**SUPPLEMENTARY MATERIALS AND METHODS**

**Reagents**

For *in vivo* experiments, rat anti mouse PD-1 (clone RMP1-14) was purchased from BioXCell (West Lebanon, NH, USA). For immunohistochemical analyses, we utilized the following primary antibodies: purified rat anti-Mouse CD45 (Clone 30-F11, BD Biosciences, San Jose, CA, USA) for the identification of all leukocytes, rat anti mouse F4/80 (Clone Cl:A3-1, BioRad, Hercules, CA, USA) for the identification of macrophages, anti arginase I (Genetex International Corp., Irvine, CA, USA), Purified rat anti-mouse Ly-6G and Ly-6C (Gr-1) (Clone RB6-8C5, BD Pharmingen) for the identification of granulocytes, purified rat anti-mouse CD45R (Clone RA3-6B2, BD Pharmingen) for the identification of B cells, mouse NKp46/NCR1 antibody (R&D systems, Minneapolis, MN, USA) for the identification of NK cells.We utilized the following antibodies for Flow Cytometry analysis: CD45 VioGreen (Miltenyi, Bergisch Gladbach, Germany), DX5 PeVio770 (Miltenyi), CD11b PE (BD Pharmingen), Ly6G PercpVio700 (Miltenyi), Ly6C APC (Miltenyi), MHC-II Alexa700 (Thermo Fisher Scientific), F4/80 PEfluo610 (Thermo Fisher Scientific), CD11c FITC (Miltenyi).

**Fluorescence in situ hybridization (FISH)**

FISH analysis was carried out by means of a commercially available dual color (ZytoLight® SPEC MDM2/CEN 12 Dual Color and ZytoLight® SPEC MDM4/1p12 Dual Color) probes specifically designed to detect *MDM2* (located on chromosomes 12q15) and *MDM4* (located on chromosomes 1q32.1) amplification, according to the manufacturer’s protocol. At least 100 tumor cells were evaluated for each sample and amplification was reported utilizing Capuzzo’s amplification criteria (1). Briefly, gene amplification was characterized by presence of tight *MDM2/4* gene clusters and a ratio of *MDM2/4* gene to chromosome of ≥ 2 or ≥ 15 copies of *MDM2/4* per cell in ≥ 10% of analyzed cells.

**Generation of anti mouse and human (Nivolumab) PD-1 antibody F(ab)2**

Anti mouse PD-1 F(ab)2 was generated using Immobilized Pepsin (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol with some modifications. The enzymatic digestion was performed for 7 hours at 37°C. Antibody concentration was determined by BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Nivolumab F(ab)2 was generated following the same protocol. The enzymatic digestion was performed for 60 minutes at 37°C. SDS-PAGE under non-reducing conditions (2) and Coomassie Blue staining (Thermo Fisher Scientific Inc.) were carried out to check the obtained digested antibody. To verify whether anti mouse PD-1 F(ab)2 was still able to bind PD-1, flow cytometry analysis was performed using the murine T lymphoblast cell line EL-4, reported to constitutively express high levels of PD-1 (3). EL-4 cells were stained with 10g/ml of anti-mouse PD-1 clone RMP1-14 (BioXCell), anti mouse PD-1 F(ab)2 or rat IgG2a isotype control clone 2A3 (BioXCell) for 30 minutes at 4°. After washing, cells were incubated with FITC-conjugated Anti-Rat IgG (H+L) Antibody, Mouse Serum Adsorbed (KPL, **SeraCare Life Sciences,** Milford, MA, USA) for 30 minutes at 4°C. Samples were then acquired with BD LSRII Fortessa™ (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (FlowJo LCC, Ashland, OR, USA) (Supplementary Figure S3F). To verify whether Nivolumab F(ab)2 was still able to bind PD-1, the capacity of F(ab)2 fragments to antagonize PD-1 staining by biotinylated Nivolumab was assessed on PD-1 expressing OKT3-activated T-cells and on PD-1 expressing latex beads (Supplementary Figure S3G). Both, anti mouse PD-1 F(ab)2 and Nivolumab F(ab)2 were tested for the capacity to bind PD-1 by enzyme-linked immunosorbent assay (ELISA, data not shown).

Cell lines

The human non small cell lung cancer (NSCLC) cell line NCI-H460 (hereafter H460, ATCC® HTB-177™) and PC9 (formerly known as PC-14) were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA) and ECACC General Cell Collection (Salisbury, UK) respectively. Cell lines were authenticated by the Fragment Analysis Facility at Fondazione IRCCS – Istituto Nazionale dei Tumori (Milan, Italy) using the GenePrint 10 System (Promega, Madison, WI, USA). Cells were maintained in RPMI 1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA) and a solution of 100 U/ml penicillin and 100 U/ml streptomycin (Lonza, Basel Switzerland) at 37°C in a 5% CO2 atmosphere. Cultures were regularly tested for Mycoplasma by using the mycoAlert Plus Kit (Lonza).

***In vivo* treatments**

All xenograft experiments were carried out using 8- to 9-week-old female athymic nude mice (from Charles River Laboratories, Calco, Italy) maintained in laminar-flow rooms at constant temperature and humidity, with food and water given *ad libitum*. Before implantation in mice, H460 cells were tested for the presence of rodent pathogens using IMPACT I PCR Profile (IDEXX BioResearch, Ludwigsburg, Germany) and resulted negative. Tumor cells (2.5\*106 cells/mouse) were injected subcutaneously (s.c.) on the mice right flank. Mice were intraperitoneally (i.p.) or peritumorally (p.t.) treated with 200 g of monoclonal antibody anti mouse PD-1 (clone RMP1-14, BioXCell) starting at day +6 and +3 after tumor injection, respectively. Anti-PD-1 antibody was administered twice a week until the end of the experiment. Control group received vehicle. For the *in vivo* experiment using anti PD-1 F(ab)2, mice were injected with H460 cells and treated with 200 g of anti mouse PD-1 F(ab)2 or received vehicle, as described above. Tumor mass was measured with a caliper and tumor volume (mm3) calculated [long diameter × (short diameter)2/2].

PDXs were established as described in (4). Experiments were carried out in groups of 4 SCID mice, bearing a PDX sample or a cell suspension (105 cells for H460 and PC9 xenografts experiments) in each flank. Mice were treated twice a week with intraperitoneal injection of 10mg/Kg Nivolumab (Opdivo, Bristol-Myers Squibb) or Nivolumab F(ab)2, or with intraperitoneal injection of 10mg/Kg clodronate filled liposomes (Bio-connect services, The Netherlands). Tumour growth was followed by caliper twice a week and results were analysed using GraphPad Prism software. Response Rate was calculated as described in (5). Hyperprogression was defined as follows: HP1 (score -2), maximal growth inhibition (MGI) vs controls < -10% and Tumor Growth Delay (TGD = Time to event treated/average time to event of controls, event= 3x starting volume) >0.75; HP2 (score -4), MGI<-10% and TGD<0.75.

**Immunohistochemistry for tumor xenografts**

For immunohistochemical analyses, tumor samples were fixed in 10% buffered formalin, paraffin-embedded and sectioned (4-µm thick). For CD45R/B220 and NKp46 staining, sections were deparaffinized and underwent heat-induced epitope retrieval at pH 9, for 40 min at 95°C (Dewax and HIER Buffer H, Thermo Fisher Scientific Inc.). Endogenous peroxidase activity was blocked by incubating sections in 3% H2O2 for 15 min. Slides were rinsed and treated with PBS containing 10% normal serum for 30 min to reduce nonspecific background staining and then incubated for 1 hour at room temperature with the appropriate primary antibodies. Sections were incubated for 30 min with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA), and then labelled by the avidin-biotin-peroxidase (ABC) procedure with a commercial immunoperoxidase kit (VECTASTAIN Elite ABC HRP Kit Standard, Vector Laboratories). The immunoreaction was visualized with 3,3’-diaminobenzidine substrate (Peroxidase DAB Substrate Kit, Vector Laboratories) for 5 min and sections were counterstained with Mayer’s haematoxylin (Diapath, Martinengo, Italy). Immunohistochemistry for CD45, F4/80 and Gr-1 were carried out as previously described (6,7). For CD45, F4/80, CD45R/B220, Gr-1 and NKp46 quantification. Digital slides were obtained from immunostained sections by using the NanoZoomer-XR Digital slide scanner (Hamamatsu), and images were captured by using the NDP.view2 Viewing software (Hamamatsu). The % positive immunostained area was evaluated using the ImageJ analysis software (http://rsb.info.nih.gov/ij/) in 3 hot spot 20x microscopic fields selected either within the tumor (intratumoral) or in the region between the tumour and surrounding host tissues (interface), or in both regions. For Arginase I staining, sections were deparaffinized and rehydrated. Antigen unmasking was performed using pH 9 Tris/EDTA buffer (Novocastra, Leica Microsystem, Buffalo Grove, IL, USA) in a PT Link Dako (Dako, Agilent, Santa Clara, CA, USA) unit at 98°C for 30 min. Sections were then brought to room temperature and washed in PBS. After neutralization of endogenous peroxidase with 3% H2O2 and Fc blocking by a specific protein (Novocastra, Leica Microsystem), samples were incubated with polyclonal rabbit anti-mouse arginase antibody for 1 hr at room temperature. Staining was revealed by a polymer detection kit (Novocastra, Leica Microsystem) and AEC (3-amino-9-ethylcarbazole, Dako, Agilent) substrate-chromogen. Slides were counterstained with Harris hematoxylin (Diapath). Sections were analyzed under a Leica DM2000 optical microscope (Leica Microsystems) and microphotographs were collected using a Leica DFC320 digital camera (Leica Microsystems). ArgI+ cells quantification was performed as previously described (8).

**Immunohistochemistry for human samples and PDXs**

Immunohistochemistry was carried out on FFPE human or patient-derived xenograft (PDX) tissue sections. Briefly, sections 2.5/3 micron-thick were cut from paraffin blocks, dried, de-waxed and rehydrated. The antigen unmasking technique was performed using Target Retrieval Solutions pH6 and pH 9 in a PT Link Dako pre-treatment module at 98°C for 30 minutes. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H2O2 and Fc blocking by a specific protein block (Novocastra UK), the samples were incubated with primary antibodies.

The following primary antibodies were adopted for IHC: CD163 (clone 10D6 Leica Biosystems, 1:100, pH6); CD33 (clone PWS44 Leica Biosystems, 1:100, pH9); CD123 (clone BR4MS Leica Biosystems, 1:100, pH9); CD138 (clone 5F7 Leica Biosystems, 1:50, pH9); Arginase-A1 (Genetex, 1:200, pH9); Myeloperoxidase (Cell Marque, 1:200, pH6) and F4/80 (clone CI:A3-1 Abd Serotec, 1:200, pH6). Staining was revealed using Novolink Polymer Detection System (Novocastra) or IgG (H&L) specific secondary antibody (Life Technologies, 1:500) and either AEC (3-Amino-9-ethylcarbazole) or DAB (3,3'-Diaminobenzidine, Novocastra) as substrate-chromogens. PD-L1 (clone 22c3 Dako, 1:50, pH8), PD1 (clone NAT105 Biocare, 1:50, pH8), FOX-P3 (clone 259D/C7 BD Pharmingen, 1:200, pH8), CD3 (Dako, 1:400, pH8), CD4 (clone 4B12 Dako, 1:300, pH8), and CD8 (clone C8144B Dako, 1:20, pH8) antibodies were incubated with a commercially available detection kit (EnVision™ FLEX+, Dako) in an automated Immunostainer (Dako Autostainer System).

For double-marker immunofluorescence, after antigen retrieval, the sections were incubated overnight at 4°C with a combination of two primary antibodies against antigens defining the epithelioid macrophage “complete phenotype” (i.e. anti-CD33 and anti-CD163; anti-PD-L1 and anti-CD33; anti-PD-L1 and anti-CD163). The binding of the primary antibodies to their respective antigenic substrates was revealed by Opal Multiplex IHC kit, which allowed for combined immunostainings using antibodies with a same made through tyramide signal amplification. After deparaffinization, antigen retrieval was performed using microwave heating and a pH6 or pH9 buffer and the first primary antibody was incubated for 1h. Immunofluorescence labeling was achieved by incubating with a specific secondary antibody followed by the addition of one selected Opal fluorophore and microwave treatment in pH6 buffer. The same procedure was repeated for the second primary antibody using a different Opal fluorophore and DAPI nuclear counterstain.

**Gene expression profiling and analysis and RT-qPCR**

RNA was extracted from FFPE samples using miRNAeasy FFPE kit (Qiagen). After quality check and quantification by 4200TapeStation (Agilent) and Qubit Fluorometer (Thermo Fisher Scientific), respectively, RNA expression was assessed using the human Affymetrix Clariom S Pico assay (Thermo Fisher Scientific). Briefly, 100 ng of total RNA was used to generate single-stranded cDNA samples for hybridization. Then, cDNA was enzymatically fragmented and biotinylated using the WT Terminal Labeling kit (Thermo Fisher), combined with the hybridization buffer, and injected into the Human Clariom S arrays targeting >20,000 well-annotated genes. Arrays were stained using the Affymetrix GeneChip Fluidics Station 450 and scanned with the 7G Affymetrix GeneChip Scanner 3000. Raw CEL files were pre-processed using the Robust Multi-Array Average (9) method implemented in the *oligo* Bioconductor package. Probe sets were annotated to HUGO gene symbols using the *clariomshumantranscriptcluster.db* package. A batch effect due to RNA quality was identified and corrected using ComBat (10). Finally probe sets were collapsed to gene symbols using the *collapseRows* function of the *WGCNA* package with “maxRowVariance” method. Differential expression analysis was performed using the *limma* package (11), adjusting p-values with the Benjamini-Hochberg false discovery rate (FDR) method. Gene Set Enrichment Analysis (GSEA) (12) was run for the HALLMARK gene set collection in pre-ranked mode using the t-statistic from *limma* as ranking metric.

RT-qPCR for 10 selected immune-related genes analysed in duplicates was performed using the TaqMan PreAmp Master Mix Kit (ThermoFisher) as by protocol. After synthesis of cDNA by RT-PCR starting from 10μl of total RNA, 14 pre-amplification cycles were performed and the product diluted 1:5 on TE Buffer to run qPCR on QuantStudio 7 Flex (Thermo Fisher Scientific). Data were elaborated using the QuantStudio Real-Time PCR software v 1.1 (Thermo Fisher Scientific) and the –ΔΔCt value was considered for further analysis considering only Ct<35 and using ACTB63 as housekeeping gene and the minimum expression value as calibrator. Unpaired two-tailed t-test was used to compare gene expression among different classes and p-values<0.05 were considered significant.

**Flow Cytometry**

Samples were dissociated with Tumor Dissociation kit (Miltenyi biotec). Murine lungs single-cell suspensions (106 cells) were incubated in staining solution containing 1% BSA, 2 mM EDTA, 7-aminoactinomycin D (7-aad) and PreCP-eFluor 710- conjugated anti murine MHC(H-2Kd) (eBioscience). Samples were acquired by FACS Calibur and analysed with FlowJo\_V10 software as described in (13). PDXs single cell suspensions (106 cells) were incubated in staining solution containing 1% BSA, 2 mM EDTA, 7-aminoactinomycin D (7-aad) FITC-conjugated anti-PD1 antibody (BD Biosciences). For analysis of immune infiltrate, cell suspensions were incubated in staining solution containing 1% BSA, 2 mM EDTA, and the corresponding antibodies described within reagents. For Nivolumab cross-reactivity analysis, cell suspensions were incubated in staining solution containing 1% BSA, 2 mM EDTA, 7-aminoactinomycin D (7-aad) and Nivolumab, biotinyilated-Nivolumab or PE murine anti PD-1 antibody. Samples were acquired by FACS Calibur and analysed with FlowJo\_V10 software.

**Statistical Analysis**

For in vivo experiments, differences between treatment groups were calculated using mixed models ANOVA as described in (14). Data were analyzed using Graph Pad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Statistical analyses of the results were performed using either unpaired T test or Mann-Whitney U test depending on data distribution (parametric or nonparametric). p-values < 0.05 were considered statistically significant.

**SUPPLEMENTARY FIGURE LEGENDS**

**Supplementary Figure S1. Analysis of gene expression and RT-qPCR data in strata of class of response: hyperprogressors (HP), progressors (P), stable disease (SD) and reponders (R)**.

(**A**) Heatmap of genes differentially expressed (p<0.01) comparing HP vs P+SD+R. (**B**) Barplot of the Normalized Enrichment Score (NES) for significant pathways (NES≥1.5, p<0.01, FDR<0.15) identified by GeneSet Enrichment Analysis (GSEA) comparing HP vs P+SD+R. (**C**) Histograms of the –Ct value of genes representative of immune subsets measured by RT-qPCR using ACTB as housekeeping gene and the maximum expression value as calibrator (\*p<0.05).

**Supplementary Figure S2**: **Fluorescence in situ hybridization (FISH)**. Amplification of MDM2/4 was assessed in a cohort of 28 FFPE NSCLC tissue by FISH analysis. Figure shows representative pictures of **(A)** MDM2/4 amplified tumors of patients with HP and **(B)** MDM2/4 amplified tumors of patients without HP

**Supplementary Figure S3**. (**A**) Absence of cross-reactivity between Nivolumab and PD-1 epitopes on murine cells. We checked human and-PD-1 expression in PD-1-expressing A20 murine reticulum cell sarcoma B lymphocytes, EL-4 murine lymphoma cells, and F1-PD-1 murine fibroblasts stably expressing PD-1 transgene by FACS. No positivity was detected using Nivolumab (red line), or biotinylated Nivolumab (blue line), whereas 99% of the cells were positive with the mouse anti-PD-1 antibody (green line). (**B**) PD-1 expression on tumor cells in PDXs and xenografts. Low percentage of PD-1+ cells were observed in all tested murine models by FACS analysis. Graph shows results of n02 for PDX111 and xenograft PC9; n=3 for PDX305; n= 4 for H460 xenograft and n=6 for PDX302 FACS analysis performed on naive tumors. (**C**) F4/80 immunostaining on PDXs. Representative images show that epithelioid/monocytoid F4/80+ myeloid cells are detected in PDX302 (HP) but not in PDX305 (NR) and PDX220 (NR). PDX111 (NR but with variable response to Nivolumab treatment) samples were characterized by foci with an elevated number of F4/80+ cells that lacked epitheliod/monocytoid morphology (right-half panel at 20x magnification) alternated with myeloid infiltrate-free areas (left half-panel at 20x magnification). (**D**) FACS analysis performed on primary PDX302 tumors, untreated or Nivolumab treated, for characterization of myeloid (CD11b, Ly6G, Ly6C, F4/80) and NK cells (CD49b). (**E**) Representative images of Arginase staining of PDX302 and PDX 305 samples. Quantification, shown in the graph as average of 5 distinct counts for each slide, confirms an increase in Arg+ in PDX302 model. (**F**) Flow cytometry analysis to test if anti-mouse-PD-1 F(ab)2 is still able to bind mouse PD-1. EL-4 cells, expressing high level of PD-1, were stained with the whole antibody (red line) or with F(ab)2 (blue line) anti-mouse PD-1. Rat IgG2a served as isotype control (black line). Our results show that F(ab)2 fragment can efficiently bind mouse PD-1. (**G**) F(ab)2 Nivolumab antagonizes PD-1 staining by biotinylated Nivolumab on PD-1 expressing T cells and latex beads. Left panel: CD3+ T cells immunosorted from PBMCs of healthy donors were activated with OKT3 (1g/ml) and cultured for 5 days. Cells were stained with biotinylated Nivolumab + PE-streptavidin with or without pre-incubation (30 min at 4°C) with F(ab)2 Nivolumab. Right pane: latex beads (5m diameter, Invitrogen) pre-coated with human recombinant PD-1 (1g/200g beads, R&D System) were stained with biotinylated Nivolumab + PE-streptavidin with or without pre-incubation (30 min at 4°C) with F(ab)2 Nivolumab. Samples were acquired by FACSCalibur and analyzed by Kaluza2.0.

**SUPPLEMENTARY REFERENCES**

1. Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst. 2005;97:643–55.

2. Castagnoli L, Iezzi M, Ghedini GC, Ciravolo V, Marzano G, Lamolinara A, et al. Activated d16HER2 homodimers and SRC kinase mediate optimal efficacy for trastuzumab. Cancer Res. American Association for Cancer Research; 2014;74:6248–59.

3. Oestreich KJ, Yoon H, Ahmed R, Boss JM. NFATc1 regulates PD-1 expression upon T cell activation. J Immunol. 2008;181:4832–9.

4. Moro M, Bertolini G, Tortoreto M, Pastorino U, Sozzi G, Roz L. Patient-derived xenografts of non small cell lung cancer: Resurgence of an old model for investigation of modern concepts of tailored therapy and cancer stem cells. J Biomed Biotechnol. 2012;2012.

5. Moro M, Bertolini G, Caserini R, Borzi C, Boeri M, Fabbri A, et al. Establishment of patient derived xenografts as functional testing of lung cancer aggressiveness. Sci Rep. 2017;

6. Grazia G, Vegetti C, Benigni F, Penna I, Perotti V, Tassi E, et al. Synergistic anti-tumor activity and inhibition of angiogenesis by cotargeting of oncogenic and death receptor pathways in human melanoma. Cell Death Dis. 2014;5:e1434.

7. Wilson NS, Yang A, Yang B, Couto S, Stern H, Gogineni A, et al. Proapoptotic activation of death receptor 5 on tumor endothelial cells disrupts the vasculature and reduces tumor growth. Cancer Cell. 2012;22:80–90.

8. Le Noci V, Tortoreto M, Gulino A, Storti C, Bianchi F, Zaffaroni N, et al. Poly(I:C) and CpG-ODN combined aerosolization to treat lung metastases and counter the immunosuppressive microenvironment. Oncoimmunology. 2015;4:e1040214.

9. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003;4:249–64.

10. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics. 2007;8:118–27.

11. Phipson B, Lee S, Majewski IJ, Alexander WS, Smyth GK. ROBUST HYPERPARAMETER ESTIMATION PROTECTS AGAINST HYPERVARIABLE GENES AND IMPROVES POWER TO DETECT DIFFERENTIAL EXPRESSION. Ann Appl Stat. 2016;10:946–63.

12. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102:15545–50.

13. Bertolini G, D’Amico L, Moro M, Landoni E, Perego P, Miceli R, et al. Microenvironment-Modulated Metastatic CD133+/CXCR4+/EpCAM- Lung Cancer-Initiating Cells Sustain Tumor Dissemination and Correlate with Poor Prognosis. 2015;

14. Liu C, Cripe TP, Kim M-O. Statistical Issues in Longitudinal Data Analysis for Treatment Efficacy Studies in the Biomedical Sciences. Mol Ther. The American Society of Gene & Cell Therapy; 2010;18:1724–30.