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1 **Antibody-Fc/FcR Interaction on Macrophages as a Mechanism for Hyperprogressive Disease**
2 **in Non-Small Cell Lung Cancer Subsequent to PD-1/PD-L1 Blockade**

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73 **ABSTRACT**

74 **Background:** Hyperprogression (HP), a paradoxical boost in tumor growth, was described in a
75 subset of patients treated with immune checkpoint inhibitors (ICI). Neither clinico-pathological
76 features nor biological mechanisms associated with HP have been identified.

77 **Methods:** Among 187 patients with non-small cell lung cancer (NSCLC) treated with ICI at our
78 Institute, cases with HP were identified according to clinical and radiological criteria. Baseline
79 histological samples from patients treated with ICI were evaluated by immunohistochemistry (IHC)
80 for myeloid and lymphoid markers. T-cell deficient mice, injected with human lung cancer cells and
81 patient-derived xenografts (PDXs) belonging to specific mutational subsets, were assessed for
82 tumor growth after treatment with antibodies against mouse and human programmed death
83 receptor-1 (PD-1). The immune microenvironment was evaluated by flow cytometry and IHC.

84 **Results:** Among 187 patients, 152 were evaluable for clinical response. We identified 4 categories:
85 32 cases were defined as Responders (21%), 42 patients with Stable Disease (27.7%), 39 cases
86 defined as Progressors (25.7%) and 39 patients with HP (25.7%). Pre-treatment tissue samples from
87 all patients with HP showed tumor-infiltration by M2-like CD163⁺CD33⁺PD-L1⁺ clustered
88 epithelioid macrophages. Enrichment by tumor-associated macrophages (TAM) was observed, even
89 in tumor nodules from immunodeficient mice injected with human lung cancer cells and with
90 PDXs. In these models, tumor growth was enhanced by treatment with anti-PD-1, but not by anti-
91 PD-1 F(ab)₂-fragments.

92 **Conclusions:** These results suggest a crucial role of TAM reprogramming, upon Fc receptor
93 engagement by ICI, eventually inducing HP and provide clues on a distinctive immunophenotype
94 potentially able to predict HP.

95

96 **STATEMENT OF TRANSLATIONAL RELEVANCE**

97 Hyperprogressive disease in lung cancer and other tumors is an urgent clinical issue reaching 1 out
98 of 5 patients treated with ICI, affecting their prognosis and leading to death in a very short time. As
99 the use of immunotherapy increases in the clinic, it is important to understand the intricacies of this
100 new treatment option in order to optimize treatment approaches. Data already published in the field
101 mainly focused on adaptive immunity without finding any characteristics to predict a priori the
102 phenomenon. Our preclinical findings underline the role of innate immunity in mediating
103 hyperprogression via Fc/FcR triggering on macrophages by anti-PD1 antibody. Accordingly, all
104 patients with HP showed tumor-infiltration by M2-like CD163⁺CD33⁺PD-L1⁺ clustered epithelioid
105 macrophages. These results, pointing to the involvement of innate immune cells in HP, provide new
106 insights into the still unknown mechanisms behind a clinical conundrum.

107

108 **INTRODUCTION**

109 The advent of immune checkpoint inhibitors (ICI) has radically changed the paradigm of care for
110 patients with non-small cell lung cancer (NSCLC). Several agents are now approved in the
111 treatment of NSCLC based on their superiority over chemotherapy (1,2). Immunotherapy with
112 antibodies targeting either the programmed death receptor 1 (PD-1) or its ligand (PD-L1) may
113 provide long-term benefits in approximately 20% of patients (2). However, in a subset of patients,
114 ICI paradoxically accelerates tumor growth, a phenomenon known as hyperprogression (HP) (3–6).
115 Studies have estimated that the prevalence of HP in patients with different cancer histotypes treated
116 with ICI may range between 9% and 29% (3–6). No significant histopathological and molecular
117 features capable of predicting *a priori* HP have been identified, with the partial exception of rare
118 *MDM2* amplification and epidermal growth factor receptor (*EGFR*) mutations (5). These
119 observations have ignited an international debate regarding whether HP is a true phenomenon or
120 only representative of a subset of patients with a particularly worse prognosis.

121 In the tumor microenvironment, the effector functions of innate immune cells may be blunted by the
122 PD-1 receptor, as observed for T lymphocytes, suggesting that these innate cells are another
123 potential target for ICI (7–9). Accordingly, it has been demonstrated that anti-PD-1 antibody can
124 exert antitumor activity in immunodeficient mice via natural killer (NK) cells (8). Conversely, it has
125 been demonstrated that, in PD-1^{-/-} NK cells or in NK cells pre-treated with anti-PD-1 antibody, the
126 production of lytic molecules such as perforins and granzymes is decreased (10). Moreover, tumor-
127 infiltrating dendritic cells (9) and monocytes (11) are reported to release the immunosuppressive
128 cytokine interleukin-10 upon anti-PD-1 treatment. Therefore, it is possible to hypothesize that in
129 some circumstances, PD-1 blockade might exacerbate immunosuppression upon interaction with
130 innate immune cells.

131 The aim of this study was to investigate, at the clinical and pathological level, the phenomenon of
132 HP in NSCLC patients and to evaluate the role of innate immunity during ICI treatment. To

133 eliminate the interference of T lymphocytes we exploited cell lines and patient derived xenografts
134 (PDXs) transplanted in immunodeficient mice.

135

136 **MATERIAL AND METHODS**

137 **Clinical Series**

138 Medical records, radiological findings, and available tumor specimens were collected from patients
139 with NSCLC treated with ICI at the Thoracic Unit of the Istituto Nazionale dei Tumori, Milan,
140 Italy, from July 2013 to December 2017. The study complied with the Declaration of Helsinki and
141 was done in accordance with good clinical practice guidelines. All samples were obtained according
142 to the Internal Review and the Ethics Boards of the Istituto Nazionale Tumori of Milan and all
143 patients provided informed consent. All experimental protocols were approved by the Ethics Boards
144 of the Istituto Nazionale Tumori of Milan (Int 22/15). Radiological evaluation (computed
145 tomography scan with or without brain magnetic resonance imaging) was performed at treatment
146 initiation and every 8 weeks thereafter. Considering that criteria to define patients with HP
147 described by previous authors (3-6) are applicable only in advanced lines, our multidisciplinary
148 team (oncologists, pneumologists, radiologists, and thoracic surgeons) created institutional clinical
149 and radiological criteria designed to identify patients with HP also in first line treatment. Patients
150 with HP or those patients defined as P were classified according to predefined criteria as follows: i)
151 Time-to-treatment failure < 2 months (Time to treatment failure is defined as the time from the start
152 of treatment with ICI to ICI discontinuation for any reason, including progression, patient
153 preference, toxicity or death); ii) Increase of $\geq 50\%$ in the sum of target lesions major diameters
154 between baseline and first radiological evaluation; iii) Appearance of at least two new lesions in an
155 organ already involved between baseline and first radiological evaluation; iv) Spread of the disease
156 to a new organ between baseline and first radiological evaluation; v) Clinical deterioration with
157 decrease in ECOG performance status ≥ 2 during the first 2 months of treatment.
158 Patients who fulfilled at least three of the clinical/radiological criteria were defined as exhibiting
159 HP, while patients with RECIST 1.1 progressive disease as best response without fulfilling at least
160 three criteria were defined as P patients. All R patients and SD patients were classified according to

161 their RECIST 1.1 best response. Only patients who underwent at least two cycles of ICI treatment
162 were included in the present analysis.

163

164 **Immunohistochemistry**

165 Immunohistochemistry was carried out on Formal-fixed Paraffin embedded human or PDX tissue
166 sections as described in Supplementary materials and methods. All the slides were analyzed under a
167 Zeiss Axioscope-A1 equipped with fluorescence module and microphotographs were collected
168 using a Zeiss Axiocam 503 Color with the Zen 2.0 Software (Zeiss, Oberkochen DE). All markers
169 were scored according to the percentage of immunoreactive cells out of the total cellularity.

170

171 **Animals Studies**

172 All xenograft experiments were undertaken using 8- to 9-week-old female athymic nude or SCID
173 mice (Charles River Laboratories, Calco, Italy). Human NSCLC cell line H460 tumor-bearing
174 athymic nude mice were treated i.p. or peri-tumorally (p.t.) with either 200 μ g of monoclonal
175 antibody anti-mouse PD-1 (clone RMP1-14, BioXCell) or saline, and with either p.t. anti-PD-1
176 F(ab)₂ or isotype control. Experiments were carried out in groups of four SCID mice, bearing a
177 PDX sample or a cell suspension (10^5 cells for H460 and PC9 xenograft experiments) in each flank.
178 Mice were treated twice weekly with an i.p. injection of 10 mg/kg Nivolumab (Opdivo, Bristol-
179 Myers Squibb) or Nivolumab F(ab)₂ fragments. Mice were maintained in the Animal Facility of the
180 Fondazione IRCCS Istituto Nazionale dei Tumori. Animal experiments were authorized by the
181 Institutional Animal Welfare Body and the Italian Ministry of Health, and performed in accordance
182 with National law (D.lgs 26/2014) and Guidelines for the Welfare of Animals in Experimental
183 Neoplasia (12). At the end of each experiment, tumors were harvested for subsequent analyses.

184

185 **Statistical Analysis**

186 Distribution of continuous and categorical biomarkers was summarized by the median as a measure
187 of central tendency and absolute frequencies, respectively. The Cochran–Mantel–Haenszel chi-
188 square test was used to detect statistical association (i.e. $P < 0.05$) in univariate analysis. The
189 median and interquartile range (IQR), follow up was estimated using the reverse Kaplan-Meier
190 method.
191

192 **RESULTS**

193 **Clinical and Pathological Evidence in Patients with Advanced NSCLC Treated with ICI**

194 From July 2013 to December 2017, 187 patients with advanced NSCLC received treatment with ICI
195 at the Thoracic Unit of the Medical Oncology Department at the Istituto Nazionale dei Tumori,
196 Milan, Italy, and 152 patients were evaluable for response. We identified 4 categories: Responders
197 (R, 32 cases, 21%), patients with Stable Disease (SD, 42 cases, 27.7%), Progressors (P, 39
198 cases, 25.7%), and patients with HP (39 cases, 25.7%). Patients' characteristics are described in
199 Supplementary Table S1. In this population, after a median follow-up of 32.7 (IQR 15.1-39.6)
200 months, 108 out of 152 patients (71%) died. Median (95% CI) Overall Survival (OS) in the overall
201 population was 11.9 (95% CI 8.8-15.5) months.

202 If we restrict the analysis to patients with HP, median OS significantly decreased to 4.4 (95% CI
203 3.4-5.4) months as compared to 17.7 (95% CI 13.4 -24.1) in non HP patients. Median OS was 8.7
204 (95% CI 5.3-13.4), 17.7 (95% CI 12.7-25.5) and not reached in P, SD and R patients, respectively.

205 Supplementary Table S2 shows the differences between P and HP according to our criteria
206 described in the Materials and Methods section. Of 187 patients, 64 were diagnosed in other centers
207 and could not be included in the present histopathological and molecular analysis. Of the remaining
208 123, 35 patients (11 with HP and 24 without HP) were evaluable for response and had tissue
209 samples suitable for a wide immunohistochemical characterization and gene expression analysis.
210 Patients' characteristics of the extensively analyzed 35 samples resembles the clinical
211 characteristics of the whole treated population

212 Immunohistochemical analysis was performed to assess the presence and distribution of tumor-
213 infiltrating immune elements. The immunophenotype of 11 patients with HP was compared to that
214 of 24 patients without HP (6 P, 11 SD and 7 R). No significant differences were observed among all
215 the clinical classes of patient with respect to the subsets of tumor-infiltrating T lymphocytes (TILs),
216 evaluated by the density of CD3⁺, CD4⁺, and CD8⁺ lymphocytes and FOXP3⁺ regulatory T cells
217 (Tregs). In addition, no differences were detected between classes of patient in the numbers of

218 CD138⁺ plasma cells (PCs), CD123⁺ plasmacytoid dendritic cells (pDCs), peritumoral and stromal
219 myeloperoxidase (MPO)⁺ myeloid cells, CD163⁺ macrophages, CD33⁺, PD-1 and PD-L1⁺ immune
220 cells. However, MPO⁺ myeloid cells within the tumor were directly correlated ($P= 0.0497$) and PD-
221 L1 expression in tumor cells was inversely correlated ($P= 0.0457$) with HP. Furthermore, a
222 statistical trend was shown for the M2 macrophage/myeloid derived suppressor cells marker,
223 Arginase-A I (ArgI) on peritumoral immune cells ($P =0.0666$) (Supplementary Table S3).

224 Gene expression profile (GEP) analysis of pre-treatment tumors did not show any relevant features
225 except for under-expression of pathways related to proliferative activity and cell metabolism in
226 patients experiencing HP after ICI (Supplementary Figures S1A and S1B). The analyses of selected
227 genes representative of immune subsets by RT-qPCR revealed overexpression of the *CD274* gene,
228 encoding for PD-L1, as the only significant marker in R patients (Supplementary Figure S1C).

229 Fluorescence in situ hybridization of *MDM2* and *MDM4* genes, carried out on in a cohort of 30
230 FFPE NSCLC tissue derived from 11 patients with HP and 17 patients without HP, revealed the
231 presence of 3 amplified tumors (2 *MDM2*, 1 *MDM4*) in patients with HP and 6 amplified tumors (4
232 *MDM2*, 1 *MDM4* and 1 *MDM2* and *MDM4*) in patients without HP (Supplementary Figure S2).

233 Notably, we noticed that, in some cases, CD163⁺ tumor-infiltrating macrophages showed
234 epithelioid morphology (alveolar macrophage-like) with the tendency to form dense clusters within
235 neoplastic foci (Figure 1A). In these cases, the same cells were found to co-express CD33 and PD-
236 L1 (Figure 1B and Figure 2). Such a peculiar morphology, aggregation, and immunophenotype
237 (CD163⁺CD33⁺PD-L1⁺) of macrophages, which we define “complete immunophenotype”, was
238 observed in all patients with HP and found to be statistically significant versus patients without HP
239 ($P<0.0001$) (Supplementary Tables S4 and S5). This complete immunophenotype was also
240 observed in one P patient, two patients with SD, and one R patient (Supplementary Table S5). All
241 other cases experiencing treatment response with stable or slowly progressive disease either lacked
242 the presence of epithelioid macrophages or showed loose clustering, or lacked some of the above
243 markers (mainly CD33⁻ and/or PD-L1) (Figure 1C and 1D).

244 **Anti Mouse PD-1 Antibody Induces Tumor Progression in Athymic Mice**

245 Histopathological analyses showed the presence of clustered CD163⁺CD33⁺PD-L1⁺ epithelioid
246 macrophages as a distinctive trait in tumors with HP. Therefore, we sought to test whether
247 macrophages are involved in the detrimental effects associated with anti-PD-1 therapy in preclinical
248 models. Athymic nude mice implanted with human H460 NSCLC cell line were treated either
249 intraperitoneally (i.p.) (Figure 3A) or peritumorally (p.t.) (Figure 3B) with anti-PD-1 antibody
250 (clone RMP1-14) or saline. Anti-PD-1 treatment increased tumor growth compared with the control
251 group, regardless of route and schedule of treatment (Figure 3A and 3B). Anti-PD-1 treatment was
252 also associated with a significant increase in CD45⁺ leukocyte infiltration at the host-tumor
253 interface, evaluated by immunohistochemistry (IHC) (Figure 3C). Such an increase was mainly due
254 to increasing numbers of intratumoral macrophages (F4/80⁺ cells) and Arginase-I⁺-expressing cells,
255 whereas the density of B lymphocytes (CD45R/B220⁺), granulocytes (Gr-1⁺) and NK (NKp46⁺)
256 cells was comparable to the control group (Figure 3C). Of note, Arginase-I was also consistently
257 expressed by the complete immunophenotype intra-tumor macrophages characterizing patients with
258 HP (Figure 1E). Tumor-associated macrophages (TAMs) can express PD-1 and the blocking of this
259 receptor restores antitumor functions (7). Thus, the detrimental boost in tumor growth may not be
260 ascribed to such receptor blockade, but rather to the Fc domain of the antibody which is reported to
261 modulate anti-PD-1 antibody functional activity (13). Accordingly, the same experiments were
262 performed using anti-PD-1 F(ab)₂ fragments. The lack of the Fc portion abrogated the increase in
263 tumor growth observed with the whole antibody (Figure 3D).

264 **Anti Human PD-1 Antibody (Nivolumab) Induces Tumor Progression of PDXs in SCID Mice**

265 To exclude a direct involvement of PD-1 expression in immune cells, we treated severe combined
266 immunodeficient (SCID) mice with anti-human PD-1 (Nivolumab) which does not cross-react with
267 the murine counterpart (Supplementary Figure S3A). Since a link between HP and *EGFR*
268 mutational status has been proposed by Kato et al. (5), we compared two NSCLC PDXs, with and
269 without *EGFR* mutation: PDX302 (P53^{C135Y}, EGFR^{L858R}, KRAS^{WT}, APC^{WT}) versus PDX305

270 (P53^{WT}, EGFR^{WT}, KRAS^{G12C}, APC^{R1114L}). On PDXs, before treatment, fluorescence-activated cell
271 sorting (FACS) analysis with human anti-PD1 antibody showed expression of PD-1 receptor on a
272 subset of tumor cells (around 1% PD-1⁺ cells, Supplementary Figure S3B). In addition, IHC
273 analysis showed that F4/80⁺ epithelioid/monocytoid elements, aggregated in clusters resembling
274 those identified in patients with HP, were appreciable only in PDX302 (Supplementary Figure
275 S3C).

276 SCID mice carrying subcutaneous PDX302 ($n = 8$), but not PDX305 ($n = 8$), showed a significant
277 increase in tumor growth rate compared with controls following twice weekly treatment with
278 Nivolumab (Figure 4A). FACS analysis in lungs of PDX302 bearers showed increased cancer cell
279 dissemination in Nivolumab-treated mice but not in controls ($3.88 \pm 1.99\%$ vs. $0.87 \pm 0.33\%$, $P =$
280 0.0286 , respectively) (Figure 4B), whereas no differences were detected in PDX305 bearers (data
281 not shown).

282 FACS analysis was also performed on primary tumors for characterization of different myeloid
283 subsets (CD11b, Ly6G, Ly6C, F4/80) and NK cells (CD49b). In PDX302-bearing mice, an increase
284 in CD11b⁺F4/80^{high} macrophages was observed in the Nivolumab-treated group versus controls
285 (Supplementary Figure S3D), whereas no significant changes occurred in other immune
286 subpopulations. Accordingly, IHC analysis performed on the same tumors revealed accumulation of
287 macrophages and Arg1⁺-expressing cells (Figure 4C and Supplementary Figure S3E). Notably, in
288 PDX302 the F4/80⁺ epithelioid/monocytoid clusters were enriched in Nivolumab-treated tumors
289 (Figure 4C).

290 Overall, these data indicate that, in the *EGFR*-mutated PDX302-bearing mice, Nivolumab triggers a
291 detrimental effect characterized by increased tumor growth, lung dissemination and the accrual of
292 macrophages, most likely M2.

293 To further confirm the detrimental effect induced by Nivolumab in other preclinical models,
294 PDX111 (P53^{C242X}, KRAS^{G12V}, CDKN2A^{E69X}, CTNNB1^{T41I}), PDX220 (wild type for all tested
295 genes), H460 (KRAS^{Q61H}, STK11^{Q37*}), and PC9 (EGFR^{L858R}, EGFR^{E746_A750del}, CDKN2A^{G67V}) were

296 xenografted in SCID mice. All tested models showed low levels of PD1-expression on tumor cells
297 (ranging from 0.6 to 4%, Supplementary Figure S3B). As with PDX302, an increase in tumor
298 growth was observed in H460- and PC9-bearing mice after Nivolumab treatment. PDX111 tumors
299 showed a variable response, whereas tumors in PDX220- and PDX305-bearing mice showed no
300 response to Nivolumab (Figure 4D).

301 To reinforce the role of the Fc domain of the antibody in boosting tumor growth, PDX302-bearing
302 SCID mice were treated with Nivolumab-F(ab)₂ fragments. No HP-like growth was observed
303 (Figure 4E) whereas in the same experiment mice treated with the entire antibody showed HP-like
304 growth, dissemination to lung (Figure 4F) and regional (iliac) lymph node metastases (Figure 3G).
305 In the group pretreated with clodronate, which reduces F4/80⁺ macrophages, impaired Nivolumab-
306 induced tumor growth was observed (Figure 4E). The F4/80⁺ macrophages also stained for CD206
307 that marks M2-like subsets and aggregated in fibrotic-like areas in Nivolumab- but not in
308 Nivolumab F(ab)₂-treated tumors. In the latter less fibrotic areas than in control mice were observed
309 (Figure 4H).

310 These results suggest that HP is sustained by Nivolumab interaction with M2-like macrophages,
311 most likely via Fc-Fc γ receptor binding (Figure 5).

312

313 **DISCUSSION**

314 Although ICI have changed the paradigm of care for patients with NSCLC, an in-depth examination
315 of the Kaplan-Meier curves from the CheckMate-026 (14), CheckMate-057 (15), CheckMate-227
316 (16), and KEYNOTE-042 (17) trials showed an excess of disease progression and death in the
317 immunotherapy treatment arms compared with chemotherapy in the first 3 months of treatment.
318 This was also underscored by the European Medicines Agency in response to the appraisal for the
319 second-line use of Nivolumab in non-squamous histologies
320 ([http://www.ema.europa.eu/docs/en_GB/document_library/EPAR -
321 _Product_Information/human/003985/WC500189765.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/003985/WC500189765.pdf)). Furthermore, the benefit of ICI in trials
322 conducted in never smokers and in patients with *EGFR* mutation-positive or Anaplastic Lymphoma
323 Kinase (*ALK*)-mutation-positive NSCLC seems unclear (1,2,5,14,15). The four most important
324 papers on the HP topic showed prevalence rates ranging from 9% to 29% throughout tumor types,
325 including NSCLC (3–6). In all these papers, the ratio of the tumor growth rates before and during
326 ICI treatment was used to identify HP, although with slightly different cut-offs. None of these
327 studies defined pathological features able to predict HP, although *MDM2/4* amplification and *EGFR*
328 alterations were proposed to be associated (5). However, we did not find any significant difference
329 in the frequency of *MDM2/4* amplification between patients with and without HP, and the role of
330 *EGFR* cannot be discussed due to the low number of *EGFR*-mutated patients included in our case
331 series (Supplementary Table S1)

332 Our definition of HP is different from that used in the above-mentioned studies, where radiological
333 imaging before, at the start and after ICI is needed to identify HP. However, in clinical practice all
334 these radiological evaluations are often unavailable, and as a consequence, the criteria used in
335 literature are unable to classify patients with HP considering that ICI are starting to be widely used
336 as first line therapy. Furthermore, both Response Evaluation Criteria In Solid Tumors (RECIST) 1.1
337 and Immune-related Response Evaluation Criteria In Solid Tumors (irRECIST) criteria, used in the

338 reported analyses, considered only changes in tumor size and did not take into account non-target
339 lesions, such as lymphangitis and pathological lesions under 10 mm. In addition, functional and
340 clinical aspects, such as deterioration in performance status, were not considered. Therefore, we
341 decided to include both clinical and radiological criteria to identify patients with HP in our series.
342 These proposed criteria might overestimate the real fraction of patients experiencing HP; for these
343 reasons, a clinical trial is ongoing within our Institute to properly validate the criteria and thereby
344 obtain the true rate of HP as well as the distinctive immunophenotype.

345 Nivolumab-treated cell lines and PDX-bearing SCID mice mirrored the clinical observation of HP
346 following treatment with ICI. Interestingly, patients and mice classified with HP share a similar
347 tumor immunophenotype. Indeed, the population of F4/80⁺CD206⁺Arginase-A1⁺ cells emerging
348 from PDXs with HP matches macrophage features of the human counterpart (Figures 1A, 4C, 4H
349 and Supplementary Figure S3C). M2-like macrophages were preferentially associated with fibrotic
350 foci in PDXs that experience HP-like tumor growth after PD-1 blockade. The recruitment of these
351 myeloid cells may promote a peculiar cancer-associated innate response that may affect tumor
352 growth. Indeed, Knipper et al. described a cross-talk between myeloid cells and fibroblasts
353 promoting skin fibrosis that could provide proliferative and pro-survival signals in cancer cells (18).
354 Prominent mitotic figures can be consistently identified in Nivolumab-treated tumor foci embedded
355 in a fibrotic stroma. In human samples, the accumulation of these cells is apparently unrelated to the
356 extent and distribution of tumor-infiltrating T-cell populations.

357 The role of the innate immune system in mediating the effects of ICI is now clearly emerging. Cells
358 of myeloid origin present in the tumor microenvironment decrease the effects of ICI via PD-L1
359 expression (19), by “stealing” anti-PD-1 antibody from the membrane of T lymphocytes that return
360 to anergy (20) or by secreting immunosuppressive molecules (21). Our study provides novel
361 evidence of negative immuno-regulatory role exerted by PD-L1⁺ macrophages enriched at tumor
362 site under treatment with ICI. Pre-treatment lesions from all patients classified as HP showed tumor
363 infiltration by clustered epithelioid macrophages characterized by a CD163⁺CD33⁺PD-L1⁺ profile.

364 Interestingly, CD163⁺ PD-L1⁺ macrophages represent a common immune landscape for different
365 tumors. PD-L1⁺ macrophages have been recently described to accumulate in tight clusters at the
366 tumor invasive margin in NSCLC (22). Macrophages expressing both PD-L1 and the “M2” marker
367 CD163 have been described in MSI-colorectal cancer (23), triple-negative breast cancer (24),
368 gastric and cervical cancer (25,26). In some of these studies, the presence of PD-L1⁺ macrophages
369 has been associated with poor prognosis (24,26) and/or with immunosuppressive function through
370 IL-10 production (27). In addition, the concurrent expression of CD163, CD33, and PD-L1 has been
371 recently described in alveolar macrophages from acute respiratory distress syndrome patients, a non
372 oncological condition present in 10% of subjects admitted to intensive care units (28). In this
373 regard, we observed an increased infiltration of M2 macrophages after anti-PD-1 administration,
374 providing evidence for their involvement in determining HP. TAMs can also express PD-1 that, if
375 neutralized, can restore M1-like properties (7). This likely excludes blockade of PD-1 signaling in
376 our models that remain rather oriented to M2. Therefore, we examined the possibility of FcR
377 engagement as a modulator of anti-PD-1 activities (13). Upon testing the F(ab)₂ moiety in
378 comparison to whole Ab, we have shown that Nivolumab without the Fc domain no longer induces
379 HP-like disease in our models.

380 The anti-mouse PD-1 clone RMP1-14, utilized for the treatment of tumor-bearing athymic mice, is
381 a rat immunoglobulin IgG2a reported to interact with the mouse inhibitory receptor, FcγRIIb (13).
382 FcγRIIb has been shown to be involved in dampening the immune response, and impairments in
383 FcγRIIb function are associated with an exacerbation of inflammatory processes (29). The anti-
384 human PD-1 antibody Nivolumab is an IgG4 isotype with reduced binding affinity to activating
385 FcγRs, thereby avoiding antibody-dependent cell-mediated cytotoxicity on PD-1⁺ immune cells
386 (30). However, Nivolumab maintains the ability to bind to the inhibitory FcγRIIb receptor (13).
387 Therefore, we suggest a possible role of FcγRIIb in the detrimental effect associated with anti-PD-1
388 therapy. However, since both human IgG4 and rat IgG2a can bind at lower affinity to other FcRs,
389 the involvement of these receptors cannot be excluded. Further studies elucidating the involvement

390 of FcRs in the development of HP are required. Very recently, a report described PD-1 expression
391 in one NSCLC case with HP and in one mouse NSCLC cell line. The latter treated with anti murine
392 PD-1 Ab underwent accelerated tumor growth (31). This interesting and logical explanation of HP
393 can't be totally supported by the low expression/prevalence of PD-1 on tumors and by F(ab)₂
394 experiments in mice. We found clusters of PD-1 expressing cells in 2 out of 11 patients with HP
395 (data not shown) and in all xenografts and PDXs, although at low levels (0.6 - 4%) and with no
396 correlation with HP-like progression.

397 In conclusion, the ongoing debate regarding whether HP is a true phenomenon or only
398 representative of patients with a particularly worse prognosis is confirmed in our preclinical models,
399 where ICI are able to boost tumor growth in a manner akin to the clinical observations of HP in
400 patients with NSCLC. Our results suggest that FcR triggering of clustered epithelioid macrophages
401 with a specific immunophenotype by ICI delivers a signaling cascade that promotes functional
402 reprogramming of these cells toward a more aggressive pro-tumorigenic behavior. This eventually
403 induces HP in a subset of patients with distinctive immune and genetic profiles (depicted in Figure
404 **5**). Our analyses, for the first time, suggest a role of innate immunity in this process. A further
405 prospective validation of the HP immunophenotype and its relationship with specific genotypes, as
406 well as the new proposed clinical criteria to classify HP, is ongoing.

407

408 **Authors' Contributions**

409 Conception and design: GS, MCG, ABardelli, SM, LR, AA, ABalsari

410 Development of methodology: VC, GC, SF, PG, CS

411 Acquisition of data: CT, MMilione, MB, MS, MMoro, GL, PG, MG, VH, CP, DS, ET, SS

412 Analysis and interpretation of data: CT, MMoro, MS, GL, GP, LP, VT, CP, DS, LS, SS

413 Writing, review and/or revision of the manuscript: ABardelli, SM, MS, MMoro, MG, CP, DS, GL,

414 MPC, CT, GA, AA, ABalsari, LR, GS, MCG

415 Study supervision: MPC, GS, MCG, GA

416

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427

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524
525
526

527 **FIGURE LEGENDS**

528 **Figure 1. Immunohistochemical analyses for CD163, PD-L1 and CD33 in representative**
529 **hyperprogressor and non-hyperprogressor cases.**

530 (A) Representative microphotographs detailing the presence of macrophages displaying epithelioid
531 morphology and expression of CD163, PD-L1, and CD33 markers (defined as *complete phenotype*)
532 in four hyperprogressor cases. (B) Double immunofluorescence staining for CD163 (green) and PD-
533 L1 (red) showing the co-expression of the two markers in epithelioid macrophages (arrows). (C)
534 Representative microphotographs detailing the presence of macrophages displaying epithelioid
535 morphology, variable clustering and the incomplete expression of the three CD163, PD-L1, and
536 CD33 markers (defining the complete phenotype of HP patients) in two cases of non-HP patients
537 (stable disease). (D) Representative microphotographs detailing the presence of myeloid elements
538 with non-epithelioid morphology (stellate or spindle-shaped cells) on CD163, PD-L1, and CD33
539 markers populating tumor infiltrates of non-HP patients (one stable disease and one response). (E)
540 Representative microphotographs relative to Arginase-A1 expression by clustered epithelioid
541 macrophages in four HP patients' infiltrates. Magnification 20x.

542 **Figure 2. CD33, CD163, and PD-L1 co-localization in clustered macrophages with epithelioid**
543 **morphology**

544 Immunofluorescence panels from prototypical hyperprogressive disease infiltrates showing the co-
545 localization of CD33, CD163, and PD-L1 in clustered macrophages with epithelioid morphology.
546 Three different combinations of double-marker stainings are shown. Green signal and red signal
547 correspond to Opal-520 and Opal-620 fluorophores, respectively. Original magnifications x100 and
548 x400.

549 **Figure 3. Anti Mouse PD-1 Antibody Induces Tumor Progression in Athymic Mice**

550 Athymic nude mice were xenografted with H460 lung cancer cell lines and treated i.p. (n=6
551 mice/group) (A) or p.t. (n=5 mice/group) (B) with 200 μ g of anti-mouse PD-1 blocking antibody
552 (red dots) or with vehicle (black dots). Red arrows indicate the days of anti-PD-1 antibody

553 treatment. Dots represent Mean \pm SEM of tumor volume for each group. **P <0.01 by Mixed
554 Models ANOVA. (C) Representative immunohistochemistry images and quantification of
555 leukocytes (CD45⁺), macrophages (F4/80⁺), Arginase-I⁺, B lymphocytes (CD45R/B220⁺),
556 granulocytes (Gr-1⁺) and NK (NKp46⁺) cells in the tumor microenvironment of H460 lung cancer
557 xenografts collected from the study illustrated in Figure 2B (p.t. experiment, n=5 mice/group).
558 Original magnification x20. *P <0.05, **P <0.01 by Mann-Whitney U test. (D) Athymic nude mice
559 were xenografted with H460 lung cancer cell lines and treated i.p. with 200 μ g anti-PD-1 F(ab)₂
560 (blue dots) or with vehicle (black dots) (n=6 mice/group). Blue arrows indicate the days of anti-PD-
561 1 antibody treatment. Dots represent Mean \pm SEM of tumor volume for each group.

562 **Figure 4. Anti Human PD-1 Antibody (Nivolumab) Induces Tumor Progression of PDXs in**
563 **SCID Mice**

564 (A) PDX302 (P53C135Y, EGFR^{L858R}, KRAS^{WT}, APC^{WT}) and PDX305 (P53^{WT}, EGFR^{WT},
565 KRAS^{G13C}, APC^{R1114L}) samples were injected in both flanks of SCID mice (n = 4). Mice were
566 treated with 10 mg/kg i.p. Nivolumab (red dots) twice weekly from day 1 after tumor implant to the
567 end of the experiment. **P < 0.01 by mixed models ANOVA. (B) Analysis of tumor cells
568 disseminated to mice lungs. Mice lungs were analyzed by FACS, after tissue dissociation to single
569 cells, for the presence of human disseminated cells. Graphs indicate the percentage of human cells
570 in Nivolumab treated and control mouse lungs. *P < 0.05 (C) Representative images showing
571 F4/80⁺ cells with epithelioid/monocytoid elements aggregated in clusters in an untreated PDX302
572 model. (D) Dot plot summarizing the results of the effects of Nivolumab treatment in all tested
573 PDX (PDX302, PDX305, PDX111, and PDX220) and xenograft models (H460 and PC9).
574 Response rate was estimated as described in the Materials and Methods section. (E) PDX302
575 (P53C135Y, EGFR^{L858R}, KRAS^{WT}, APC^{WT}) samples were injected in both flanks of SCID
576 mice (n = 4). Mice were treated with 10 mg/kg i.p. Nivolumab, Nivolumab F(ab)₂ or clodronate
577 plus Nivolumab twice weekly (once weekly for clodronate injection) from day 1 after tumor
578 implant to the end of the experiment. *P < 0.05. (F) Analysis of tumor cells disseminated to mice

579 lungs. Mice lungs were analyzed by FACS, after tissue dissociation to single cells, for the presence
580 of human disseminated cells. Graphs indicates the percentage of human cells in Nivolumab-,
581 Nivolumab F(ab)₂- or Clodronate plus Nivolumab treated and control mouse lungs. *P < 0.05
582 compared to control; #P < 0.05 compared to Nivolumab-treated (G) Representative IHC images of
583 mouse iliac lymph nodes show tumor cell dissemination with the presence of a metastatic nodule
584 (indicated by the asterisk) only in Nivolumab-treated mice (H/E: hematoxylin/eosin staining; CK:
585 pan-cytokeratin staining). (H) H&E and Masson's trichrome staining showing the presence of
586 fibrotic areas with consistent matrix deposition in Untreated, Nivolumab- and Nivolumab F(ab)₂-
587 treated representative cases. IHC analysis for CD206⁺ macrophages highlighting the enrichment in
588 macrophages in Nivolumab treated tumor.

589
590 **Figure 5. Hypothesized mechanism through which macrophages and ICI are involved in**
591 **determining HP**

592

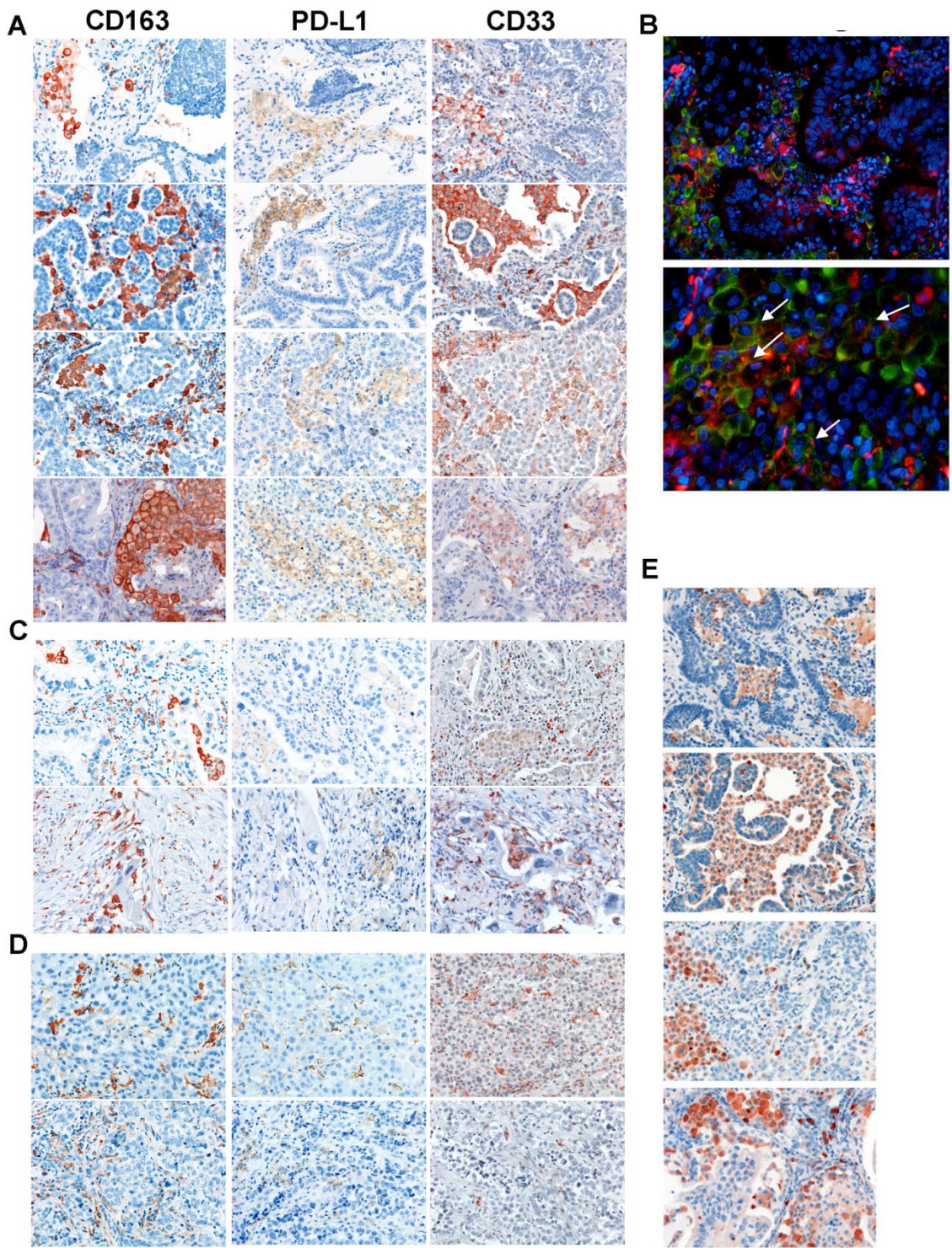


Figure1

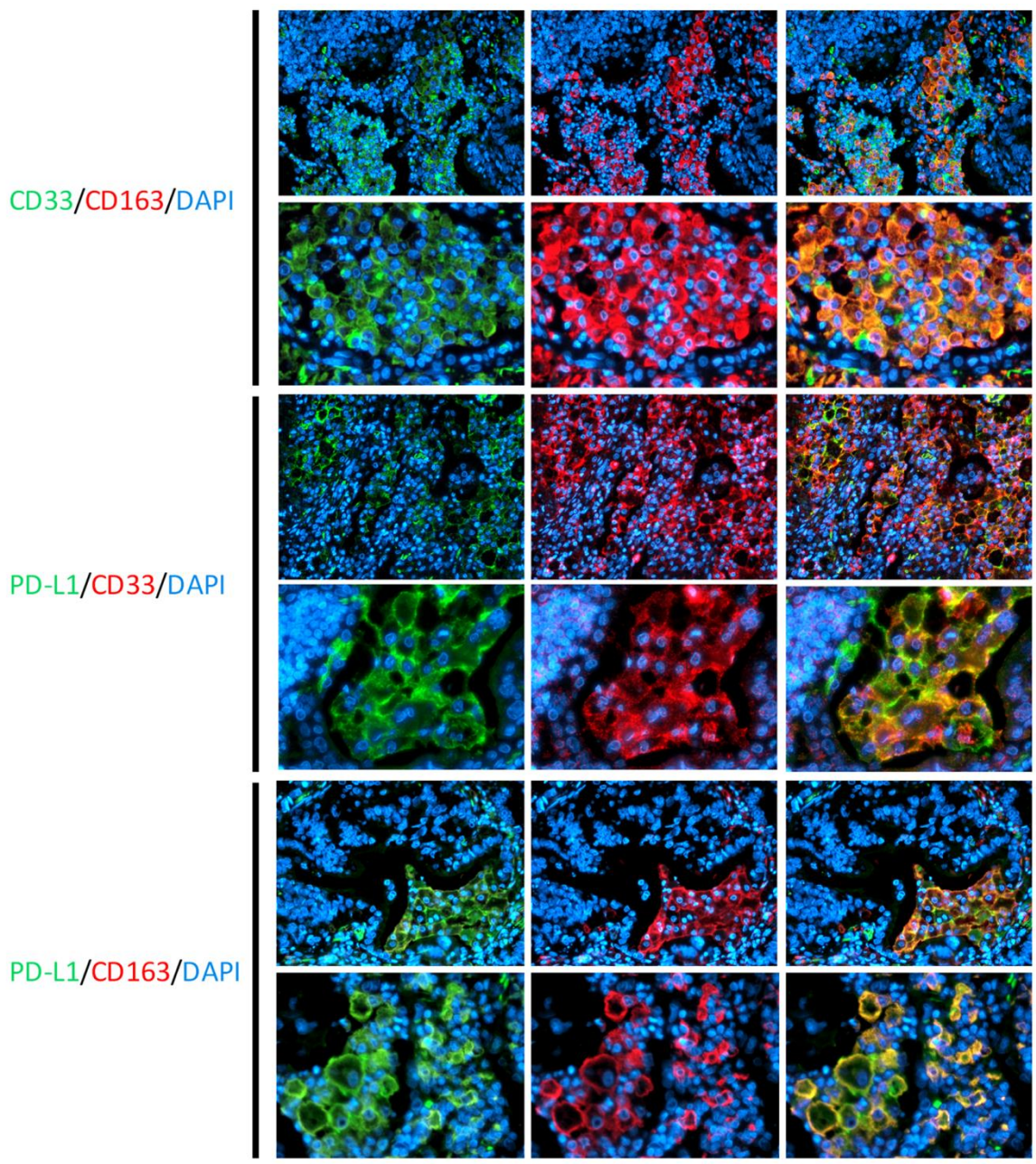


Figure2

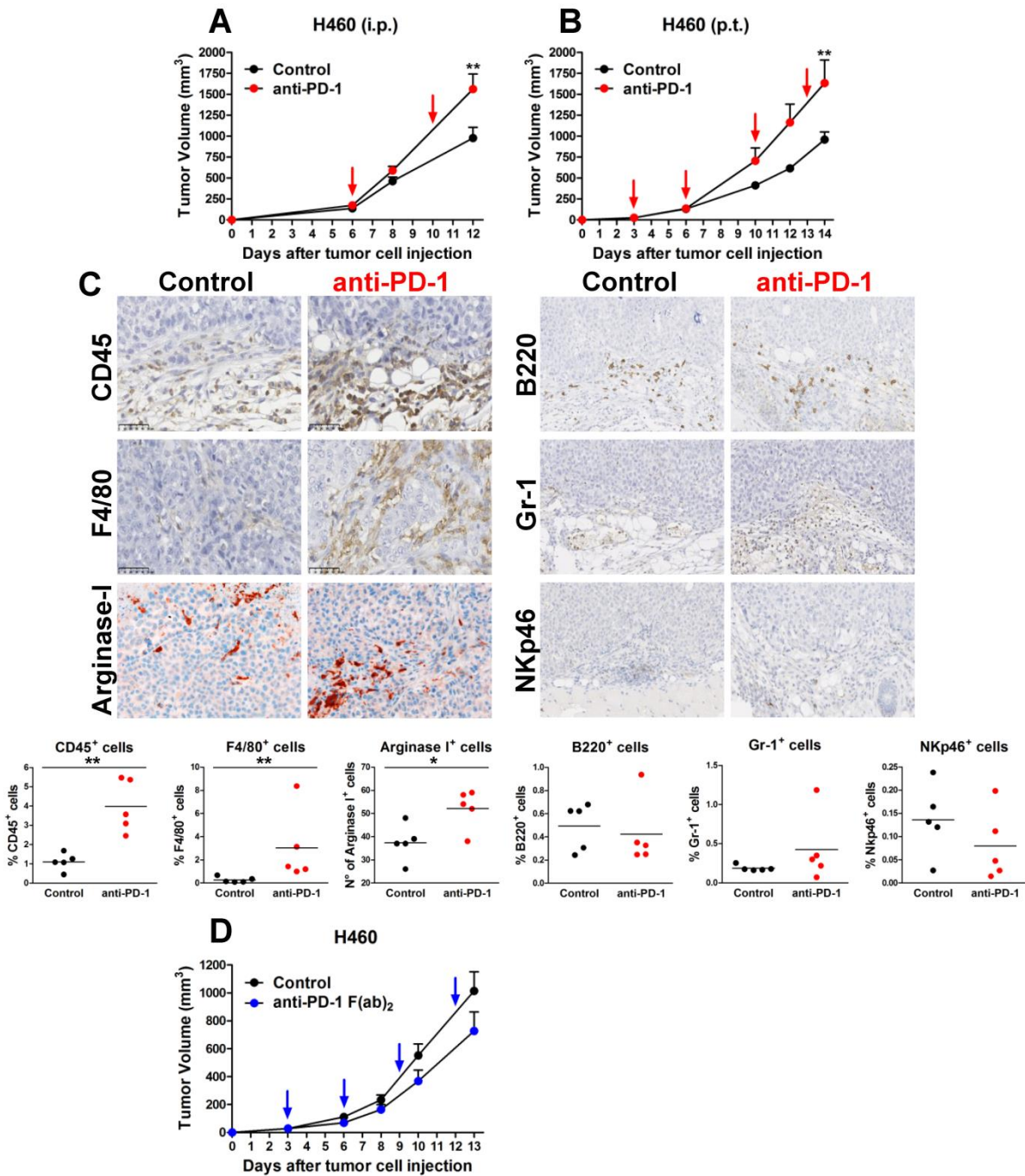


Figure3

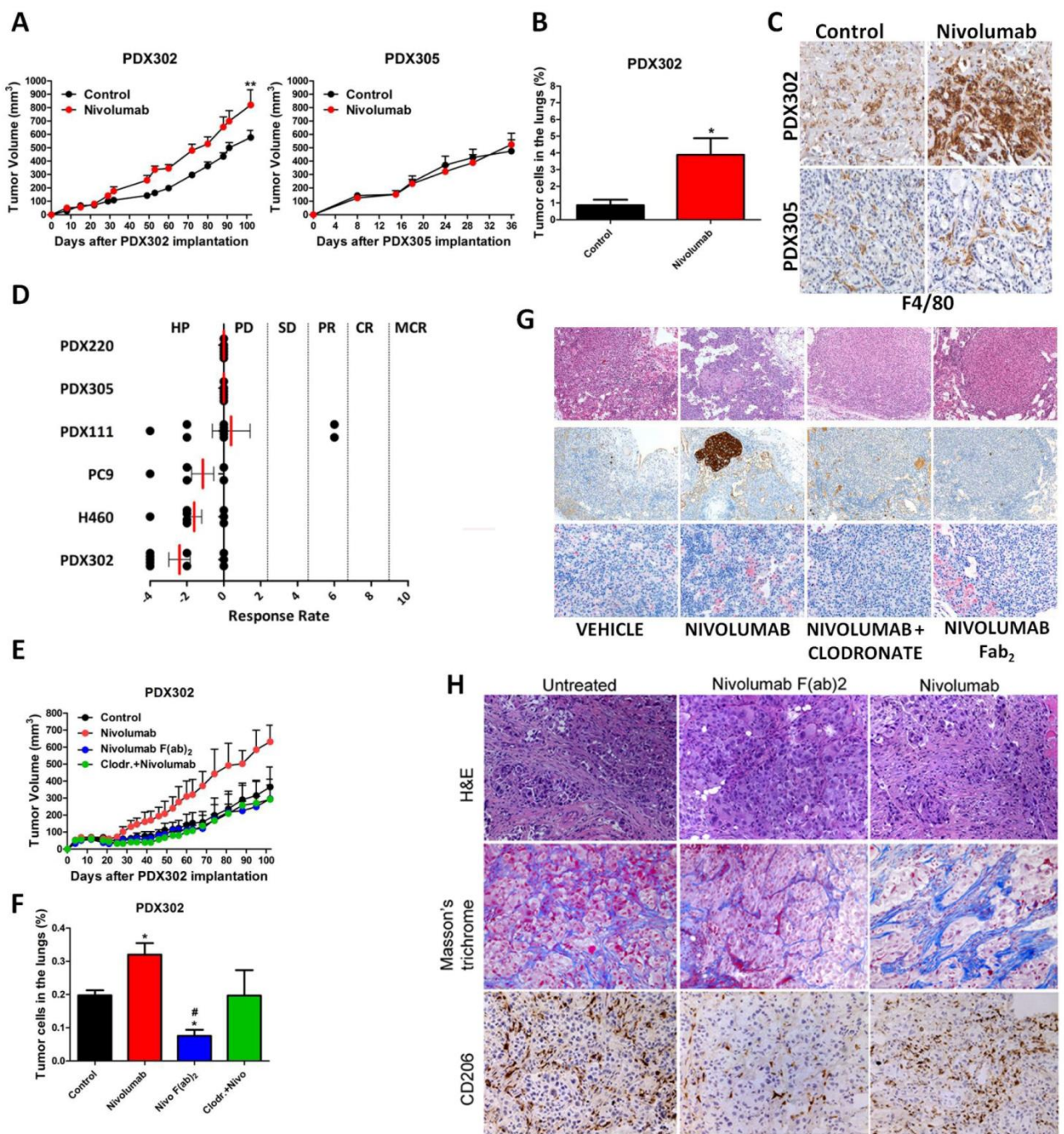


Figure 4

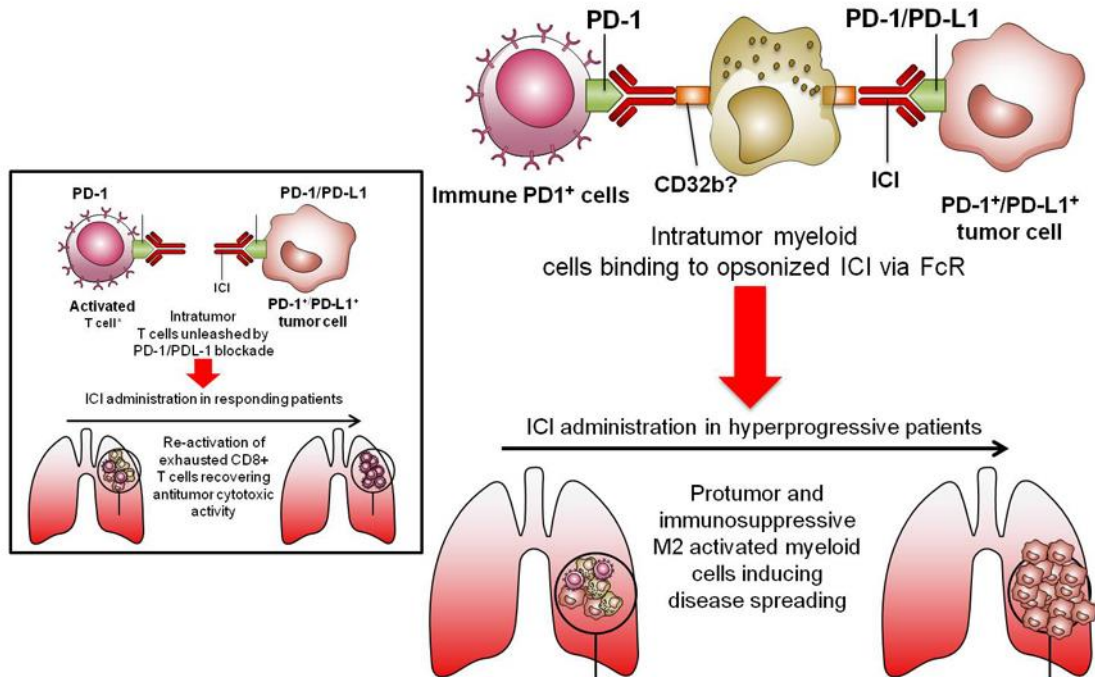


Figure 5

Clinical Cancer Research

Antibody-Fc/FcR Interaction on Macrophages as a Mechanism for Hyperprogressive Disease in Non-Small Cell Lung Cancer Subsequent to PD-1/PD-L1 Blockade

Giuseppe Lo Russo, Massimo Moro, Michele Sommariva, et al.

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