor (BRAFI, dabrafenib or vemurafenib) ± MEK inhibitor (MEKI, trametinib or cobimetinib) and received as a second line treatment immunotherapy (nivolumab, pembrolizumab or ipilimumab-nivolumab). |
| Recruitment | The retrospective study includes 54 BRAFV600 mutated patients treated with immunotherapy between 01.01.2011 and 29.02.2019 at the Lausanne University Hospital, Switzerland. The cohort cohort is representative of the patient populations that are followed at the Oncology Clinic of Lausanne University Hospital. |
| Ethics oversight | The retrospective analysis of BRAF V600 mutant melanoma patients from Lausanne University Hospital, Switzerland and was conducted in accordance with the Declaration of Helsinki, the Swiss legal requirements and the principles of Good Clinical Practice. Patients signed the CHUV General consent and accepted the use of their data for research purposes or did not explicitly refuse the use of personal data (following Art. 34 HRA). Patients did not receive compensation. The protocol was approved by the Research Ethics Committee of Canton de Vaud, Switzerland (Protocol No. 2019-00448). |

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

For flow cytometry of whole tumours, tumours were dissected, cut into small pieces and dissociated for 1.5 h on 37 degree using Collagenase A (1 mg/ml, Roche) in PBS. Single cell suspensions were strained through a 70 μm nylon mesh, washed in FACS buffer (0.5 % BSA, 2 mM EDTA) and incubated for 10 min on 4 degree with anti-mouse Fc-Block CD16/CD32 antibody (clone 2.4G2, BD Pharmingen). Cells were subsequently stained with antibodies detecting immune cells in FACS buffer for 30 min on 4 degree. For flow cytometry of cultured cells, cells were detached using 0.05% Trypsin, inhibited with full medium and stained in FACS buffer (0.5% BSA, 2mM EDTA).

Instrument

- BD LSR Fortessa for analysis
- BD FACS Aria for sorting

Software

- DIVA software for acquisition
- FlowJo V10.7.1 for analysis

Cell population abundance

For sorting of GFP+ and CD45+ cells, cells were reanalyzed at the sorting machine and used at a purity of above 90% for sequencing. For Smartseq, no purity confirmation was performed, since the cells were directly sorted into lysis buffer.

Gating strategy

For all gating strategies a singlet discrimination and live-dead cell exclusion was performed. Subsequently, we gated on CD45+ cells and then on individual immune cell populations.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.