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Crizotinib-induced immunogenic cell death in non-small cell lung cancer

Peng Liu, Liwei Zhao, Jonathan Poi, Sarah Levesque, Adriana Petrazzulo, Christina Pfirschke, Camilla Engblom, Steffen Rickelt, Takahiro Yamazaki, Kristina Iribarren, Laura Senovilla, Lucillia Bezu, Erika Vaccelli, Valentina Sica, Andréa Melis, Tiffany Martin, Xia Lin, Heng Yang, Qingqiong Li, Jinfeng Chen, Sylvère Durand, Fanny Aprahamian, Deborah Lefevre, Sophie Broutin, Angelo Paci, Amaury Bongers, Veronique Minard-Colin, Eric Tartour, Laurence Zitvogel, Mikael J. Pittet, Oliver Kepp & Guido Kroemer

Immunogenic cell death (ICD) converts dying cancer cells into a therapeutic vaccine and stimulates antitumor immune responses. Here we unravel the results of an unbiased screen identifying high-dose (10 µM) crizotinib as an ICD-inducing tyrosine kinase inhibitor that has exceptional antineoplastic activity when combined with non-ICD inducing chemotherapeutics like cisplatin. The combination of cisplatin and high-dose crizotinib induces ICD in non-small cell lung carcinoma (NSCLC) cells and effectively controls the growth of distinct (transplantable, carcinogen- or oncogene induced) orthotopic NSCLC models. These anticancer effects are linked to increased T lymphocyte infiltration and are abolished by T cell depletion or interferon-γ neutralization. Crizotinib plus cisplatin leads to an increase in the expression of PD-1 and PD-L1 in tumors, coupled to a strong sensitization of NSCLC to immunotherapy with PD-1 antibodies. Hence, a sequential combination treatment consisting in conventional chemotherapy together with crizotinib, followed by immune checkpoint blockade may be active against NSCLC.
Several druggable oncoproteins are tyrosine kinases that become activated due to mutations or gene amplifications. In consequence, small inhibitory molecules and antibodies targeting such tyrosine kinases (when they are expressed on the cell surface) have been developed and introduced into the clinics. Indeed, tyrosine kinase inhibitors (TKI) targeting oncoproteins such as c-ABL (activated in Philadelphia chromosome-positive chronic myeloid leukemia, CML)1, BRAF (activated in melanoma)2, ERBB2 (activated in a fraction of breast cancers)3, EGFR (activated in a sizable portion of non-small cell lung cancers, NSCLC)4, KIT (activated in gastrointestinal stromal tumors, GIST)5, or VEGF (activated in renal cancers and others)6, have been approved for the routine treatment of cancer patients.

The development of anti-neoplastic TKIs has been largely driven by the cell-autonomous view that (i) cancer is a genetic and epigenetic cellular disease and (ii) anticancer drugs should target specific characteristics of transformed cells to eliminate them or to reduce their growth7. At odds with this vision, however, imatinib is also evidence that cisplatin (CDDP), mitomycin C (MitoC) or GIST expressing activated ERBB2 (activated in a fraction of breast cancers)3, ERBB3 (activated in melanoma)2, as a prominent hallmark) causing progression-free or overall survival post-chemotherapy17. There is autophagic response that allows the cells to release ATP during epigenetic cellular disease and (ii) anticancer drugs should target the cell-autonomous view that (i) cancer is a genetic and (ii) immune checkpoint blockade, to treat NSCLC that lack genetic rearrangements leading to the activation of ALK or ROS1.

Results
Identification of (R)-crizotinib as a potential ICD inducer. We used a fully automatized high-content, medium throughput fluorescence-based screening procedure22 to identify potential ICD inducers among two libraries of tyrosine kinase inhibitors (TKIs), namely the Public Chemogenomic Set for Protein Kinases with more than 500 compounds25 (Fig. 1a) and an in-house collection of FDA-approved TKIs (32 compounds) that are currently used in the oncological armamentarium (Fig. 1b). Human osteosarcoma U2OS cells expressing CALR-red fluorescent protein (RFP), green fluorescent protein (GFP)-LC3 or HMGB1-GFP fusion proteins were treated with each of these compounds at a concentration that is high enough to induce off-target effects (10 μM). Roboticized fluorescence microscopy and automated high-content image analysis were used to determine surrogate markers of ICD such as the redistribution of CALR-RFP into peripheral dots, the generation of autophagy-associated GFP-LC3 puncta in the cytoplasm and the exodus of HMGB1-GFP from the nucleus into the cytosol, as well pyknosis of nuclei counterstained with DAPI. Of note, both screens yielded an overlapping set of TKIs that were able to induce ICD characteristics to a similar level as the control, the anthracycline mitoxantrone (MTX), namely, (R)-crizotinib, foretinib, canertinib, lestaurtinib and ceritinib (Fig. 1a, b). This panel of TKIs indeed induced signs of ICD such as immunofluorescence-detectable CALR exposure and the release of ATP and HMGB1 into the culture supernatant in several human cancer cell lines (U2OS, cervical carcinoma HeLa, colorectal cancer HCT-116) and in murine fibrosarcoma MCA205 cells (Supplementary Fig. 1a–c).

The aggregate analysis of the screening experiments (Fig. 1) ranked (R)-crizotinib first among FDA-approved TKIs. Therefore, we decided to concentrate our efforts on (R)-crizotinib. Of note, (R)-crizotinib was more efficient than its enantiomer (S)-crizotinib in inducing CALR-RFP redistribution and pyknosis in U2OS cells (Fig. 1c, d), as well as in mouse fibrosarcoma MCA205 and lung adenocarcinoma TCI cells (Fig. 1d). When combined with poor ICD inducers such as cisplatin (CDDP) and mitomycin C (MitoC), both of which lack the ability to cause CALR exposure13,14, (R)-crizotinib (but not (S)-crizotinib) was able to reestablish CALR exposure to the same level as that observed for MTX. This result was obtained in several cancer cell lines including U2OS, MCA205 and TC1 cells (Fig. 1d). (R)-crizotinib and several other clinically used ALK inhibitors (such a certinib, entrectinib and others) induced the phosphorylation of eIF2α (Supplementary Fig. 1d) and ICD hallmarks in U2OS cells (Supplementary Fig. 1e–g).

As expected, (R)-crizotinib (but not (S)-crizotinib) was more efficient in inducing apoptosis and ICD hallmarks (CALR exposure, ATP and HMGB1 release) when added to a human
NSCLC cell line that is positive for the EML4-ALK fusion protein (H2228 cells) than when administered to cells that are negative for the ALK activating translocation (H1650 cells) (Fig. 2a–h).

However, at high doses of 5–10 µM (R)-crizotinib was active on cells lacking the EML4-ALK fusion protein (Fig. 2a, c, e, g). This contrasts with the fact that metabolic effects such as glycolysis and mitochondrial respiratory inhibition by (R)-crizotinib (Supplementary Fig. 2a–f), or downregulation of hexokinase-2 were (Supplementary Fig. 2g, h) exclusively detectable in EML4-ALK-positive H2228 cells. (R)-crizotinib induced eIF2α phosphorylation, a sign of endoplasmic reticulum stress associated with CALR exposure, in U2OS cells (Supplementary Fig. 3a) and in both H1650 and H2228 cells, though with distinct kinetics (Supplementary Fig. 3b, c).

In U2OS cells, the depletion of several (R)-crizotinib-inhibited tyrosine kinases, namely ALK, JAK2, MET and ROS1 by individual small interfering RNAs (siRNAs) or pools of such siRNAs (validated by RT-PCR. Supplementary Fig. 3d) triggered several hallmarks of ICD to the same level as did (R)-crizotinib (Fig. 2i–l). Thus, depletion of ALK, JAK2, MET, and ROS1 induced significant eIF2α phosphorylation (Fig. 2i), redistribution of CALR-RFP to dots (Fig. 2k) and HMGB1-GFP release into the

![Figure 1](https://example.com/figure1.png)

**Fig. 1** Identification of (R)-crizotinib as a novel immunogenic cell death inducer. **a** Human osteosarcoma U2OS cells stably co-expressing calreticulin (CALR)-RFP and HMGB1-GFP or GFP-LC3 were treated with the compounds from the Public Chemogenomic Set for Protein Kinases at a concentration of 10 µM for 8 h, 12 h and 32 h, followed by the assessment of CALR exposure (CALR-RFP redistribution), autophagy (GFP-LC3 granularity) and HMGB1 release (decrease of nuclear HMGB1-GFP intensity), respectively. Nuclear pyknosis was monitored as an indicator for cell death. The data was normalized to calculate Z scoring (mean, n = 4, Supplementary data 1) and the effects of the agents are hierarchically clustered in a heatmap. Top hits are separately displayed with red and blue reporting positive and negative values, respectively. **b** A collection of tyrosine kinase inhibitors (final concentrations 5, 10 µM) was screened following the same approach. Z score was calculated for each agent (mean, n = 4, Supplementary data 2). **c** Representative images of (R)-crizotinib (Criz) induced ICD phenotypes are depicted and the scale bars equals 10 µm. **d** ICD inducing effects of (R)/(S)-crizotinib in combination with cisplatin (CDDP; 150 µM) and mitomycin C (MitoC; 150 µM). Human osteosarcoma U2OS cells, murine fibrosarcoma MCA205 cells, as well as murine NSCLC TC1 cells were treated with mitoxantrone (MTX; 2 µM), CDDP, MitoC alone or in combination with increasing concentrations (1, 5, 10 µM) of (R) or (S)-crizotinib, for 24 h before determination CALR exposure (by flow cytometry), ATP secretion (ATP-dependent luminescence kit), and HMGB1 release (ELISA). Results are normalized as log2 and shown as a heatmap in which each rectangle represents the mean of triplicate assessment, statistical significances are indicated as *p < 0.001 comparing to controls using Student’s t-test.

| Compound | U2OS | MCA205 | TC-1 |
|----------|------|--------|------|
| Foretinib | +     | +      | +    |
| (R)-Crizotinib | +     | +      | +    |
| Ceritinib | +     | +      | +    |
| Lestaurtinib | +     | +      | +    |
| GW622475 | +     | +      | +    |
| GSK1669921 | +     | +      | +    |
| GSK1558669 | +     | +      | +    |
| GW779439 | +     | +      | +    |
| SB-601436 | +     | +      | +    |
| SB-601273 | +     | +      | +    |
| Alatinib | +     | +      | +    |
| GW567142 | +     | +      | +    |
| Canertinib | +     | +      | +    |
| Bosutinib | +     | +      | +    |
| GSK260205 | +     | +      | +    |
| GSK1649598 | +     | +      | +    |
| GW440146 | +     | +      | +    |
| GSK1660437 | +     | +      | +    |
| GSK1804250 | +     | +      | +    |

**Table 1** Compound efficacy in U2OS, MCA205, and TC-1 cells following treatment with (R)-crizotinib.
cytoplasm (Fig. 2l). In contrast, only the depletion of JAK2 was as efficient as (R)-crizotinib in inducing the formation of GFP-LC3 punctae (Fig. 2l). We knocked down several other genes coding for tyrosine kinases that are not inhibited by (R)-crizotinib (such as BTK, EGFR, ERBB, HCK) and found that these manipulations induced less CALR exposure, ATP release and HMGB release than treatment with (R)-crizotinib (Supplementary Fig. 3e–g). Thus, it appears that the inhibition of a specific set of TKIs by (R)-crizotinib participates in ICD induction. Confirming a prior report28, (R)-crizotinib also induced a higher expression of class I and class II MHC molecules (Supplementary Fig. 3h–k). In contrast, (R)-crizotinib failed to significantly modulate mRNA expression of ALK, JAK2, MET, and ROS1 (Supplementary Fig. 3l–n).

(R)-crizotinib plus cisplatin induces ICD in vivo. As previously reported13,14,20, in vitro MTX-treated TC1 cells can be injected subcutaneously (s.c.) to protect immunocompetent C57BL/6 mice against rechallenge with live tumor cells of the same kind injected two weeks later into the opposite flank (Fig. 3a, b). This contrasts with CDDP (Fig. 3c, e) or MitoC (Fig. 3d, e)-treated TC1 cells that largely failed to induce ICD in this in vivo assay. (R)-crizotinib-treated TC1 cells formed tumors at the injection site (not shown), correlating with the fact that, in contrast with chemotherapeutic agents such as CDDP, (R)-crizotinib alone was unable to completely abolish the clonogenic potential of tumor cells (Supplementary Fig. 4). However, TC1 cells became immunogenic when they were cultured in vitro with either CDDP combined with (R)-crizotinib (Fig. 3c, e) or MitoC together with (R)-crizotinib (Fig. 3d, e). The gene editing-mediated invalidation of the Anxa1 or Hmgb1 genes in TC1 cells was compatible with the survival of these cells, as well as the formation of tumors in vivo. However, in contrast to WT cells, Anxa1−/− or Hmgb1−/− cells treated with CDDP plus (R)-crizotinib in vitro failed to elicit anticancer immune responses against TC1 cells (Fig. 3f, g). Similarly, CALR...
neutralization with a blocking antibody or the destruction of extracellular ATP with apyrase, abolished the immunogenicity of TC1 cells killed by CDDP plus (R)-crizotinib (Fig. 3h, i).

Very similar results were obtained when this in vitro/in vivo protocol for assessing ICD was applied to MCA205 cells. Again, (R)-crizotinib was highly efficient in inducing ICD when combined with CDDP or MitoC (Supplementary Fig. 5a–d) and knockout of Anxa1 or Hmgb1, blockade of CALR or hydrolysis of ATP abolished the immunogenicity of MCA205 cells (Supplementary Fig. 5e–h). Direct comparison of (R)-crizotinib and (S)-crizotinib in these vaccination assays led to the conclusion that the (R) enantiomer is more efficient in inducing ICD than its stereoisomer (Supplementary Fig. 5b–d).

We conclude that (R)-crizotinib acts as a TKI that favors the induction of ICD in a variety of cancer cells, independently of their ALK status.
Improvement of chemotherapy by (R)-crizotinib. Driven by the consideration that (R)-crizotinib induces ICD when combined with CDDP or MitoC, we investigated the therapeutic potential of such a combination regimen in immunocompetent mice. In conditions in which established s.c. MCA205 cancers failed to reduce their growth in response to mono-therapy with either intratumoral (i.t.) administration of (R)-crizotinib (Supplementary Fig. 6a) or systematic treatment with CDDP (Supplementary Fig. 6b) or MitoC (Supplementary Fig. 6c), the combination therapy of (R)-crizotinib administered i.t. (but less so (S)-crizotinib) together with systemic CDDP or MitoC resulted in a significant delay in disease progression (Supplementary Fig. 6b–d). The growth of transplanted MCA205 tumors was also efficiently controlled when either CDDP or MitoC were combined with systemic (intraperitoneal) injections of (R)-crizotinib (Supplementary Fig. 6e, f), which reached a concentration of ~10 μM in plasma and tissues (Supplementary Fig. 6g). None of these anti-neoplastic effects on MCA205 cancers were observed when the tumors evolved in nu/nu mice that lack thymus-dependent T lymphocytes (Supplementary Fig. 6h, i). TC1 cancers growing subcutaneously on immunocompetent animals reduced their growth in response to the combinations of these drugs (Supplementary Fig. 6k–m). Moreover, knockout of either Anxax or Hmgbl in MCA205 and TC1 cell abolished the tumor-growth reducing effect of the combination therapy with CDDP plus (R)-crizotinib (Supplementary Fig. 6j, m).

Even more spectacular effects were obtained against established orthotopic NSCLC TC1 tumors expressing luciferase to facilitate the monitoring of lung cancers in immunocompetent mice (Fig. 4a, b). In this model, systemic therapy with (R)-crizotinib, CDDP or MitoC alone had no significant therapeutic effects. However, the combination of (R)-crizotinib with CDDP or MitoC caused the (at least temporary) disappearance of more than 50% of the cancers, as well as an increase in overall survival (Fig. 4c–h, Supplementary Fig. 7a). Mice that stayed tumor-free for more than 3 months after the combination treatment with (R)-crizotinib plus CDDP were challenged with both TC1 and MCA205 cells injected into opposite flanks (Fig. 4i). Importantly, those mice that had been cured from established TC1 lung cancers failed to develop s.c. TC1 lesions, but did develop MCA205 cancers, which are antigenically different (Fig. 4j, k). As a further proof that the observed therapeutic effects are indeed mediated by the cellular immune system, the best combination therapy (namely, (R)-crizotinib plus CDDP) completely lost efficacy against TC1 lung cancers developing in athymic nu/nu mice (Fig. 4l–n, Supplementary Fig. 7b), or when CD4+ or CD8+ T lymphocytes were depleted by the injection of suitable antibodies (Supplementary Fig. 7c–e). Blockade of CD11b partially compromised the efficacy of the treatment with (R)-crizotinib plus CDDP (Supplementary Fig. 7f). We next investigated the possibility to combine (R)-crizotinib with CDDP for the treatment of oncogene and carcinogen-induced lung cancers. In the model of Kras-activated, Trp53-deleted (KP) lung cancer (in which a Cre-encoding adenovirus is instilled into the trachea of mice bearing a mutant Kras antigen downstream of a LOX-Stop-LOX cassette, as well as Cre-excisable Trp53)30, the combination therapy was more efficient in reducing tumor burden than either (R)-crizotinib or CDDP alone (Fig. 5a–d). This applies as well to a model of urethane-induced NSCLC treated from the moment of micro-computed tomography detection (Fig. 5e). Again, the numbers of neoplasias, as well as the total tumor burden were reduced by co-treatment with (R)-crizotinib and CDDP (Fig. 5f–i).

Of note, (R)-crizotinib, alone or in combination with CDDP, increased the infiltration of Kras-induced NSCLC by CD8+ cytotoxic T lymphocytes (CTL), yet had no major effect on the local frequency of Foxp3+ regulatory T cells (Treg), meaning that it improved the CD8/Foxp3 ratio in the tumor bed (Supplementary Fig. 8a–h). Moreover, crizotinib favored the infiltration of cancers by interferon-γ (IFNγ)-producing T lymphocytes in urethane-induced NSCLC (Supplementary Fig. 8i–k). In addition, s.c. MCA205 cancers treated with (R)-crizotinib, alone or together with CDDP, exhibited a significant increase (p < 0.001, ANOVA test) in the local presence of activated dendritic cells (DC) expressing CD11c and CD86 (Supplementary Fig. 9a, d), CTL (Supplementary Fig. 9b, e). This was accompanied by a (though non-significant) reduction in Treg (Supplementary Fig. 9c, f), translating into a significant increase (p < 0.0001) in the CD8/ Foxp3 ratio that was more pronounced (p < 0.01) for the combination regimen than for (R)-crizotinib alone (Fig. 6a). Cytoluciferometric analyses (Supplementary Fig. 10) confirmed crizotinib-induced changes in the immune infiltrate including an increase in inflammatory macrophages (Supplementary Fig. 9g), NKT cells (Supplementary Fig. 9i, j), but no change in the local presence of activated NK cells (Supplementary Fig. 9h), or granulocytic or monocytic myeloid derived suppressor cells (MDSC) (Supplementary Fig. 9k). In addition, (R)-crizotinib tended to increase the frequency of IFNγ producing CD4+ and CD8+ T cells (Fig. 6b, c, Supplementary Fig. 11), as well as that of interleukin-17 (IL-17) producing CD4+ T lymphocytes (Fig. 6d, Supplementary Fig. 11). RNAseq analyses led to the identification of a set of genes that are significantly upregulated by the combination of CDDP and (R)-crizotinib. Among this set, genes that contribute to IFNγ production and its biosynthesis, as well as T cell activation markers were identified (Supplementary Fig. 12a, b). Significant reduction of Foxp3 (p < 0.001, t-test) and increased infiltration of Kras-induced NSCLC by CD8+ and CD4+ (p < 0.05, t-test) Gr1+ (p < 0.001, t-test) CD11c+ (p < 0.05, t-test) CD54+ (p < 0.05, t-test) cells were observed in the tumors treated with (R)-crizotinib plus CDDP, compared with either (R)-crizotinib or CDDP alone (Supplementary Fig. 12c). Notably, (R)-crizotinib treatment, both alone or in combination with CDDP, increased the infiltration of Kras-induced NSCLC by CD8+ pump (p < 0.001, t-test) and CD4+ Gr1+ (p < 0.001, t-test) CD11b+ (p < 0.001, t-test) cells, compared with either (R)-crizotinib or CDDP alone (Supplementary Fig. 12d, e). As a consequence, the frequency of FOXP3+ cells was reduced (p < 0.001, t-test) and the ratio of CD8+ to CD4+ FOXP3+ cells was increased (p < 0.001, t-test) in the tumors treated with (R)-crizotinib plus CDDP, compared with either (R)-crizotinib or CDDP alone (Supplementary Fig. 12f).
systemic neutralization of the type-1 interferon receptor (IFNAR) abolished the efficacy of the combination therapy against subcutaneous orthotopic cancers (Supplementary Fig. 12a–c). Neutralization of IL-12b failed to affect therapeutic efficacy of the combination therapy (Supplementary Fig. 12d–f), although its RNA level was found significantly upregulated in RNA Seq analysis (Fig.6e, f) and crizotinib caused a small increase in IL-12 secretion by bone-marrow derived dendritic cells (BMDC) in vitro (Supplementary Fig. 12g–i).

Of note, the (R)-crizotinib effects apparently depend on the tumor microenvironment, because in vitro treatment of naïve CD4+ T cells in the presence of the TKI and suitable differentiation factors (Supplementary Fig. 13a) failed to affect the expression and secretion of major cytokines (IFNγ, IL-4, IL-9,
IL-17) and Foxp3 by all major CD4+ T cell subsets (Supplementary Fig. 13b–i). Similarly, systemic treatment of tumor-free mice with (R)-crizotinib, alone or in combination with CDDP (Supplementary Fig. 13j), failed to affect the expression of these factors by splenic CD4+ and CD8+ T lymphocytes (Supplementary Fig. 13k–p), neither affected those factors by lymph nodes’ CD4+ and CD8+ T lymphocytes (data not shown).

Altogether, these results indicate that (R)-crizotinib can be combined with chemotherapeutic agents to stimulate IFNγ-dependent anticancer immune responses in various models of NSCLC.

(R)-crizotinib synergizes with immune checkpoint blockers. In vitro treatment of several human (A549, HCT116, U2OS) or mouse cancer cell lines (CT26, MCA205, TC1) with crizotinib induced the expression of PD-L1 protein within 24 h (Supplementary Fig. 14). In tumor bearing mice, crizotinib increased the expression of the PD-1 (and LAG-3 but neither TIM-3) on circulating CD4+ and CD8+ T lymphocytes (Supplementary Figs. 15, 16). Treatment of mice with (R)-crizotinib in combination with CDDP caused an increase in the mRNA levels of PD-1, PD-L1, and CTLA-4 within the tumor (Fig. 6i). Moreover, (R)-crizotinib alone or with CDDP induced PD-1 expression on tumor-infiltrating CD4+ Foxp3− but not in CD4+Foxp3+(Treg) cells bearing the exhaustion marker ICOS (Fig. 6m, n). As a result, we investigated whether immune checkpoint blockade (ICB) would be able to improve the therapeutic outcome. For this we evaluated all possible combinations of (R)-crizotinib, CDDP-based chemotherapy and dual ICB therapy with PD-1 and CTLA-4-targeting antibodies on s.c. MCA205 fibrosarcomas (Supplementary Fig. 17a, b) or s.c. TC1 (Supplementary Fig. 17a). Based on the consideration that IC induction can sensitize cancer to subsequent ICB treatment31, ICB was performed after the treatment with (R)-crizotinib and CDDP by three injections of CTLA-4 and PD-1 blocking antibodies, starting from 8 days after the administration of the small molecules (Fig. 7a). While immune checkpoint inhibitors on their own had no therapeutic effects, the combination of ICB with (R)-crizotinib (and optionally with CDDP) improved therapeutic outcome, causing complete regression of >60% of s.c. MCA205 fibrosarcomas (Supplementary Fig. 17a, b) and reducing the growth of s.c. TC1 cancer (Fig. 7b, c).

Next, we applied this chemo-immunotherapeutic protocol to established orthotopic TC1 Luc NSCLC. The small molecule (R)-crizotinib and/or CDDP were administered upon bioluminescence-based lung cancer diagnosis, followed by the initiation of immunotherapy one week later (Fig. 7d). Cure rates (evaluated as complete disappearance of a diagnostic pulmonary bioluminescence signal) and long-term survival reached >88% for the triple combination therapy consisting of (R)-crizotinib, CDDP and ICB (Fig. 7e–i). Of note, comparison of different immunotherapies (CTLA-4 blockade alone, PD-1 blockade alone, or combination therapy) led to the conclusion that PD-1 (not CTLA-4) blockade was sufficient to achieve this high cure rate (Fig. 7f). Supplementary Fig. 17c–f). The combination of (R)-crizotinib, CDDP and PD-1 blockade achieved a 100% cure rate (15 out of 15 mice) in yet another orthotopic NSCLC model, namely Lewis lung carcinoma-1 (LLC1) tumors forming after intrathoracic injection (Supplementary Fig. 17g, h). Only (R)-crizotinib, not (S) crizotinib, was able to sensitize TC1 lung cancers to cure with PD-1 blockade (Supplementary Fig. 17i, j). Mice that had been cured from orthotopic TC1 lung cancers by the combination of (R)-crizotinib, CDDP and PD-1 blockade became resistant against s.c. rechallenge with TC1 tumors (Supplementary Fig. 17k–n).

Cancer patients do not tolerate simultaneous administration of crizotinib and PD-1 blockade due to hepatotoxicity32. Similarly, mice receiving simultaneous treatment with such agents exhibited signs of liver toxicity that were not observed when PD-1 blockade was started one week after crizotinib treatment (Supplementary Fig. 18a–d). This correlated with the capacity of (R)-crizotinib (alone or in combination with CDDP) to transiently induce PD-L1 expression in the liver (but not in other tissues) (Supplementary Fig. 18e–h).

Altogether, these results demonstrate that (R)-crizotinib can be useful in boosting anticancer chemotherapy effects, as well as subsequent responses to ICB-based immunotherapy.

Discussion

The incidence of NSCLC treated with crizotinib is rather low, because only 1% of these cancers have ROS1 rearrangements33 and only 4–5% harbor ALK genetic rearrangements34. Such NSCLC patients, who typically are non-smokers with adenocarcinomas, have been treated with crizotinib in first line (for ALK-positive cancers only) and, once the tumors become crizotinib-resistant and relapse, with other TKI inhibitors such as ceritinib35,36. Typically, crizotinib resistance is accompanied by secondary mutation of ALK, amplification of the ALK fusion gene or aberrant activation of other oncogenic kinases such as KIT or EGFR7. Although crizotinib mediates occasional effects against NSCLC bearing activating MET mutations, it is generally not efficient for the treatment of NSCLC which lacks oncogenic kinases that might be inhibited by crizotinib38. Hence the available evidence suggests that crizotinib is acting on-target, by inhibiting ALK, ROS1 or MET to mediate its antineoplastic effects.

Here, we propose that crizotinib also has an off-target effect that becomes manifest in the context of cisplatin-based chemotherapy and that may be taken advantage of to treat NSCLCs that bear none of the canonical crizotinib targets (Supplementary Fig. 19, graphical abstract). Indeed, when combined with CDDP (or another chemotherapeutic agent such as mitomycin C), crizotinib is effective against s.c. and orthotopic TC1 or LLC1 NSCLC, as well as against more realistic NSCLC models that were either induced by chemical carcinogenesis or by the conditional activation of mutant Kras and deleted Trp53. In all these models, crizotinib combined with CDDP caused signs of an anticancer
immunotherapy with antibodies blocking CTLA-4 and PD-1 (or PD-1)
Indeed, crizotinib plus CDDP sensitized tumors to immu-
the intratumoral upregulation of CTLA-4, PD-1, and PD-L1.
importantly, the combined action of crizotinib and CDDP led to
lysis of the immune in
system curtailed the ef
immune response, as indicated by an increase in the T cell
response to
activating chromosome translocations, the drug was usually
treated with either solvent (Sol) or the
combination of (R)-crizotinib (Criz) with cisplatin (CDDP) are shown
b. Lung weight was used as proxy of tumor burden and is depicted for
tumor-free (Ctrl) and treated tumor-bearing mice (c). Hematxoylin-eosin
based tumor area quantification on lung lobe sections of KP mice is
presented (d). Results are expressed as mean ± s.e.m. *p < 0.05;
**p < 0.001 as compared to Sol, #p < 0.05 as comparing indicated groups
using Student’s t-test, n = minimum of 6 mice per group. Friend Virus B
(FVB) mice were used as a spontaneous mouse model of lung
adenocarcinoma, tumor induction and drug treatments were performed
according to the scheme demonstrated in e. Tumor development was
monitored with microCT photographing and all mice were euthanized to
obtain lung lobes for further histology. Representative stereo microscope
scans of lung lobes (f), as well as quantified neoplasia numbers (g, h) and
tumor sizes (i) are reported. Results are expressed as mean ± s.e.m.
**p < 0.01; ***p < 0.001 as compared to Sol.
alone), allowing to induce a durable immune response, as well as
complete cure against the established and transplantable
NSCLC.
As to the mechanisms through which crizotinib favors the
induction of antitumor immune responses, it appears that (R)-
crizotinib (the TKI) is more efficient than its stereoisomer (S)-
crizotinib (the inhibitor of nudix hydrolase 1, NUDT1, a
nucleotide pool sanitizing enzyme)39. By virtue of simultaneous
inhibiting several tyrosine kinases (namely ALK, ROS1, MET,
and JAK2), (R)-crizotinib combined with CDDP (or mitomycin
C) induces all the molecular and functional hallmarks of ICD in a
variety of human and mouse NSCLC cell lines, even if such cells
lack activating mutations of ALK or ROS1. This is speci-
fic for the drug combination, meaning that crizotinib, CDDP or mitomycin
C do not induce ICD on their own; this effect was only obtained
crisotinib was combined with the chemotherapeutics.
Removal of any of the known DAMPs that are required for ICD
including ANXA1, ATP, CALR, or HMGB1 abolished the capacity
crisotinib plus CDDP-treated NSCLC cells to elicit an
antitumor activity in vaccination assays. Hence, it
appears that the combination treatment induces a canonical ICD
pathway that resembles that induced by oxaliplatin or
anthracyclines.
In the clinical trials that led to the approval of crizotinib for the
treatment of a subcategory of NSCLC bearing ALK and ROS1
activating chromosome translocations, the drug was usually
compared to, but not combined with, CDDP35,38. Hence, there is
no information on the possible benefits of the combination
therapy in NSCLC. Only a minority of NSCLC with ALK rearran-
gerements (ALK+) that had been treated with crizotinib
responded to PD-1 blockade39,40. Preclinical evidence indicates that
ICD inducing cytoktoxictants (such as oxaliplatin and cyclo-
phosphamide, especially if combined) can sensitize NSCLC to
CTLA-4/PD-1 blockade31. Importantly, nivolumab was particu-
larly efficient among those NSCLC patients who demonstrated a
response to first-line chemotherapy42, suggesting that this may
apply to patients as well. Moreover, distinct chemotherapeutic
regimens can be advantageously combined with ICBs for the
treatment of metastatic NSCLC\textsuperscript{43,44}. Based on these premises, it might be attempted to extrapolate the results obtained in the mouse models described here to design a clinical trial in which NSCLC patients would receive standard chemotherapy in combination with crizotinib (and this independently of the status of ALK and ROS1), followed by ICB. Of note, in mice crizotinib could be safely administered without any obvious toxicity to reach a concentration of 10 \mu M in plasma, lung tissues and tumors, which is the dose that is effectively inducing ICD hallmarks in cancer cells that lack ALK rearrangements. Moreover, no toxicity was observed when crizotinib and chemotherapy were given first and immunotherapy was administered one week later when crizotinib levels had declined below detection limits. Accordingly, PD-L1 mRNA expression increased in the liver 2 days after crizotinib administration, yet returned to basal conditions one week later. Hence, a sequential regimen may avoid the toxicity that has led to the premature termination of a clinical trial in which crizotinib was co-administered with the PD-1 targeting antibody nivolumab\textsuperscript{32}.

**Methods**

**Cell culture conditions and chemical products.** Unless specified, all wild-type cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockefeller, MD, USA) and were maintained at 37 °C under 5% CO\textsubscript{2} in
**Fig. 6** (R)-crizotinib induces immune infiltration in established tumors. MCA205 tumors-bearing mice received injections (i.p.) of solvent (Sol), cisplatin (CDDP), (R)-crizotinib (CDDP), or combinations of (R)-crizotinib (1, 5, or 10 µM), alone or in combinations, for 6 h. Following 15 min before adding 400 µL staining buffer supplemented with 2 µg/mL 4′,6-diamidino-2-phenylindole (DAPI) and then immediately subjected to flow cytometry. The frequency of CTLa-4+/PD-1−, CTLA-4−/PD-1+ or CTLA-4−/PD-1− cells was determined among the indicated subsets. Statistical significance was calculated by two-way ANOVA test for multiple comparisons, *p < 0.05; **p < 0.01; ***p < 0.001 compared to Sol; #p < 0.05; ##p < 0.01; ###p < 0.001 as compared to Sol; ##p < 0.05; ###p < 0.01; ####p < 0.001 as compared to CDDP; εp < 0.05; γp < 0.01; δp < 0.001 as compared to (R)-Cinz.

Media supplemented with 10% (v/v) fetal bovine serum (FBS), 10 U mL−1 penicillin sodium and 10 µg mL−1 streptomycin sulfate. Human osteosarcoma U2OS cells, their derivatives expressing GFP-LC3, CALR-RFP and HMGBl-GFP, human cervical adenocarcinoma HeLa cells and murine methylcholanthrene induced fibrosarcoma MCA205 cells were cultured in Dulbecco’s modified eagle medium (DMEM); murine non-small-cell lung carcinoma (NSC11) TC1 cells, LLC1 cells, as well as their derivatives expressing firefly luciferase (Lum), human NSC11 cell lines NCI-H2228 and NCI-H1650 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium; human colon adenocarcinoma HCT-116 cells were cultured in McCoy’s 5 A medium. AnnexinA1-deficient (AnxA1−/−) MCA205 and TC1 cell lines were generated by means of the CRISPR/Cas-mediated gene editing (sc-240871-KO-2, Santa Cruz Biotechnology), as per manufacturer’s recommendations. All culture media and supplements were bought from Gibco (Carlsbad, CA, USA) and plastic materials came from Corning (Corning, NY, USA). The Published Kinase Inhibitor Set (PKIS) and the complementary set, PKIS2 were obtained from SGC-UNC (Structural Genomics Consortium-UNC Eshelman School of Pharmacy at the University of North Carolina, Raleigh-Durham, NC, USA). Human osteosarcoma U2OS cells stably expressing GFP-LC3 or HMGBl-GFP/CALR-RFP were seeded in 384-well black imaging plates (Greiner-bio-one, Kremsmünster, Austria) overnight at 4 °C. After 3 additional washing steps plates were incubated with an Alexa Fluor 488-conjugated goat-anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA) diluted 1:250 in flow cytometry buffer containing 2 µg/mL 4′,6-diamidino-2-phenylindole (DAPI) and the plate was subjected to flow cytometry (BD LSFRx, BD Biosciences, San Jose, CA, USA). Data were further processed with the FlowJo software (Tree Star Inc., Ashland, OR, USA) to assess the percentage of CALR+ DAPI+ cells. ATP concentrations in the supernatant of cells upon the indicated treatment were measured by means of an ENLITE Enzyme Linked Immunoassay (ELISA) kit (Promega, Fitchburg, WI, USA), based on the ATP dependent luciferin–conversion, that yields detectable bioluminescence, according to the manufacturer’s protocol. HMGBl concentrations in the supernatant of cells following the indicated treatment were measured by means of an enzyme-linked immunoassorbent assay (ELISA) kit (Shino test corporation, Tokyo, Japan), according to the manufacturer’s protocol. Luminescence and absorbance were measured using a SpectraMax i3 multi-mode microplate reader (Molecular Devices).

**High throughput screening.** Human osteosarcoma U2OS cells stably expressing GFP-LC3 or HMGBl-GFP/CALR-RFP were seeded in 384-well black imaging plates (Greiner-bio-one, Kremsmünster, Austria) at a density of 1500 cells per well and allowed to adapt for 24 h. The following day, compounds from the PKIS were added at a final concentration of 10 µM, or the 30 tyrosine kinase inhibitors were added to the HMGB1-GFP positive tumors of 5 and 10 µM. The APP activity was determined with the free software image J (https://imagej.nih.gov/ij/). For the measurement of the FITC-Annexin V detection kit I (BD Biosciences) following the manufacturer’s instructions. Total RNA (2.5 µg from each sample) was then reverse transcribed into cDNA with the Maxima First Strand cDNA Synthesis Kit (Life Technologies). Expression of the interested gene was analyzed by means of SYBR® Green based Quantitative PCR using the Power SYBR® Green PCR Master Mix in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). qPCR-RT data were normalized to the expression levels of the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). Information of PCR primers is depicted in Supplementary table 1.

Quantification of cell death by flow cytometry. Cell death was assessed by means of the FITC-Annexin V detection kit I (BD Biosciences) following the manufacturer’s protocol. Briefly, cells treated in 6-well plates were collected and washed in PBS before the cell pellet was resuspended in 50 µL staining buffer containing FITC-conjugated AnnexinV antibody. Samples were then incubated in the dark for 15 min before adding 400 µL staining buffer supplemented with 2 µg mL−1 DAPI. Acquisitions were performed on a CyAn ADP cytofluorometer ( Beckman Coulter, Indianapolis, USA), and data were statistically evaluated using FlowJo.

**In vitro validation of CALR exposure, ATP release and HMGBl release.** U2OS, MCA205 or TC1 cells were seeded in 6 well plates and allowed to adapt for 24 h. Following 15 min before adding 400 µL staining buffer supplemented with 2 µg mL−1 DAPI. Acquisitions were performed on a CyAn ADP cytofluorometer ( Beckman Coulter, Indianapolis, USA), and data were statistically evaluated using FlowJo.

**In vitro analysis of PD-L1 and MHC Class I/II expression by flow cytometry.** Cells were seeded in 96-well plates (10,000 cells per well for murine cells) in 100 µL growth medium and let adapt for 24 h before treatment. Cells were then treated by different concentrations of crizotinib, alone or in combination with cisplatin, for another 24 h, recombinant murine IFNα (2000 IU) or IFNγ (100 ng mL−1) was used as positive controls. Post-treated cells were collected in 96-well V-shape plates (Greiner-bio-one, Kremsmünster, Austria) over-night at 4 °C. After 3 additional washing steps plates were filled with 50 µL PBS per well and subjected to automated image acquisition and subsequent image analysis. For automated fluorescence microscopy, a robot-assisted Molecular Devices IXM XL Bioloader (Molecular Devices, Sunnyvale, CA, USA) equipped with a Sola light source (Lumencor, Beaverton, OR, USA), adequate excitation and emission filters (Semrock; Inc.) and a 16-bit monochrome charged-coupled device (CCD) camera (PCO, Kelheim, Germany) and a 20x Planapo objective (Nikon, Tokyo, Japan) was used to acquire a minimum of 4 view fields of each well. Following images were processed and segmented with the MetaXpress software (Molecular Devices) to analyze GFP-LC3 granularity, CALR-RFP granularity at the cellular membrane, the decrease of nuclear granularity as well as nuclear shrinkage to indicate induced apoptotic cell death. Data mining was conducted using the freely available software R (https://www.r-project.org). Data was intra-plate normalized as the ratio to plate means for each data point, while Z-scores were employed for inter-plate normalization.

**In vitro validation of CALR exposure, ATP release and HMGBl release.** U2OS, MCA205 or TC1 cells were seeded in 6 well plates and allowed to adapt for 24 h. Following 15 min before adding 400 µL staining buffer supplemented with 2 µg mL−1 DAPI. Acquisitions were performed on a CyAn ADP cytofluorometer ( Beckman Coulter, Indianapolis, USA), and data were statistically evaluated using FlowJo.

**Quantitative RT-PCR.** Total RNA extraction of cells was performed with the GeneJET RNA Purification Kit (Life Technologies). Total RNA extraction of established tumors was performed with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and RNALater RNA Stabilization Reagent (Qiagen) following the manufacturers’ instructions. Total RNA (2.5 µg from each sample) was then reverse transcribed into cDNA with the Maxima First Strand cDNA Synthesis Kit (Life Technologies). Expression of the interested gene was analyzed by means of SYBR® Green based Quantitative PCR using the Power SYBR® Green PCR Master Mix in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). qRT-PCR data were normalized to the expression levels of the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). Information of PCR primers is depicted in Supplementary table 1.
**Fig. 7** Immunogenic chemotherapy of (R)-crizotinib sensitizes NSCLC tumors to checkpoint blockades. 

**a-c** Treatment of subcutaneous (s.c.) TC1 tumors with injections of solvent control, CDDP alone or in combination with (R)-crizotinib. Isotype monoclonal antibodies (mAbs) or anti-PD-1 mAbs (αPD-1) combined with anti-CTLA-4 mAbs (αCTLA-4) were injected on day 8, 12, and 16 (schedule in **a**). Tumor growth was monitored (**b, c**) and expressed as surface (mean ± s.e.m., **b**) or size at endpoint (box plot, **c**). Tumor-free mice are reported in **h**. Representative images of tumor development are shown in **e**; average (mean ± s.e.m.) bioluminescence signals are reported in **f**; final values at endpoint are reported as box plot in **g**. The percentage of tumor free mice is reported in **h**; overall survival is reported in **i**. Data in **e**–**i** include dual checkpoint blockade (αCTLA-4/αPD-1). The effects of dual as compared to single checkpoint blockade at day 70 are shown in **j**. Statistical significance was calculated by means of the ANOVA Type 2 (Wald test) (**b, f**), ANOVA test for multiple comparisons (**c, g**), Likelihood ratio test (**h, i**) or χ² test (**j**). *p < 0.05, **p < 0.01, ***p < 0.001 as compared to control group with isotype; #p < 0.05, ##p < 0.01, ###p < 0.001 as compared to control group with the combination of αPD-1 and αCTLA-4; †††p < 0.001 comparing isotype and αCTLA-4 and αPD-1 combination, n = minimum of 10 animals per group.
Pharmacokinetic of (R)-crizotinib kinetics in murine plasma. Female C57Bl/6 mice (8 weeks) received a single i.p. injection of (R)-crizotinib (50 mg Kg⁻¹, dissolved as described above) before blood sampling via cardiac puncture. Blood was collected in Microtainer® Capillary Blood Collector (BD bioscience) containing lithium heparin and was spun to obtain plasma. Plasma was mixed with extraction buffer (methanol, H₂O, chloroform; 9:1:1, v/v) at a ratio of 1:10 (v/v) vortexed and centrifugated (10 min, at 15,000 × g, 4 °C). Then extracts from the supernatant were dried by evaporation at 40 °C in a Pavia (M) centrifugal concentrator (Techne DB3, Staffordshire, UK). The dried extract was re-solubilized with MilliQ water for analysis by liquid chromatography (LC) mass spectrometer (MS). The concentration of crizotinib was calculated based on a crizotinib calibration curve (lower limit of quantification (LLOQ), fixed at 0.0222 µM) and above upper limit of quantification (ULOQ, fixed at 44.4 µM) using the area of ion signal. For crizotinib, the collision energy (CE) was set at 39.0 V, and collision exit potential (CXP) was set at 39.0 V and collision exit potential (CXP) at 12 V. For selumetinib ISTD, which was used as an internal standard, the collision energy (CE) was set at 37.0 V, and collision exit potential (CXP) at 18 V. The transitions monitored for quantitation were m/z 450.1 > 260.1 for crizotinib and m/z 457.1 > 361.1 for the selumetinib ISTD. A 2.5 µL sample was injected on a column Kinetex Polar C18 (2.1 mm x 0.1 mm particle size 2.6 µm) from Phenomenex, protected by a guard column C18 (5 mm x 2.1 mm) and heated at 40°C by a Pelletier oven. The gradient mobile phase consisted of water with 0.2% acetic acid (A) and Methanol (B). The flow rate was set to 0.4 mL min⁻¹. Initial condition was 95% phase A and 5% phase B. Molecules were then washed using a gradient from 95% phase B to 0% phase B in 9 min and equilibrated using 95% mobile phase B for 2 min. Then the autosampler was kept at 4 °C. The Analyst (Version 1.7) software was used to operate the mass spectrometer. Peak detection, integration and quantification of the analytes were performed using the MultiQuant quantitative software (Version 3.0.3).

Antitumor vaccination assays and chemotherapy studies with established cancer models. All mice were maintained in the animal facilities of Gustave Roussy Campus Cancer in a specific pathogen-free, temperature-controlled environment with 12 h light/dark cycles and received food and water ad libitum. All animal experiments were performed in compliance with the EU Directive 63/ 2010 and specific ethic protocol (Protocols 2016_082 that was approved by the Ethical Committee of the Gustave Roussy Campus Cancer, CEEA IRCIV/IGR no. 26, registered at the French Ministry of Research) Six- to eight-week-old male wild-type C57BL/6 mice were obtained from ENVIGO France (Gannat, France). Six to seven-week-old female athymic nude (nu/nu) mice were obtained from Harlan France (Gannat, France).

To establish transplanted s.c. tumors, half a million wild type MCA205, TC1, as well as their Anxa1⁻/⁻ or Hmgbl⁻/⁻ derivatives were xenotransplanted to the left flank of immunocompetent C57Bl/6 mice (8-weeks-old female, 1 x 10⁶ cells per mouse). In the negative control group, 2 x 10⁶ of the cellular suspension was s.c. injected to the left flank of immunocompetent C57Bl/6 mice (8-weeks-old female, 1 x 10⁶ cells per mouse). Pbs was injected as a negative control. Two weeks later, all mice were confirmed tumor-free in the vaccination flank and living cancer cells of the same types (1 x 10⁶ cells per mouse for MCA205 cells and its derivatives, 2 x 10⁵ per mouse for TC1 cells and its derivatives) were injected in the right flank of vaccinated mice. Tumor growth was regularly monitored for the following weeks and the absence of tumors was considered as an indication of antitumor vaccination.
perpendicular dimension × n/t) reached around 20–25 mm² (defined as day 0), mice received the treatments described below. Tumor surface was then monitored every 2 days and animals bearing neoplastic lesions that exceeded 250 mm² were euthanized.

To establish the orthotopic TC1 NSCLC model, wild type TC1 Luc cells (5 × 10⁵ in 100 μL PBS) were intravenously injected to wild type C57BL/6 mice or athymic nude (nu/nu) mice. Tumor incidence and development were monitored by in vivo photonic imaging of tumor cells’ luciferase activity. About 7 days after injection, tumor incidence in the lung was detected at an exposure time of 4 min, and mice were assigned to different groups for treatment as described below.

To establish LCL1 orthocultically, C57BL/6 mice were anesthetized with 3% isoflurane and euthanized in a position of dorsal decubitus. The fur on the back of surgery was removed to avoid contamination and 70% ethanol and iodide were applied. A linear incision was made on the chest wall of each mouse and 2 × 10⁵ LCL Luc cells in 50 μL PBS containing 20% matrigel matrix (Corning, Ref# 356231) were injected percutaneously into the left lung of the animals using 0.3-ml insulin syringes with 30 G hypodermic needles. The skin incisions were closed with a surgical skin clip. Tumor incidence and tumor growth were monitored by in vivo photonic imaging of tumor cells’ luciferase activity. About 5 days after injection, tumor incidence in the lung was detected at an exposure time of 2 min, and mice were assigned to different treatment groups as described below.

For the acquisition of bioluminescence images, mice received an i.p. injection of luciferase substrate (Beetle Luciferin powder salt; Promega) at a dose of 3 mg per mouse, and 8 min (for TC1 model), or 12 min (for the LCL1 model) post luciferin inoculation, photons were acquired on a Xenogen IVIS 50 bioluminiscence in vivo imaging system (Caliper Life Sciences Inc., Hopkinton, MA, USA). In vivo imaging was conducted every 4–5 days with an exposure time starting with 4 min, which was then gradually increased to 3 min, 2 min, 1 min when photon saturation occurred. Tumor bearing mice showing photon saturation at 1 min of exposure time were euthanized.

All treatments on mice bearing transplanted cancers are designed as following: Solvent for chemicals (Sol): 10% Tween-80, 10% PEG400, 4% DMSO, 76% ddH₂O. Solvent for chemicals (Sol): 10% Tween-80, 10% PEG400, 4% DMSO, 76% ddH₂O. Mice were treated with the supporting software EZ-SCAN and the number of images recorded was 180. All mouse experiments were approved by the Animal Care and Use Committee of the University of California, Los Angeles. Related animal experiments were approved by the Pittet Lab at the Massachusetts General Hospital, Harvard Medical School, USA.

To establish the U87MG xenograft glioma orthotopic model in immunodeficient mice, U87MG cells (5 × 10⁶) were implanted intracranially into 5-week-old male NOD/SCID mice (Leica, Wetzlar, Germany) and captured on poly-L-lysine-coated slides (Fisher, Scientific, Pittsburgh, PA, USA). For immunofluorescence staining of tissue sections, slides were washed three times with TBS containing 0.05% Triton-X-100 (TBST) to remove remaining O.C.T. After blocking non-specific binding of antibodies with 5% fetal bovine serum (FBS) plus 1% bovine serum albumin (BSA) at room temperature for 1 h, samples were stained with specific primary antibodies in a humidified chamber (overnight at 4°C). If necessary, Alexa Fluor®-conjugated secondary antibodies (Molecular Probes-Life Technologies Inc.) were used at 1:200 dilution after washing three times for 5 min with TBST. After additional washing steps, samples were covered with cover slides using Fluoroshield™ with DAPI (Sigma-Aldrich), which allowed nuclear counterstaining. For each sample, 10 viewfields from different sections of different layers were captured with a HR-S8 Flouroscope Microscope (Leica). Image analysis was performed with the LAS X software (Leica).
A dissociated bulk tumor cell suspension was resuspended in RPMI-1640, sequenced in gentleMACS C Tubes (Miltenyi Biotech; Bergisch Gladbach, Germany) containing 1 mL RPMI-1640 medium. The samples were kept on ice until dissociation using the gentleMACS dissociator with a Millenyi mouse tumor dissociation kit (Millenyi Biotech) according to the manufacturer’s protocol. The dissociated bulk tumor cell suspension was resuspended in RPMI-1640, sequentially passed through 70 and 30 μm nylon cell strainers (Millenyi Biotech) and washed twice with cold PBS. To test IFNA and IL-17 production, cells were resuspended with ionomycin (1 μg/mL) and washed twice with cold PBS. To test IFNγ and IL-17 production, cells were resuspended with ionomycin (1 μg/mL), Dyex (Invitrogen) and incubated with antibodies specific for CD3ε/CD3ζ, for FC block as described below, and pre-incubated with fluorescent antibodies specific for CD3ε, CD4, CD8, CTLA-4, LA-3, PD-1, and TIM-3 (detailed information are provided in the following antibody list).

Isolation and phenotyping of tumor-infiltrating lymphocytes. Subcutaneous MCA205 or TC1 cancers were established and tumor bearing mice were treated as described above. Animals were sacrificed and tumors were excised and immediately collected in gentleMACS C Tubes (Milleny Biotech, Germany) containing 1 mL RPMI-1640 medium. The samples were kept on ice until dissociation using the gentleMACS dissociator with a Millenyi mouse tumor dissociation kit (Millenyi Biotech) according to the manufacturer’s protocol. The dissociated bulk tumor cell suspension was resuspended in RPMI-1640, sequentially passed through 70 and 30 μm nylon cell strainers (Millenyi Biotech) and washed twice with cold PBS. To test IFNγ and IL-17 production, cells were resuspended with ionomycin (1 μg/mL, Sigma-Aldrich) plus PMA (50 ng/mL, Sigma-Aldrich) in the presence of BD GolgiPlug (1:1000, BD Bioscience) in CTL-Test Medium (Cellular Technology Limited, Cleveland, OH, USA) containing 2 mM L-Glutamine for 6 h before the following steps. Prior to surface staining of fluorescent antibodies, samples were incubated with LIVE/DEAD® Fixable Yellow Dead Cell dye (Invitrogen) to discriminate viable cells from damaged/dead cells, and incubated with antibodies against CD16/CD32 (clone 2.4G2, BD Biosciences) to block Fc receptors. Finally, cells were incubated with a panel of fluorescence-conjugated antibodies (surface staining) before permeabilization and fixation with Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) for the staining of intracellular Foxp3, IFNγ, and IL-17. Data were acquired on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed by means of Flowjo software. Absolute counts of tumor-infiltrating lymphocytes were obtained taking in account the harvest volume of the tumor, total volume of the dissociated tumor cell suspension (cell concentration typically set to 250 mg mL⁻¹ in PBS), proportion of the whole cell suspension stained (typically 200 μL containing 50 mg of bulk tumor cell suspension) and proportion of the stained cell suspension run through the flow cytometer (typically ~300 out of 400 μL of the stained cell suspension). Detailed gating strategy for the flow cytometric analysis can be found in corresponding Supplementary Figures.

Antibodies for immunofluorescence and flow cytometry analysis. Anti-CD3 antibody (ab5690) and Anti-CD11c antibody (clone 3.9, ab11029) were purchased from Abcam (Cambridge, UK). Alexa Fluor® 488-conjugated anti-mouse CD86 antibody (clone GL-1), Alexa Fluor® 488 or Alexa Fluor® 594-conjugated anti-mouse CD3ε/CD3ζ antibody (clone 53-6.7), APC anti-mouse CD63 (clone 145-2C1), Alexa Fluor® 488-conjugated anti-mouse Foxp3 antibody (clone MF-14), and PE-Cy7-conjugated anti-mouse CD152/CTLA-4 antibody (clone UC10-149) were purchased from Biolegend. BV421-conjugated anti-mouse CD278/ICOS (clone 7E.1G9), FITC or PE-conjugated anti-mouse CD4 (clone 53-6.7), FITC anti-mouse CD4 (clone RM4-4), PE and APC anti-mouse IFNγ (XMg12.1), PE Rat anti-mouse IL4 (1B11), BV605 anti-mouse IL17A (TC11-18H10), PE-Cy7-conjugated anti-mouse CD11c (clone HI3) were purchased from BD Bioscience (San Jose, CA, USA). PerCP-Cy5.5 or Pacific Blue anti-mouse CD4 (clone RM4-4), PE-Cy7-conjugated anti-mouse CD25 (clone PC6.2), BV421-conjugated anti-mouse CD28 (clone 45-3.6.7), APC anti-mouse IFNγ (XMg12.1), PE-Cy7-conjugated anti-mouse CD3g (clone 17.A2), APC-eFluor780, APC-Cy7-conjugated anti-mouse CD279/PD-1 (clone J43), PE-conjugated anti-mouse IL-17A (clone eBio17B7), PerCP-Cy5.5-conjugated anti-mouse CD3e (clone 145-2C11), FITC-conjugated anti-mouse LAG-3 (clone eBio897F7), PE-conjugated anti-mouse LAG-3 (clone J43), APC-conjugated anti-mouse TIM-3 (clone 8B.2C12), APC-conjugated anti-mouse TIM-3 (clone 8B.2C12), Pacific blue-conjugated anti-mouse CD11b (clone M1/70.15), Alexa Fluor® 488-conjugated anti-mouse Ly-6C (clone HK1.4), and PE-conjugated anti-mouse Ly-6G (clone 1A8-Ly6G) were purchased from eBioscience (San Diego, CA, USA).

RNA-Seq (RNA sequencing) analyses of chemotherapy impacted gene expression. To prepare RNA samples for RNA-sequencing analysis, s.c. tumors were established by injecting 5 × 10⁶ WT MCA205 cells (in 100 μL PBS) into the right flank of WT C57BL/6 mice. When tumors became palpable (approximately 7 days post cell injection), mice received 0.25 mg Kg⁻¹ i.p. CDDP in 200 μL solvent, 50 mg Kg⁻¹ i.p. (R)-crizotinib in 200 μL solvent, 0.25 mg Kg⁻¹ i.p. CDDP dissolved together with 50 mg Kg⁻¹ i.p. (R)-crizotinib in 200 μL solvent, or an equivalent volume of solvent at day 0 and day 2. At day 10 tumors were harvested and immediately immersed in the RNA later RNA Stabilization Reagent (Qiagen, Hilden, Germany) and total RNA was extracted with the RNeasy Plus Mini Kit (Qiagen) following the manufacturers’ instructions. RNA-Seq data analysis was performed by GenoSpicce Technology (www.genospicce.com). Sequencing, data quality, reads repartition (e.g., for potential ribosomal contamination), and insert size estimation were performed using FastQC, Picard-Tools, Samtools, and rseqc. Reads were mapped using STARv2.4.0m on the mm10 Mouse genome assembly. Gene expression regulation study was performed as already described[48]. Briefly, for each gene present in the Mouse FAST DB v2016.1 annotations, reads aligning on constitutive regions (that are not prone to alternative splicing) were counted. Based on these read counts, normalization and differential gene expression were performed using DESeq2[49] on R (v.3.2.5). Only genes expressed in at least one of the two compared experimental conditions were further analyzed. Genes were considered as expressed if their rpkm value was greater than 95% of the background rpkm value based on intergenic regions. Results were considered statistically significant for corrected p-values ≤ 0.05 and fold-changes ≥ 1.2.

Assessment of hepatotoxicity. MCA205 cells were s.c. inoculated into the right flank of C57BL/6 mice. When tumor became palpable mice received (R)-crizotinib alone (50 mg Kg⁻¹ i.p.) or in combination with CDDP (0.25 mg Kg⁻¹ i.p.), together with anti-PD-1 antibody (10 mg Kg⁻¹ i.p.) in a simultaneous (schedule A) or sequential (schedule B) way. Schedule A consisted of (R)-crizotinib and CDDP administration combined with anti-PD-1 at day 0, 2, and 4 followed by (R)-crizotinib and CDDP at day 6 to simulate continuous crizotinib administration. Schedule B consisted of (R)-crizotinib and CDDP administration at day 0 and 2 followed by anti-PD-1 at day 8, 12, 16. The mice were observed daily and body weight was recorded as one proxy for toxicity. Blood and liver were sampled 3 days after the last antibody administration. Serum alanine transaminase activity (ALT; Fluorometric) (Abcam, ab105134) following the manufacturer’s instructions. Livers were fixed in 4% FFA and subjected to hematoxylin and eosin staining for morphology observation.

Statistical analyses. Unless specified, results were expressed as mean ± s.e.m. Statistical tests included unpaired one-tailed and two-tailed Student’s t-tests using Welch’s correction and one-way ANOVA followed by multiple comparison tests. When applicable, two-way ANOVA followed by multiple comparison tests were used for analysis of interleaved box whiskers plot and scatter dots plots; comparison of tumor growth curves was performed by means of the ANOVA Type 2 (Wald test); Comparison of survival curves was performed with the Likelihood ratio test. P-values of 0.05 or less were considered to denote significance (p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant). In vivo data was analyzed using the freely available TumGrowth software (https://github.com/kroemerlab/TumGrowth)[48].

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Author contributions

P.L., L.Z., J.P., S.L., A.D.F., T.Y., K.I., L.R.B. and E.V. performed most of the experiments; V.S. performed the Seahorse analysis; C.P. and C.E. performed the in vivo experiments with the KP lung cancer model; S.R. performed the immunohistochimistry of KP mice samples; A.M. and T.M. tested the immune effects of crizotinib on the mouse immune system.
system, X.L., H.Y., Q.L., J.C. performed the in vivo experiments with urethane induced lung cancer model; L.S. performed the intercostal injection for the establishment of LLC1 orthotopic lung cancer model; S.D., F.A., D.L., S.B.A., N.P. and A.B. measured drug concentrations; O.K. and G.K. conceived the study; V.M.-C., E.T., L.Z., L.A., Y.M., M.J.P., designed (parts of) the study; P.L., L.Z., O.K., and G.K. wrote the paper.

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