1   **Title**: Senescence rewires microenvironment sensing to facilitate anti-tumor immunity

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3   **Running title**: Senescence enhances tumor cell sensitivity to host IFN-γ

4

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38 **ABSTRACT**

39 Cellular senescence involves a stable cell cycle arrest coupled to a secretory program that, in

40 some instances, stimulates the immune clearance of senescent cells. Using an immune

41 competent liver cancer model in which senescence triggers CD8 T cell-mediated tumor rejection,

42 we show that senescence also remodels the cell surface proteome to alter how tumor cells

43 sense environmental factors, as exemplified by Type II interferon (IFN-γ). Compared to

44 proliferating cells, senescent cells upregulate the IFN-γ receptor, become hypersensitized to

45 microenvironmental IFN-γ, and more robustly induce the antigen presenting machinery—effects

46 also recapitulated in human tumor cells undergoing therapy-induced senescence. Disruption of

47 IFN-γ sensing in senescent cells blunts their immune-mediated clearance without disabling the

48 senescence state or its characteristic secretory program. Our results demonstrate that

49 senescent cells have an enhanced ability to both send and receive environmental signals, and

50 imply that each process is required for their effective immune surveillance.

51

52 **SIGNIFICANCE**

53 Our work uncovers an interplay between tissue remodeling and tissue sensing programs can be

54 engaged by senescence in advanced cancers to render tumor cells more visible to the adaptive

55 immune system. This new facet of senescence establishes reciprocal heterotypic signaling

56 interactions that can be induced therapeutically to enhance anti-tumor immunity.

57

58

INTRODUCTION

Cellular senescence is a stress response program characterized by a stable cell cycle arrest and a secretory program capable of remodeling the tissue environment (1). In normal tissues, senescence contributes to tissue homeostasis during wound healing; however, in aged or damaged tissues, the aberrant accumulation of senescent cells can cause chronic inflammation and reduced tissue regenerative capacity (2-4). In cancer, senescence has been shown to mediate both beneficial and detrimental effects on tissue biology. On one hand, senescence provides a barrier to oncogene-initiated tumorigenesis and contributes to the anti-tumor activity of some cancer therapies (5,6). On the other, the persistence of senescent tumor cells post therapy can produce a tissue environment that promotes relapse and metastasis (7,8). The molecular underpinnings of these opposing biological outputs remain poorly understood.

One facet of the senescence program that is likely to contribute to such diverse biology is the senescence-associated secretory phenotype [SASP; ref. (9)]. SASP is activated through a global chromatin remodeling process that evolves over time and is controlled by key epigenetic regulators such as BRD4 and pro-inflammatory transcription factors such as NF-κB and C/EBP-β (10-12). This, in turn, leads to the induction of genes that encode tissue remodeling proteins such as matrix metalloproteinases, growth factors, and fibrolytic factors known to play crucial roles in wound healing process (3,13,14). Other SASP components include chemokines and cytokines that can alter the composition and state of immune cells within the tissue, leading to the immune-mediated targeting and clearance of the senescent cells themselves (15,16). Nonetheless, the aberrant accumulation of senescent cells in many pathological contexts implies that immune mediated clearance is not a universal outcome of senescence or the SASP and raises the possibility that additional mechanisms dictate the paradoxically beneficial and detrimental effects of senescence in tissue biology and immune surveillance (17-19).

3

83   Certainly, senescence-associated immune surveillance can have potent anti-cancer

84   effects, though the precise effector mechanisms vary with tissue and cell type (10,15,16,20). In

85   mouse models of hepatocellular carcinoma (HCC), liver tumor cells triggered to senesce are

86   eliminated by immune-dependent mechanisms engaged by wild-type p53 (15). In agreement,

87   *TP53* is frequently mutated in human HCC, particularly in the "Proliferation class" tumors

88   showing the worst prognosis (21,22). Though immunotherapy and TP53-targeting drugs are

89   emerging as promising strategies to improve disease outcomes, the molecular basis for

90   response and resistance remains unknown (23-25). Therefore, understanding the mechanisms

91   by which senescent liver tumor cells become visible to the immune system may facilitate

92   strategies to elicit anti-tumor immunity in *TP53*-mutated HCC that may extend to other tumor

93   types.

94   Here, we set out to establish principles that modulate the immune recognition and

95   clearance of senescent cells to identify actionable senescence mechanisms that may be

96   exploited to improve the immune control of cancer. To this end, we developed a novel

97   'senescence-inducible' model in which liver cancer cells can be selectively switched to a

98   senescent state through genetic modulation of endogenous p53. We reasoned this would mimic

99   the effects of therapies that trigger senescence (26,27) while avoiding the confounding effects of

100   senescence-inducing therapies on immune cells or other components of the tissue environment.

101   Using this model and then extending to other systems, we reveal that, in addition to the SASP,

102   senescence drives a major remodeling of the cell surface proteome and signaling programs in a

103   manner predicted to fundamentally alter the way cells sense and respond to environmental

104   signals, exemplified herein through a hypersensitivity to microenvironmental type II IFN (IFN-γ).

105   This process enables a more robust upregulation of the antigen processing and presenting

106   machinery in senescent tumor cells that renders them susceptible to immune surveillance *in*

107   *vivo*. Thus, our results reveal a rewired tissue-sensing program in senescent cells that acts in

108 concert with SASP to boost their immunogenic potential, thereby facilitating immune-mediated

109 tumor rejection.

110

111 **RESULTS**

112 **A p53-restorable immunocompetent tumor model to study senescence surveillance**

113   To study how senescence reprograms cellular and tissue states, we exploited

114 hydrodynamic tail vein injection (HTVI) technique (28) to generate a senescence-inducible liver

115 cancer model controlled by a tumor-specific, restorable p53 short-hairpin RNA (shRNA).

116 Specifically, adult liver hepatocytes of immunocompetent Bl/6 mice were transfected *in vivo* with

117 a sleeping beauty SB13 transposase vector and two transposon constructs (encoding

118 NrasG12D-IRES-rtTA and TRE-tRFP-shp53, or "NSP") which integrate in the genome. In this

119 Tet-On system, endogenous p53 is suppressed in the presence of doxycycline (Dox) through

120 the activation of inducible shRNA linked to RFP (**Fig. 1A**), enabling genetic control of

121 senescence in established tumors. Consistent with the co-occurrence of mutations that

122 inactivate *TP53* and activate cell proliferation signaling pathways (e.g. PI3K/AKT and

123 RAS/MAPK cascades) in human liver tumors, the cooperation between oncogenic RAS and

124 suppression of p53 led to hepatocyte transformation, with most mice developing tumors with

125 poorly differentiated features 5-8 weeks after HTVI. Transcriptional profiling revealed that these

126 murine tumors resemble the 'Proliferation´ class of human HCC (Supplementary Fig. S1A-S1F),

127 which is the typical class of human HCC harboring *TP53* mutations (21,22,29).

128   Based on previous work (15), we anticipated that p53 reactivation in the above system

129 would trigger senescence and engage anti-tumor immunity. Accordingly, Dox withdrawal

130 triggered dramatic tumor regressions over the course of several weeks, leading to prolonged

131 animal survival (**Fig. 1B and 1C**). Analysis of the tumors at 14 days post Dox withdrawal

132 revealed the expected downregulation of the p53-shRNA (as visualized by the linked RFP

reporter) and accumulation of senescence-associated-β-galactosidase (SA-β-gal) without any

notable effects on the RAS-effector p-ERK (**Fig. 1D**). Similarly, an increase in SA-β-gal activity

and SASP-associated transcriptional profiles, together with a concomitant proliferative arrest,

were observed in explanted tumor cells 6-8 days following p53 restoration (Supplementary Fig.

S2A-S2H). Of note, engraftment of these cultures (kept on Dox to maintain p53 silencing) into

Dox-fed immunocompetent mice produced synchronous and focal secondary tumors that

regressed with similar kinetics as the primary tumors upon Dox withdrawal (**Fig. 1B**;

Supplementary Fig. S3A-S3E). Control experiments using a Tet-Off system or incorporating a

constitutive p53 shRNA ruled out the possibility that Dox itself had any effect on tumor behavior

in our model (Supplementary Fig. S3F and S3G). Therefore, this system allows for the efficient

induction of senescence in tumor cells without resorting to therapies that can also alter the host

immune system. Given its added flexibility, we used the orthotopic transplant model (hereafter

referred to as 'NSP') for many of the mechanistic studies described below.

As anticipated, the marked tumor regressions noted above were immune mediated.

Hence, NSP tumors that arose following transplantation into immunocompromised Nude and

Rag2$^{-/-}$Il2rg$^{-/-}$ (R2G2) mice underwent a prominent cytostatic response but failed to regress, with

R2G2 animals showing the most profound defects (**Fig. 1E-1G**; Supplementary Fig. S3H and

S3I). As Nude mice are defective in adaptive immunity whereas R2G2 are also compromised

for aspects of innate immunity, these results imply that the adaptive immune system is essential

for efficient tumor regression in the model and establish a well-controlled experimental context

to explore the mechanistic basis for these effects.

Senescence triggers a switch from tumor immune evasion to immune recognition

To characterize the tumor suppressive paracrine effects of senescence, we next

characterized the immune microenvironments of tumors harboring p53-suppressed (referred to

as "proliferating') and p53-restored (referred to as "senescent") tumor cells after one week of

6

158 Dox withdrawal, a time when senescence is established but tumors have not yet regressed

159 (Supplementary Fig. S4A; Fig. S2-S3). Lesions harboring senescent tumor cells showed a ~1.8-

160 fold increase in total CD45+ immune cells compared to proliferating controls [**Fig. 2A;** refs.

161 (15,16)]. Immunophenotypic and histologic analyses (at day 9 post-Dox withdrawal) revealed

162 that this involved a prominent increase in the percentage of lymphocytes (B cells, CD4+ and

163 CD8+ T cells) and a decrease in the percentage of Gr-1+ myeloid-derived suppressor

164 cells/neutrophils (CD11b+Gr1+Ly6C low) (**Fig. 2B;** Supplementary Figs. S4B). While the

165 fraction of macrophages as a percentage of total CD45 population remained unchanged, the

166 absolute numbers were markedly increased (**Fig. 2A and 2B**; Supplementary Fig. S4C-S4E).

167 Within the T cell population, accumulating CD8 T cells showed markers of antigen experience

168 (CD44+, CD69+) and harbored an increased population of effector cells (CD44+CD62L-) [**Fig.**

169 **2C**; ref. (30)]. This overall remodeling of immune environment led to a significant increase in the

170 CD3:neutrophil ratio for tumors harboring senescent cells (Supplementary Fig. S4F), effects

171 consistent with similar increases in the CD3:neutrophil ratio which have been associated with

172 immune reactivity in human liver tumors (31). The remodeling could be clearly visualized using

173 three dimensional (3D) imaging after tissue clearing [**Fig. 2D**; Supplementary Fig. S4G and S4H;

174 Supplementary video S1; ref. (32)].

175 To pinpoint the specific immune cell types responsible for immune surveillance of

176 senescent tumor cells, we generated parallel cohorts of mice harboring orthotopic NSP tumors

177 and examined the impact of depleting various immune cell populations on tumor regressions

178 after Dox withdrawal. Whereas blocking antibodies targeting neutrophils/monocytes (Gr1), NK

179 cells (NK1.1), and CD4+ T cells (GK1.5) had no effect, depletion of CD8+ T cells (2.43) and

180 macrophages (using liposomal clodronate which selectively targets macrophages

181 (CD11b+F4/80+) but not cDC (CD11b-CD11c+MHC-II+CD103+); ref. (33,34)) markedly

182 impaired tumor regression (**Fig. 2E;** Supplementary Fig. S4I).

7

183   To characterize how p53-driven tumor senescence results in productive anti-tumor

184   immunity, we performed single-cell RNA-seq (scRNA-seq) analysis of freshly isolated CD45

185   cells from proliferating and senescent NSP tumors early after Dox withdrawal (8 days)

186   (Supplementary Fig. S5A and S5B), and used the differential abundance testing algorithm Milo

187   (35) to capture cell state shifts within the immune cell types mediating this process

188   (Supplementary Fig. S5C-S5F). In line with their contribution to tumor regression, the CD8+ T

189   cell and macrophage subpopulations showed marked changes in quantity and state.

190   Concerning T cells, proliferating (p53-suppressed) tumors were significantly enriched in CD8+ T

191   states exhibiting high expression of both dysfunction markers (*Tox, Tigit, Lag3, Ctla4,*

192   Pdcd1/*PD1, Cd160*) and activation markers (*Prf1*) (**Figs. 2F and 2G**; Supplementary Fig. S5G,

193   Supplementary Table S1). These CD8+ T cells also showed high levels of *Tnfrsf9*, a marker

194   known to delineate T cells subsets that have the capacity to become reinvigorated in human

195   HCC and other cancer types (36,37). In stark contrast, in senescent (p53-reactivated) lesions,

196   CD8 T populations appeared highly activated, showing low levels of dysfunction markers and

197   high expression of effector cytokines (e.g. *Ifng, Tnf,* Supplementary Table S1). Accordingly,

198   transcriptional profiling of bulk tumor tissues showed immune active and cytotoxic signatures in

199   senescent tumors undergoing regression [Supplementary Fig. S5H; ref. (38)].

200   Changes to the macrophage compartment provided further evidence that tumor cell

201   senescence triggered an immune evasion-to-surveillance switch. Hence, scRNA-seq,

202   immunophenotyping and histology indicated that tumor-associated macrophage phenotypes

203   transitioned from F4/80^{low};CD11c^{high} states (cluster 8), including immune-suppressive PDL1+

204   populations (characteristic of human HCC tumors with poor prognosis (39,40)), to

205   F4/80^{high};CD11c- states (cluster 0), defined by high expression of an antigen-presentation gene

206   signature (Supplementary Figs. S5E-S5J; S6A and S6B; Supplementary Table S1). Of note,

207   these senescence-associated F4/80^{high};CD11c- macrophages were particularly sensitive to the

208   liposomal clodronate treatment (Supplementary Fig. S6C-S6E), which also resulted in a

209   significant reduction in the fraction of active CD8 but not CD4 T cells (Supplementary Fig. S6F

210   and S6G), indicating a CD8-dependent immune response involving cooperativity with

211   macrophages. Accordingly, histological analyses confirmed that accumulating CD8+ T cells and

212   F4/80+ macrophages were frequently co-enriched following senescence induction in tumors

213   (**Fig. 2H;** Supplementary Fig. S4D). Collectively, these biological and molecular analyses

214   support a model where tumor cell senescence induces an abrupt switch from immune evasion

215   to immune surveillance mediated by changes in macrophages and CD8+ T cell states, leading

216   to productive anti-tumor immunity and, ultimately, tumor rejection.

217

218   **Senescence remodels tissue sensing programs and cell-surfaceome landscape**

219   We next set out to exploit the above model to understand the molecular mechanisms

220   responsible for rendering senescent tumor cells visible to the immune system. Senescence

221   induction involves a chromatin remodeling program that silences proliferative genes and

222   activates many genes encoding SASP factors, with the latter program being largely dependent

223   on the enhancer reader, BRD4 (10). We therefore performed transcriptional profiling

224   experiments on NSP cells under proliferating (p53-suppressed) versus senescent (p53-restored)

225   conditions in the absence and presence of JQ1, a drug that inhibits BRD4 function

226   (Supplementary Table S2). Consistent with expectations, p53 restoration dramatically reduced

227   the expression of proliferative genes and induced the expression of well-known SASP factors

228   [**Fig. 3A;** Supplementary Fig. S7A; ref. (7)], including several cytokines known to stimulate T

229   cells (*Cxcl16, Il18*) or macrophage activation and recruitment (*Csf2*, encoding protein GM-CSF)

230   or previously linked to senescence (*Igfbp7, Igfbp3, Pdgfa*). As anticipated from previous work

231   (10), many of the upregulated SASP-encoding transcripts (~65%) were BRD4-dependent

232   (Supplementary Fig. S7B). Similarly, a range of growth factors and immune modulators were

233   secreted from the senescent cells as assessed by multiplexed cytokine assays, including the T

234   cell and macrophage attractants CCL5, CXCL9, and GM-CSF as well as the vasculature

235 remodeling factor VEGF (Supplementary Fig. S7C). Therefore, senescence in p53-restored

236 NSP tumor cells is associated with a robust SASP, consistent with the marked remodeling of the

237 immune ecosystem characterized above.

238 Strikingly, examination of the subcellular localization for differentially expressed genes

239 (DEGs) revealed that senescent tumor cells not only increased their expression of secreted

240 ('extracellular', EC) SASP factors, but also displayed major changes in the expression levels of

241 transcripts encoding surface proteins ('plasma membrane', PM) (**Fig. 3B**). Indeed, 25% of total

242 upregulated DEGs encoded PM proteins, a significant enrichment that deviated from the

243 random distribution (15%) (**Fig. 3B**). Dynamic PM-DEGs were linked to protein tyrosine kinase

244 signaling transduction (*Nrp1, Egfr*), cytokine receptor activity (*Ifngr1*), ECM receptors (*Itgb3,*

245 *Cd44*) and ion transporters (*Slc12a1*, *Slc24a3*) and captured known senescence-associated

246 molecules (*Cd44, Vcam1, and Itgb3*), suggesting senescent cells may have an enhanced

247 capability to interact with and sense their environment [**Fig. 3C**; Supplementary Fig. S7D; refs.

248 (41-43)].

249 Interestingly, the senescence-associated increase in the expression of many of these

250 PM proteins was blunted by JQ1, suggesting that their induction may be part of the broader

251 chromatin remodeling program coupled to SASP [**Fig. 3D**; ref. (10)]. Of note, profound changes

252 in the transcription of genes encoding PM proteins also occurred in p53-deficient NSP tumor

253 cells treated with the senescence-inducing drug combination trametinib and palbociclib

254 [Supplementary Fig. S7E-top panel; Supplementary Table S2; ref. (20)] and in a series of 13

255 genetically-diverse *TP53* wild-type and *TP53* mutant human cancer lines–derived from liver,

256 breast, lung, and colon cancers induced to senesce by various triggers [**Fig. 3E**; Supplementary

257 Fig. S7F; ref. (44)] This was particularly robust for upregulated (but not downregulated) PM-

258 DEGs, reminiscent of effects observed for extracellular (EC) SASP factors (**Fig. 3E**;

259 Supplementary Fig. S7E-bottom panel). Therefore, the markedly altered expression of cell

260　　surface proteins we observed in our model extends beyond p53-induced senescence and may

261　　be a hallmark of the senescent state.

262　　　　To validate the global remodeling of PM factors in senescence at the protein level, we

263　　performed surface proteomics on isogenic proliferating and senescent NSP tumor cells, using a

264　　biotin-labeling enrichment method, where cell surface proteins were labeled with membrane-

265　　impermeable biotin, purified, and subjected to mass spectrometry [**Fig. 3F;** Supplementary Fig.

266　　S7G; ref. (45)]. A strong correlation between biological replicates under each condition was

267　　observed (Supplementary Fig. S7H), with detected proteins being enriched for annotated

268　　plasma membrane proteins by 60% after induction of p53-induced senescence. Of 887 proteins

269　　that were reproducibly detected, more than 50 % were differentially expressed. Most

270　　differentially expressed proteins correlated well with the directionality observed in our

271　　transcriptional profiling data, although some were differentially expressed without a

272　　corresponding change in transcript levels (Supplementary Fig. S7I).

273　　　　Annotated cell surface proteins detected by mass spectrometry upon senescence

274　　induction included several previously linked to senescence (e.g. CD44, VCAM1), various growth

275　　factor and cytokine receptors (e.g. EGFR, ICAM1 and IFNGR1), and other, less characterized

276　　factors (**Fig. 3F-3H**; Supplementary Fig. S7J and S7K). Of note, the set of cell surface-enriched

277　　proteins identified in our model showed limited overlap with those identified in human fibroblasts

278　　undergoing oncogene-induced senescence (46), suggesting heterogeneity between cell types

279　　or senescence triggers. Regardless, these results show that in addition to a rewiring in their

280　　secretory program, senescent cells undergo profound changes in the content and abundance of

281　　cell surface proteins, and imply that senescent cells acquire distinctive microenvironment-

282　　sensing traits that may influence their state and fate in vivo.

283

Senescent cells are primed to sense IFN-γ and amplify IFN-γ signaling

To identify pathways that might functionally influence how senescent cells sense their environment, we mined transcriptional and proteomic datasets for senescence-associated changes linked to anti-tumor immunity. Interestingly, GO analysis revealed that Type II interferon-gamma (IFN-γ) response (47) was among the top 5 annotated pathways enriched during senescence and dependent on cell state-specific enhancer programs (i.e. JQ1-sensitive; i.e. "C1" of Fig. 3D) (Supplementary Fig. S8A). Among the altered transcripts, we noted several positive regulators of IFN-γ signaling, including the IFN-γ receptor subunit IFNGR1 (one of the most significantly upregulated proteins from our proteomic data) and multiple interferon effectors [*Irf1*, *Irf7* and *Irf9*; refs. (47,48)] (**Fig. 4A-4C**; Supplementary Fig. S8B and S8C). Besides these Brd4-sensitive upregulated genes, transcripts encoding negative regulators of IFN-γ signaling (*Ptpn2*, *Socs1* and *Socs3*) were significantly decreased [**Fig. 4C**; refs. (49,50)]. Similar changes were noted in NSP tumor cells treated with different senescence inducers (**Fig. 4C**; Supplementary Fig. S8D-S8G) and, more broadly, in a panel of 13 human breast, lung, liver and colon derived cancer cell lines triggered to senesce [**Fig. 4D**; ref. (44)]. Therefore, changes in the expression of Type II IFN signaling components are a general feature of senescent cells, independent of cell type, cell genotype, species, and nature of the senescence inducer.

The concurrent increase in IFN-γ signaling effectors and decrease in negative regulators led us to hypothesize that senescent cells become primed to sense IFN-γ within their environment. To test this hypothesis directly, we treated proliferating and senescent NSP cells with recombinant IFN-γ and performed immunoblotting analyses of JAK-STAT signaling activation. While IFN-γ dramatically increased the baseline levels of STAT1 in both states, senescent cells accumulated more phosphorylated STAT1, irrespective of the senescence trigger (**Fig. 4E**; Supplementary Fig. S8H). Additionally, we also found an increased level of phosphorylated JAK1 in p53-restored senescent cells, further supporting our finding on a more active JAK-STAT signaling pathway in senescent cells sensing IFN-γ (Supplementary Fig. S8I).

12

310 As predicted from transcriptional analyses, senescence also triggered a decrease in PTPN2

311 protein (51), irrespective of the presence of exogenous IFN-γ (**Fig. 4E**). Thus, senescent cells

312 more efficiently activate IFN-γ signaling in response to limiting concentration of IFN-γ in the

313 environment.

314

315 **Senescence and extracellular IFN-γ cooperatively upregulate the antigen processing and**

316 **presentation machinery**

317   To better understand the functional contribution of IFN-γ sensing to the senescence

318 program, we next compared the phenotypic and transcriptional states of proliferating and p53-

319 restored senescent NSP tumor cells treated with recombinant IFN-γ at a low (50 pg/mL) or

320 higher (1 ng/mL) dose. While the addition of exogenous IFN-γ to proliferating or senescent

321 tumor cells had negligible effect on the viability, proliferation, or SASP gene expression of either

322 cell type at the doses tested (**Fig. 5A**; Supplementary Fig. S9A-S9D), marked changes in IFN-γ

323 pathway gene expression linked to the senescent state were observed. Specifically, supervised

324 clustering of the Hallmark "IFN-γ response signature" across proliferating and senescent cells

325 revealed three DEG modules: (i) genes that were downregulated during senescence

326 irrespective of IFN-γ (including the aforementioned negative regulators); (ii) genes that were

327 upregulated during senescence irrespective of IFN-γ and, interestingly, (iii) a substantial set of

328 DEGs that are cooperatively induced by the combination of senescence and IFN-γ (**Fig. 5B**).

329 Therefore, senescence triggers quantitative and qualitative changes in the transcriptional

330 response to IFN-γ.

331   One well-established output of IFN-γ signaling regulating cells' susceptibility to adaptive

332 immune surveillance is an increased capacity for antigen presentation mediated by MHC class I

333 molecules (MHC-I) (47,52). Indeed, many of the genes upregulated in senescent cells (class ii

334 genes) or hyper-induced in the presence of exogenous IFN-γ (class iii genes) included

13

components of the antigen presentation machinery. Among the genes induced during
senescence (class ii genes) were *Tap1*, transporters associated with antigen processing, and
Psme1, a proteosome factor associated with antigen processing (53). Those hypersensitive to
exogenous IFN-γ (class iii genes) included *Nlrc5*, a transcriptional co-activator of MHC-I genes
(54), the MHC-I assembly factor *Tapbp*, and the MHC-I subunit *B2m*. Two other class iii genes
were components of the immunoproteasome (*Psmb8, Psmb9*), whose actions can alter the
repertoire of presented peptides when overexpressed and are associated with an improved
tumor response to immune checkpoint blockade (55). This amplified output of IFN-γ in
senescent cells was confirmed by RT-qPCR and was retained at even higher levels of
exogenous IFN-γ (**Fig. 5C**, Supplementary Table S3). Consistent with the multifactorial process
described above, this effect was not observed in proliferating tumor cells, even those
overexpressing an IFNGR1 cDNA and/or treated with IFN-γ (Supplementary Fig. S10A-S10D).

Also consistent with the gene expression changes described above, senescent tumor
cells more robustly upregulate MHC-I in response to low levels of exogenous IFN-γ compared to
proliferating counterparts. Hence, while cell surface levels of MHC-I of both proliferating and
senescent cells were low at baseline and induced by exogenous IFN-γ, senescent cells showed
a significant increase of MHC-I protein expression (**Fig. 5D**). Similar synergies were observed
for cell surface HLA expression (identical to MHC-I in mice) in human cancer cells from liver and
other cancer types triggered to senescence with nutlin, which engages a p53-dependent
senescence program (56), or trametinib/palbociclib, which preferentially targets tumor cells with
an activated MAPK pathway [Supplementary Fig. S11A-S11D; ref. (20)]. Of note, the
combinatorial effects of drug treatment and IFN-γ on HLA expression required senescence
induction and did not occur in liver tumor cells that failed to senesce owing to a spontaneous or
engineered p53 mutation (irresponsive to nutlin) or a non-hyperactivated MAPK pathway
(irresponsive to trametinib/palbociclib). Furthermore, even though type I and type II IFN

360   response pathways include overlapping components, exogenous IFN-β treatment could not

361   substitute for IFN-γ in producing a robust MHC-I induction in senescent cells nor a strongly

362   differential induction between proliferating and senescent cells in our p53 restoration model

363   (Supplementary Fig. S11E and S11F). These data imply that murine and human cells triggered

364   to senesce acquire an increased capacity for antigen processing and presentation in the

365   presence of limiting quantities of IFN-γ.

Senescent tumor cells hyperactivate the IFN-γ signaling pathway *in vivo*

367   To determine the *in vivo* consequences of the rewiring of IFN-γ signaling identified in

368   senescent cells, we next adapted an IFN-γ sensing (IGS) reporter system to directly visualize in

369   intracellular IFN-γ signaling activation in real time (57). This reporter consists of a series of

370   consensus interferon gamma-activated sequences, which has specificity to type II IFN over

371   other signals (57), followed by a cDNA sequence encoding ZsGreen1 fluorescent protein and is

372   linked to a constitutively expressed RFP transgene to visualize transduced cells (**Fig. 6A**). NSP

373   tumor cells expressing this construct were RFP positive and showed a dose-dependent

374   increase in ZsGreen1 signal upon treatment with IFN-γ *in vitro* that increased following p53

375   induction or following treatment with senescence-inducing drugs (**Fig. 6B;** Supplementary Fig.

376   S12A and S12B).

377   We next used this system to monitor signaling activity following senescence induction in

378   tumors. Reporter-transduced tumor cells (on Dox) expressing constitutive RFP were injected

379   into the livers of Dox-fed syngeneic recipients and, upon tumor manifestation, doxycycline was

380   removed to induce p53 expression and trigger senescence as above (see Figures 1 and 2).

381   Regressing tumors were isolated 9 days post Dox withdrawal for 3D imaging of reporter activity

382   and parallel assessment of IFN-γ signaling in comparison to proliferating controls (from mice

383   maintained on Dox). As illustrated in Fig. 6C, proliferating tumor cells showed little, if any,

384   reporter expression, whereas tumor cells triggered to senesce *in vivo* displayed a more

385   prominent ZsGreen1 signal (**Fig. 6C and 6D**; Supplementary video S2). This effect coincided

386   with a specific increase in levels of IFN-γ protein (but not Type I IFN) in tumor tissue extracts

387   (**Fig. 6E**; Supplementary Fig. S12C).

388   To test whether the altered composition of immune cells in senescent tumors (Fig. 2;

389   supplementary Fig. S5-S6) contributed to the enhanced signal of the IFN-γ sensing (IGS)

390   reporter, we performed *in vitro* co-culture assays allowing exposure of senescent or proliferating

391   tumor cells to equal number of activated CD8 T cells, which we identified via scRNA-seq data

392   as the predominant cellular source of IFN-γ *in vivo* (**Fig. 6F-6H**). Senescent cells still showed a

393   significant increase of ZsGreen1 signal as compared to proliferating controls (**Fig. 6I**).

394   Consistent with a non-cell-autonomous signaling activation, IFN-γ was not detected in

395   conditioned media from NSP tumor cells under proliferative or senescent conditions

396   (Supplementary Fig. S12D), yet IFN-γ was readily detected upon co-culture with CD8 T cells, an

397   effect that was further enhanced by the addition of macrophages and associated with increased

398   MHC class I on senescent cells as well as increased activation of CD8+ T cells (Supplementary

399   Fig. S12E-S12J). Collectively, these data support a model whereby heterotypic interactions

400   between senescent tumor cells and immune cells sensitizes the tumor to exogenous IFN-γ,

401   leading to enhanced antigen presentation and efficient immune surveillance.

402   **IFN-γ signaling in senescent tumor cells is necessary for immune surveillance**

403   Our results imply that the immune-mediated clearance of senescent NSP tumor cells

404   involves the combined effects of SASP, known to stimulate immune cell recruitment (10,20,58),

405   together with a previously underappreciated capacity of senescent cells for enhanced sensing

406   and response to extracellular signals, as shown here with IFN-γ. To test the contribution of the

407   senescence-associated IFN-γ sensing program to the immune surveillance of senescent tumor

408   cells, we examined how disruption of the IFNGR in the tumor cells, or IFN-γ depletion in the

409   host, impacts the clearance of NSP tumor cells upon senescence induction. Indeed, tumor

410 regression (but not senescence *per se)* (Supplementary Fig. S13A-S13D) was impaired upon

411 knock-out (KO) of IFNGR1 (**Fig. 7A and 7B**; Supplementary Fig. S14A-S14C), an effect that

412 was even more pronounced for IFNGR-intact tumors engrafted into *Ifng-/-* mice (**Fig. 7C and 7D**)

413 and associated with the expected loss of surface MHC-I in tumor cells (Supplementary Fig.

414 S14D and S14E).

415   Consistent with the known contribution of IFN-γ signaling and tumor cell MHC-I to CD8+

416 engagement (59), tumors that lacked IFNGR1 or that developed in *Ifng-/-* recipients contained

417 fewer CD8+ T cells than their WT counterparts in both proliferating and senescent states

418 (Supplementary Fig. S14F), while still inducing robust immune infiltrate including abundant

419 macrophages (**Fig. 7E and 7F**; Supplementary Fig. S14G). Regardless, the impaired

420 senescence surveillance phenotype was not simply a result of this decrease in CD8+ T cells.

421 Co-culture assays providing uniform exposure of IFNGR1-KO and WT tumor cells to CD8 T

422 cells and macrophages still showed IFNGR1-dependent killing of senescent tumor cells

423 (Supplementary Fig. S15A-S15E)—a dependence that required the presence of both T cells

424 and macrophages and that not was observed in proliferating tumor cells under the same

425 conditions. Taken together, these data indicate that enhanced ability of senescent cells to sense

426 microenvironmental IFN-γ acts in concert with SASP-stimulated immune cell recruitment to

427 enable mutually reinforcing heterotypic interactions among tumor cells, macrophages, and

428 activated T cells that improve antigen presentation and immune surveillance, leading to potent

429 tumor regressions.

430

DISCUSSION

Enabled by a murine tumor model in which cancer immune evasion versus senescence surveillance is under tight genetic control, we reveal how senescent cells dramatically alter their ability to both send and receive environmental signals (Supplementary Fig. S16). Consistent with known senescence programs, p53-driven senescence induction led to the silencing of proliferative genes and induced the SASP. However, we also observed a profound effect on gene expression for plasma membrane proteins, including a range of growth factor receptors and cytokine receptors that are predicted to drastically alter how senescent cells respond to environmental signals. Importantly, while we employed a liver cancer model as our primary experimental system, a similar rewiring in the expression of cell surface sensors and gene programs sensitizing to environmental signals was observed in a broad range of murine and human tumor cells treated with senescence inducing agents, implying that the altered sensing program is a general hallmark of the senescent state.

One of the prominent sensing pathways altered in senescent cells involves type II IFN signaling. In our liver cancer model and across all senescent states we examined, senescence is accompanied by cell-intrinsic transcriptional and protein expression changes predicted to enhance signaling from exogenous IFN-γ. Indeed, senescent cells more robustly activated IFN-γ effectors in response to IFN-γ *in vitro* and *in vivo*, and both an intact IFN-γ effector pathway and IFN-γ in the environment are required for efficient CD8 T cell-mediated clearance of senescent tumor cells. While pathway analysis of senescent cell transcriptomes invariably identifies type II IFN signaling as an enriched feature, overlaps between type I and type II signaling components and the fact that IFN-γ is typically not detected as a SASP factor have left mechanistic questions regarding type II IFN signaling in senescence largely unexplored. Our studies demonstrate that such enrichment in IFN signaling signatures of senescent cells reflects an enhanced capacity for IFN-γ sensing whose output is most prominent *in vivo*.

18

456   Perhaps the most well-established output of type II IFN signaling involves its ability to

457   induce the antigen presentation machinery. Indeed, IFN-γ induced cell surface expression of

458   MHC-I (or HLA in human cells) in our model under both proliferating and senescence conditions.

459   However, IFN-γ-induced MHC-I upregulation was more pronounced in senescent cells, an effect

460   that correlated with increased expression of the transporter associated with antigen processing,

461   other antigen processing factors, and structural components of MHC-I. A similar hypersensitivity

462   to IFN-γ in inducing MHC-I/HLA was observed in human liver and lung cancer cell lines

463   triggered to senesce. These results imply that the senescence program can enhance antigen

464   presentation in non-immune cells, thereby facilitating tumor immunosurveillance.

465   Our results support a model whereby the ultimate impact of senescent cells on tissue

466   biology is dictated by the combined effects of how they send and receive environmental signals.

467   Not only do senescent cells induce the SASP, which triggers tissue remodeling and alters the

468   cell state and composition of immune cells in the environment, but they also dramatically alter

469   their surfaceome, leading to a differential ability to sense environmental factors, herein

470   exemplified by IFN-γ. Importantly, disruption of IFN-γ signaling had no effect on senescence

471   induction or the SASP in our system yet impaired subsequent tumor regressions, indicating that

472   altered environmental sensing acts in concert with the SASP to determine the ultimate output of

473   the senescence program – in this case, immune surveillance. These effects appear to be part of

474   a coordinated epigenetic program, as both the SASP and sensing programs show a prominent

475   dependence on the chromatin remodeling factor BRD4.

476   While the mechanism of immune surveillance in our model depends on cooperative

477   effects of CD8+ T cell and macrophage populations reflective of a transition from an "immune

478   cold" to an "immune hot" tumor microenvironment, other innate or adaptive immune cell types

479   may recognize and clear senescent cells in different contexts or, alternatively, immune

480   surveillance may not occur at all (18,19). Undoubtedly, some of these distinctions reflect

481   heterogeneity in SASP factor secretion (13,14), though our results raise the possibility that the

482   extent and nature of altered environmental sensing may also influence how senescent cells

483   affect tissue biology. While knockout of IFN-γ sensing (via *Ifngr1* knockout) and deletion of

484   MHC-I (B2M-KO) in senescent tumor cells impaired their immune surveillance *in vivo*, it did not

485   completely abolish tumor regression after senescence induction, indicating that IFN-γ sensing in

486   senescent cells is not the only pathway contributing to tumor regression. Regardless, the fact

487   that senescent cells can respond differently to environmental signals implies that their ultimate

488   molecular state in tissues will be different than in cell culture, highlighting the need to better

489   characterize the process *in vivo*.

490   Our results may help explain the paradoxical effects of senescence biology in physiology

491   and disease and have implications for the effective use of senescence-modulating therapeutics.

492   For example, in our model, the difference between tumor senescent cell clearance and

493   persistence was determined, at least in part, by the presence of environmental IFN-γ and the

494   integrity of the type II IFN signaling in the senescent cells. This suggests that variation in the

495   ability of senescent cells to recruit and sense IFN-γ secreting immune cells or other immune cell

496   types could profoundly affect senescent cell clearance, such that decreased environmental IFN-

497   γ or diminished type II IFN signaling could enable senescent cell persistence within tissues. In

498   the context of cancer, therapies that induce tumor cell senescence –a cytostatic program– can

499   trigger immune-mediated tumor regression or re-sensitize tumors to immune checkpoint

500   blockade, yet these are not the universal outcomes. As such, heterogeneity in the SASP (which

501   can vary between tumor cell types and senescence inducers) or IFN-γ sensing and output

502   (perhaps affected by deletion or mutation of IFN-γ pathway or HLA components (60) or the

503   reversible transcriptional mechanisms uncovered here) may influence the effectiveness of such

504   therapies in patients. Consistent with this notion, therapy-driven induction of specific SASP

505   profiles predicts patient outcomes in a subgroup of ovarian cancer patients (61). By contrast,

506   strategies to enhance the immune surveillance of senescent cells by increasing their sensitivity

507   to IFN-γ (e.g. with PTPN2 inhibitors) may help bias program output towards tumor cell rejection.

508   We envision that investigating this and other tissue remodeling and sensing programs in pre-

509   and post-treatment tumor biopsies (e.g. through transcriptomic or proteomic profiles) may

510   expose new response biomarkers and/or combination strategies to improve the clinical

511   management of cancer.

MATERIALS AND METHODS

Cell culture and drug treatment

p53-restorable mouse liver cancer cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (GIBCO) on plates that were collagen-coated (PurCol, Advanced Biomatrix, 0.1 mg/ml) for 30' at 37 C and maintained by the addition of 1 µg/ml doxycycline to suppress p53 expression. In order to restore p53 expression and therefore induce senescence, doxycycline-containing media was replaced with doxycycline-free media for 6 to 8 days. Cells were replated every 2 to 3 days to wash off doxycycline. Several cell lines have been generated and NSP is predominantly used for the study given the robustness of senescence phenotype upon p53 restoration. For human liver cell lines, HepG2 and SK-Hep1 were cultured with EMEM and SNU447 was cultured in RPMI-1640 in non-coated, tissue culture treated plates, all supplemented with 10% FBS and 1% penicillin and streptomycin. For human lung cancer cell lines, A549, H460 and H2030 were cultured in DMEM in non-coated, tissue culture treated plates supplemented with 10% FBS and 1% penicillin and streptomycin. All human cell lines were obtained from ATCC. Both murine and human cell lines were tested with Mycoplasma regularly every 6 months. The concentration and regimen of drug treatment in cancer cell lines were as followed. For perturbing BRD4-dependent transcriptional programs, cells were treated with 500 nM of JQ-1 (S7110, Selleck Chem) for 48 h prior to harvest, starting JQ-1 at day 6 after restoring p53 (off-Dox), when NSP cells are fully senescent. For drug-induced senescence experiments, p53-suppressed (on-Dox) NSP cells were treated with trametinib (25 nM, S2673 Selleck Chem) + Palbociclib (500 nM, S1116, Selleck Chem), Nutlin (10 µM, S1061, Selleck Chem) or Cisplatin (1 µM), changed every 2-3 days, during 7 days. The concentration of DMSO corresponded to the drug treatment and does not exceed 1:1,000 dilution of total media volume, which shows no discernable toxicity to cultured cells. For IFN-γ of proliferating or senescent populations, the indicated doses of mouse or human recombinant IFN-γ were administrated to murine and human cancer cell lines respectively after 24 h of cell

22

538 seeding and cells were harvested after 24 h of IFN-γ treatment for phenotypic or molecular

539 analyses.

540

541 **Cell culture and drug treatment**

542 <u>Primary liver tumor generation and isolation of liver cell lines</u>

543 All mouse experiments were approved by the Memorial Sloan Kettering Cancer Center (MSKCC;

544 New York, NY) Internal Animal Care and Use Committee. Mice were maintained under specific

545 pathogen-free conditions, and food and water were provided ad libitum. C57BL/6N female mice

546 aged 8-9 weeks old were injected via hydrodynamic tail vein injection (HTVI) with a sterile 2 ml

547 (or 1/10 of mouse body weight) 0.9% NaCl solution containing 5 μg of pT3-EF1a-NrasG12D-

548 IRES-rtTA (Tet-On system) and 20 μg of pT3-TRE-tRFP-shp53 transposon vectors along with 5

549 μg CMV-SB13 transposase (5:1 ratio) through the lateral tail vein. Doxycycline was

550 administered to mice via 625 mg/kg doxycycline-containing food pellets (Harlan Teklad) at least

551 4 days before injection. The tumor was harvested at 5-7 weeks after injection for cell line

552 isolation. To derive cancer cell lines from primary liver tumor, tumors were minced and digested

553 with 5ml of digesting solution, containing 1 mg/ml collagenase IV (C5138, Sigma-Aldrich) and

554 0.3% Dispase II (Roche 04942078001) in DMEM, at 37 °C for 30 mins with occasional vortexing.

555 The cells were spun down to remove the supernatant and plated on collagen-coated plate.

556 Independent cell lines were passaged at least 7-8 passages to remove fibroblasts and obtain

557 homogenous population. For those experiments involving bioluminescence tracking of tumor

558 growth the transposon construct pT3-EF1a-NrasG12D-IRES-rtTA-IRES-Luc was used. In the

559 Tet-OFF system setting, the transposon construct pT3-EF1a-NrasG12D-IRES-tTA was used to

560 co-inject with pT3-TRE-tRFP-shp53 vector into mice under normal diet to allow p53 hairpin

561 expression. To restore p53 in the liver tumor, the mice were subjected to doxycycline diet. For

562 constitutive p53 knockdown model, transposon constructs pT3-EF1a-NrasG12D and pT3-EF1a-

563 tRFP-shp53 were used.

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564

<u>Orthotopic transplant experiments</u>

Both C57BL/6 mice were predominantly used for the animal study for the HTVI tumor generation and orthotopic liver injection experiments in the immunocompetent setting. C57BL/6N strain was mainly used except for the matching control strain with IFNG KO mice (Jax, #002287) that was in the C57BL/6J background. No difference was observed in terms of tumor growth or senescence surveillance phenotype between C57BL/6N and J strain. Female mice were used in the experiment for the convenience of cage separation. All *in vivo* experiments were performed with age-matched (8-13 weeks old) cohorts. For the orthotopic liver tumor injection, NSP tumor cells were trypsinized and filtered twice using 40 μm strainer to reduce cell doublets followed by pelleting and were prepared in 20 μl of 1:1 DMEM to Matrigel ratio and injected using 31-gauge needle to the left lobe of the mouse liver following the standard microsurgery institutional practice. Due to the engraftment differences in mice of different strains- C57BL/6, Nude and R2G2 (Envigo) mice- different amounts of tumor cells were injected. Specifically, $5x10^5$, $8x10^4$ and $5x10^4$ cells were injected respectively in each strain to have comparable tumor size around 2 weeks after injection. Mice were then randomized based on the similar size of tumor and assigned to different groups for the subsequent experimental design.

Lentiviral and retroviral production and transduction

Lentiviruses were generated by co-transfection of viral vectors (1.5 μg) with packaging plasmids psPAX2 (0.75 μg) and pCMV-VSVG (0.25 μg) (Addgene) into 293T cells with 90% confluency in a 6-well plate. Retroviruses were generated by co-transfection of viral vectors (2 μg) with pCMV-VSVG (0.25 μg) (Addgene) into Phoenix-gp cells with 90% confluency in a 6-well plate. Polyethyleneimine (PEI) was added during co-transfection with a ratio of total DNA:PEI = 1:3 to

589 facilitate the binding of the plasmid to the cell surface. Viral containing supernatants were

590 cleared of cellular debris by 0.45 µm filtration. Target cells were exposed to viral supernatants

591 and mixed with 4 µg/ml polybrene for overnight before being washed, grown for 24 h in fresh

592 media, then subjected to antibiotic selection or fluorescence-based cell sorting.

593

594 **Lentiviral and retroviral vectors**

595 Murine liver cancer cells were infected with retroviral vector MSCV-Luc2-IRES-GFP (62) to

596 enable bioluminescence imaging. For visualization and staining of liver tumor cells *in vivo*, tumor

597 cells were infected with either the following lentiviral vectors specified in the figure legends,

598 pRRL-SFFV-GFP-mirE(shRen)-PGK-puromycin (SGEP was a gift from Johannes Zuber,

599 Addgene #111170) or pRRL-EFS-GFP-shRen (generated through replacing SFFV with EFS

600 promoter and removing antibiotic selection marker puromycin), to label the cells with GFP. For

601 visualization of IFN-γ sensing, tumor cells were infected with the lentiviral IGS reporter construct

602 described below.

603

604 **Genetic manipulation of cell line using CRISPR/Cas9**

605 In order to knock out specific genes in mouse and human liver tumor cell lines, the plasmid

606 pSpCas9(BB)-2A-GFP (PX458) (PX458 was a gift from Feng Zhang, Addgene #48138) in which

607 a sgRNA targeting either an intergenic region of chromosome 8 (Ctrl) or the specific gene of

608 interest was cloned. Cells were transiently transfected by PEI (2 µg plasmid and 6 µl PEI in 6

609 well plate with 60% confluency). Transfected cells were subsequently FACS sorted by GFP

610 positivity 36-48 h post-transfection. For Ifngr1 and B2m KO experiment, PX458 transfected cells

611 were first stained with IFNGR1 (2E2, biotin) followed by Streptavidin-APC staining, and MHC-I

612 (H-2k^b^; AF6-88.5.5.3) antibody respectively and negative cells were sorted. Sorted population

613 were further tested with IFN-γ to evaluate KO efficiency by using MHC-I induction as a proxy. In

614 order to generate p53 KO human tumor cells, cells were electroporated following manufacturer's

25

615   instructions. Briefly, cells were trypsinized, washed in PBS once, and counted and then

616   resuspended in Neon Buffer R. In parallel, 1 µg of Cas9 (ThermoFisher) and 1 µg of sgRNA

617   were complexed for 15 min at room temperature to form the Cas9 RNP complex, which was

618   then mixed with the cell aliquot. The cell/RNP mixture was electroporated (1400 V pulse voltage,

619   20 ms pulse width, 2 pulses) using Neon electroporation system (Thermo Fisher). The cells

620   were recovered for 3 days with further selection through nutlin treatment (10 µM, Selleck

621   Chemicals S1061) for 5-7 days to enrich p53 KO cells. The sgRNA sequence used in the

622   experiments are: Ifngr1: TGGAGCTTTGACGAGCACTG, B2m: AGTATACTCACGCCACCCAC,

623   Ctrl: GACATTTCTTTCCCCACTGG and TP53: CGCTATCTGAGCAGCGCTCA.

624

625   **Co-culture assays**

626   In order to isolate CD8$^+$ T cells from spleens of female OT-I mice (Jackson laboratory), spleens

627   were mechanically disrupted by passing them through a 70 µm cell strainer and centrifuged at

628   1500 rpm x 5 minutes. Red blood cells were lysed with ACK lysis buffer (Quality Biological) for 5

629   minutes. Total splenocytes or CD8+ T cells FACS sorted on a Sony MA900 were then activated

630   with CD3/CD28 Dynabeads (one bead/T cell, Thermo Fisher) and cultured in presence of IL-2

631   (2 ng/ml; Biolegend), IL-7 (2.5 ng/ml; Peprotech), IL-15 (50 ng/ml; Peprotech) and 2-

632   mercaptoethanol (5.5uM, Fisher Scientific) in complete RPMI-1640 media supplemented with

633   10% FBS and 100 IU/ml penicillin/streptomycin for 5-6 days (passage cells every 2-3 days) prior

634   to co-culture assays with mouse liver tumor cells. For Kupffer cells isolation, BL/6 male mice

635   aged 8-14 weeks were first subjected to liver perfusion as previously described (63). After

636   perfusion, the liver was removed and homogenized and then digested with protease solution

637   (0.5 mg/ml type XIV protease, Sigma, P5147) supplemented with DNAse I (0.2 µg/ml, Roche,

638   10104159001) for 15 minutes at 37C with constant stirring. This suspension was then

639   centrifuged at 50 g for 3 minutes to remove the hepatocyte pellet. The supernatant was then

640   transferred and centrifuged 580 g for 5 minutes at 4C. Next, the pellet was washed with HBSS

26

641 to remove residual protease solution and centrifuged at 580 g for 5 minutes at 4C to pellet the

642 cells again. The pellet was then resuspended with FACS buffer and subjected to α-F4/80

643 isolation according to the manufacturer instruction (Miltenyi Biotec, 130-110-443). After isolation,

644 the purity of Kupffer cells was confirmed with F4/80 staining through flow cytometry.

645 Murine liver tumor cells NSP were transduced with retrovirus expressing PresentER-SIINFEKL

646 construct (GFP) (PresentER-SIINFEKL (GFP) was a gift from David Scheinberg, Addgene

647 #102944) to express the peptide 257-264 from chicken ovalbumin, which is presented by H-2Kb

648 on the cell surface. Transduced cells were further selected with puromycin to obtain > 95% GFP

649 positivity. Tumor cells were cultured in presence or absence of doxycycline for 6 days in order to

650 induce senescence. 1,000 proliferating or 2,000 senescent tumor cells were plated in the

651 individual well of a 96 well collagen-coated plate. For those experiments where Kupffer cells

652 were added, they were isolated on the same day and plated at the indicated ratio 6 h after

653 plating the tumor cells. 24 h after plating tumor cells, previously activated OT-I T cells were

654 added at the indicated ratio. Co-cultures were imaged over time using an INCell 6000 high-

655 content imager (GE Healthcare Life Sciences), with a 488 nm and a 633 nm laser excitation to

656 visualize tumor cells and T cells (stained by CellTracker Deep Red Dye, Invitrogen C34565)

657 respectively, using a 10x objective. Images were captured at indicated time points, starting after

658 the seeding of T cells onto tumor cells/Kupffer cells co-cultures. Images for each channel were

659 saved during the experiment and subsequently analyzed using Columbus image analysis

660 software. GFP+ tumor cells were identified and segmented from background using an intensity-

661 based threshold method. T cells were identified using the same threshold method as the tumor

662 cells. Number of the GFP+ tumor cells was quantified and normalized to the untreated control to

663 calculate the killing index.

664

665 _IFNGR1 KO and WT tumor cell mixture in co-culture experiment_

666 For IFNGR1 WT vs. KO mixture experiment (supplementary figure S15E), cells were mixed and

667 kept on or off Dox for 6 days before starting the co-culture experiment using a 24-well plate by

668 plating 7,000 and 14,000 proliferating and senescent cells respectively with the same protocol

669 described above. The percentage of IFNGR1 WT vs. KO cells and absolute number (through

670 counting beads) were measured by flow cytometry.

671

672 *Effect of cell-cell contact between macrophages and CD8 T cells in co-culture experiment*

673 To measure the effect of direct contact of macrophages with CD8 T cells, we used a transwell

674 plate (Costar 12mm transwell, 0.4 µm pore, #3460) to separate macrophage and CD8 T+tumor

675 cells by plating macrophages at the bottom well while the CD8 T cells and tumor cells were

676 plated on the upper well. As a comparison, 24-well plate with 3 cell types co-cultured together

677 were used. After 48 h, T cells were collected and stained for antibodies and subjected to flow

678 cytometry.

679

680 **Proliferation and SA-β-gal assays**

681 For colony formation assays, 2,500 mouse liver cancer cells or 10,000 human liver cancer cells

682 were plated in each well of a 6-well plate. Cells were cultured for 6 days, then fixed with 4%

683 formaldehyde, and stained with crystal violet. Detection of SA-β-gal activity was performed as

684 previously described at pH 5.5 for mouse cells and tissue and pH 6 for human cells (20). For *in*

685 *vivo* SA-β-gal staining, fresh frozen tissue sections were fixed with 0.5% glutaraldehyde

686 followed by standard SA-β-gal staining as above described. Sections were counterstained with

687 eosin. For population doubling curves, cells were washed with PBS, trypsinized, and 100,000

688 cells were plated in triplicates in 6-well plates in presence or absence of doxycycline. Every 48 h

689 cells were counted and 1×10^5 cells were replated. Population doublings for each 48 h period

690 were calculated by dividing the final cell number to initial cell number.

691

Whole mount immunostaining and tissue clearing

To detect T cells and neutrophils in the NSP liver tumors, we performed whole mount immunostaining and tissue clearing (with benzyl alcohol, benzyl benzoate, BABB) of excised tumors as previously described (32). At the indicated time points, mice were euthanized by carbon dioxide inhalation and liver tumors collected and fixed in 4% paraformaldehyde in PBS at 4ºC overnight. Tissues were washed three times with PBS for 10' at room temperature and preserved in 0.05% azide in PBS at 4 ºC before processing. Then, the tissues were permeabilized in methanol (MetOH) gradients in PBS (PBS > 50% MetOH > 80% MetOH > 100%MetOH, 30 min in each solution), bleached with Dent's bleach (15% H_2O_2, 16.7% dimethyl sulfoxide [DMSO] in MetOH) for 1h at room temperature, and rehydrated through descending MetOH gradients in PBS (80% MetOH > 50% MetOH > PBS, 30 min in each solution). Tissues were next incubated in blocking buffer (0.3% Triton X100, 0.2% BSA, 5% DMSO, 0.1% azide and 25% FBS in PBS) for 24h at 4ºC on a shaker and then stained with antibodies (rat anti-CD3 [clone 17A2, cat#100202, Biolegend, RRID:AB_312659]; goat anti-myeloperoxidase [goatMPO, AF3667, R&D Systems, AB_2250866], and [hamster anti-CD31, 2H8, MA3105, Thermo Fisher, RRID:AB_223592] all diluted 1:200 in blocking buffer), for 3 days at 4ºC on a shaker. Tissues were next washed for 24 h in washing buffer (PBS with 0.2% Triton X100 and 3% NaCl), and stained with secondary antibodies (donkey anti-rat-AF488 [A212008, Invitrogen] and donkey anti-goat AF647 [A21447, Invitrogen] diluted at 1:400 in blocking buffer) for 2 days at 4ºC with shaking. Tissues were then washed for 24 h in washing buffer and thereafter stained with goat anti-hamster-AF568 (goat anti-hamster IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 568, A21112, Thermo Fisher, diluted at 1:400) and (1:1000) in blocking buffer for 2 days at 4ºC, on a shaker. Tissues were then washed for 24 h in washing buffer and thereafter dehydrated in MetOH gradients in dH_20 using glass containers (50% MetOH > 70% MetOH > 90% MetOH > 3x 100% MetOH, 30 min for each step). Tissues were next cleared for 30 min in 50% MetOH and 50% BABB (benzyl alcohol, benzyl benzoate, mixed 1:2) followed by clearing 1

29

718   h in 100% BABB. Finally, the tissues were imaged on an SP8 Microscope (Leica). Visualization

719   and quantification was performed with Imaris software (Bitplane). In separate experiments, 3D

720   imaging after tissue clearing was used to detect the ZsGreen1, IFN-γ sensing (IGS) reporter.

721   For these experiments, we used the <u>CUBIC </u>tissue clearing protocol that maintains the

722   fluorescence from fluorescent proteins (64). Tissues were excised and fixed as stated above,

723   and then were soaked in CUBIC-I solution in a 15 mL conical tube container. CUBIC-I was

724   prepared mixing 108 ml of ddH$_2$O with 75g of Urea (Sigma, U5128), 75g of N,N,N',N'-Tetrakis(2-

725   Hydroxypropyl)ethylenediamine (Sigma, 122262) and 42ml of Triton X-100 (Sigma, X100).

726   Samples were maintained at 37ºC on a shaker for 7 days, changing the media every other day,

727   until clear. The samples were then counterstained for DAPI in CUBIC-1 (1:1000) for 24h and

728   washed in CUBIC-I overnight. Images were acquired and analyzed as described above.

729

730   **Western blotting**

731   Cell were lysed with RIPA buffer (50 m Tris PH 7.4, 150 mM NaCl, 0.5 % sodium deoxycholate,

732   0.1% SDS; 1mM EDTA; 1% NP-40) supplemented with phosphatase and protease inhibitor

733   (5872, Cell Signaling Technology) and protein concentration was determined by BCA assay.

734   Samples were boiled for 5 minutes and 20 to 30 µg of protein were separated by SDS-PAGE,

735   transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) according to standard

736   protocols and probed with the relevant primary antibody overnight at 4°C. Membranes were

737   then incubated with horseradish peroxide (HRP)-conjugated) anti-rabbit IgG or anti-mouse IgG

738   secondary antibodies (1:10,000, GE Healthcare Life Science) at room temperature and proteins

739   were detected using Pierce ECL Western Blotting Substrate (34095, Thermo Fisher Scientific).

740   Antibodies were diluted as follows: p53 (CM5) (1:500, NCL-L-p53-CM5p, Leica Biosystems,

741   RRID:AB_2895247), p21 (F-5) (1:500, sc-6246, Santa Cruz Biotechnology, RRID:AB_628073),

742   phosphor-STAT1 (Tyr701) (1:500, #9167, Cell Signaling Technology, RRID:AB_561284),

743   STAT1 (1:1,000, #14994, Cell Signaling Technology, RRID:AB_2737027), JAK1 (1:1000, #3344,

744   Cell Signaling Technology, RRID:AB_2265054), phosphor-JAK1 (1:1000, #3331, Cell Signaling

745   Technology, RRID:AB_2265057), TC-PTP (PTPN2, 1:1000, ab180764, Abcam,

746   RRID:AB_2722704). Protein loading was measured using a monoclonal β-ACTIN antibody

747   directly conjugated to horseradish peroxidase (1:20,000; A1978, Sigma-Aldrich,

748   RRID:AB_476692), NUCLEOLIN (1:5000, ab22758, Abcam, RRID:AB_776878) or VINCULIN

749   (1:2,000, ab129002, Abcam, RRID:AB_11144129). ECL developed blots were imaged using a

750   FluorChem M system (Protein Simple).

751

752   ***In vitro* multiplexed ELISA**

753   Conditioned media samples (duplicates collected in complete DMEM 48 h after seeding) from

754   proliferating or senescent NSP tumor cells (6 to 8 days after doxycycline withdrawal) were

755   centrifuged at 1500 rpm for 3 minutes and filtered through 0.2 um filter to remove cell debris.

756   Samples concentrations were normalized by diluting in complete DMEM according to cell count.

757   Aliquots (50 µl) of the conditioned media were analyzed using multiplex immunoassays

758   designed for mouse (Mouse Cytokine/Chemokine Array 31-Plex) from Eve Technologies.

759   Biological replicates from two independent experiments were performed to determine cytokine

760   levels. Heatmaps display relative cytokine expression values normalized to geometric means of

761   individual cytokines from both proliferating and senescent samples.

762   **Measurement of IFN-γ in *in vivo* tumor lysates and *in vitro* conditioned medium**

763   BD cytometric bead array Mouse Th1/Th2 cytokine kit (Cat# 551287, BD Biosciences) was used

764   to determine the IFN-γ levels. Flash frozen tissues were lysed in RIPA buffer and homogenized

765   using TissueLyser II (Qiagen) followed by protein concentration measurement determined by

766   BCA assay. 100 µg of tissue lysate were used for subsequent measurement following standard

767   manufacturer instructions of CBA kits. For in vitro conditioned medium measurement, 50 out of

768   200 µl of conditioned medium collected from the 96 well of co-culture experiments were used.

769

Plasma membrane-enriched mass spectrometry

To capture differential cell surface proteome changes induced by senescence, we adapted the protocol from previous published study (45) and followed the manufacturer instruction (Pierce Cell Surface Protein Isolation Kit #89881) to enrich cell surface proteins of proliferating and senescent cells through biotin-based labeling followed by pull-down purification. In brief, we plated 1 and 3 15 cm plates of proliferating and senescent cells (6 days after doxycycline withdrawal) with an initial seeding of 7×10^5 and 2×10^6 million cells respectively and collected the cells 2 days later, with the cells approximatively at 85% confluency. Before harvesting the cells, cells were incubated with biotin solution for 30 minutes at 4C to allow the surface protein labeling. Cells were then washed with cold PBS and scraped down followed by lysis (buffer provided in the kit). Lysates were centrifuged and the clarified supernatant was used for purification of biotinylated proteins on NeutrAvidin Agarose. Supernatant was incubated with NeutrAvidin Agarose for 2 h at room temperature in the closed column to allow biotinylated proteins binding. Column containing Agarose slurry was washed to remove unbound proteins. The proteins were then digested in situ in the column overnight using 4µg of trypsin (Promega, V5111) per column at 37C on a rotor. Digested proteins were further desalted by C18 Stagetip and subjected to liquid chromatography–mass spectrometry (LC-MS/MS) followed by proteins identification through Proteome Discover (Thermo Scientific) according to protocols previously described (45). Non-biotinylated cell lysates were also included and served as background controls.

Protein identification

The LC-MS/MS .raw files were processed using Mascot and searched for protein identification against the SwissProt protein database for human/mouse (please adjust the species accordingly). Carbamidomethylation of C was set as a fixed modification and the following variable modifications allowed: oxidation (M), N-terminal protein acetylation, deamidation (N and

796   Q), and phosphorylation (S, T and Y). Search parameters specified an MS tolerance of 10 ppm,

797   an MS/MS tolerance at 0.080 Da and full trypsin digestion, allowing for up to two missed

798   cleavages. False discovery rate was restricted to 1% in both protein and peptide level.

799   Normalized protein intensities were obtained using Scaffold (4.8.4).

800

801   **RNA preparation and High throughput RNA-sequencing analysis**

802   For *in vitro* liver cell lines RNA preparation, total RNA was extracted using TRIzol (Thermo

803   Fisher Scientific) following the manufacturer's instructions. For *in vivo* bulk tumor RNA-seq,

804   proliferating tumor (p53 Off) was harvested 7-10 day after randomization point and senescent-

805   induced tumor (p53 On) was harvested 12 days after p53 restoration, allowing similar size of

806   tumor at harvest. To extract tissue RNA, freshly isolated tumor chunk was first stored in RNA-

807   later solution (AM7024, Thermo Scientific) to preserve RNA integrity until extraction and

808   RNeasy kit (74106, Qiagen) was used to purified tissue RNA following the manufacturer

809   instructions. Purified polyA mRNA was subsequently fragmented, and first and second strand

810   cDNA synthesis performed using standard Illumina mRNA TruSeq library preparation protocols.

811   Double stranded cDNA was subsequently processed for TruSeq dual-index Illumina library

812   generation. For sequencing, pooled multiplexed libraries were run on a HiSeq 2500 machine on

813   RAPID mode. Approximately 10 million 76bp single-end reads were retrieved per replicate

814   condition. Resulting RNA-Seq data was analyzed by removing adaptor sequences using

815   Trimmomatic (65), aligning sequencing data to GRCm38 – mm10 with STAR (66), and genome

816   wide transcript count was quantified using featureCounts (67) to generate raw count matrix.

817   Differential gene expression analysis was performed using DESeq2 package (68) between

818   experimental conditions, using 3 independent biological replicates (independent cultures of NSP

819   tumor cells) per condition, implemented in R (http://cran.r-project.org/). Differentially expressed

820   genes (DEGs) were determined by > 2-fold change in gene expression with adjusted P-value <

821   0.05. For heatmap visualization of DEGs, samples were z-score normalized and plotted using

822   'pheatmap' package in R. Functional enrichments of these differential expressed genes were

823   performed with enrichment analysis tool Enrichr (69). Gene expressions of RNA-Seq data were

824   clustered using hierarchical clustering based on one minus Pearson correlation test. Subtype

825   specific gene signatures were derived (22) and used as inputs for signature score calculation

826   using R package singscore (70).

827

828   **Public dataset transcriptomic analyses**

829   Signature of different human liver cancer subtype was obtained from previous study (22). In

830   brief, the top 200 over-expressed and under-expressed gene transcripts among each tumor

831   subtype were selected as their signature. To analyze the transcriptomic changes of genes

832   encoding plasma membrane and extracellular factors distinguishing senescent and proliferating

833   tumor cells, transcriptomic data of a series of human tumor cell lines triggered to senesce was

834   used according to the previously published study (44) and obtained from the website

835   https://ccb.nki.nl/publications/cancer-senescence/. The expression of selected genes was

836   compared between senescent and the corresponding proliferating cells among individual cell

837   lines and normalized to determine the fold change. Information about protein subcellular

838   localization was derived from the Compartments_knowledge_based database (71), with the

839   genes assigned to specific subcellular localization when the criteria score is >= 3. The Cancer

840   Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) data set, including p53 mutational

841   status, transcriptomic profiles, and patient survival, were downloaded using R package

842   TCGAbiolinks (72,73). Senescence signatures derived from our mouse models were used as

843   input for computing signature scores using ssgsea method in R package GSVA (74). These

844   signature scores were used to separate patients into high and low groups, and log rank test was

845   used to test the differences in survival between these two groups.

846

847   **Gene set enrichment analysis (GSEA)**

34

848 GSEA was performed using the GSEAPreranked tool for conducting gene set enrichment

849 analysis of data derived from RNA-seq experiments (version 2.07) against signatures in the

850 MSigDB database (http://software.broadinstitute.org/gsea/msigdb), signatures derived herein,

851 and published expression signatures in organoid models and human samples. The metric

852 scores were calculated using the sign of the fold change multiplied by the inverse of the p-value.

853

854 **Reverse transcription and quantitative PCR**

855 Total RNA was isolated from mouse liver tumor cell line using TRIzol (Thermo Fisher Scientific)

856 following the manufacturer's instructions. cDNA was obtained from 500 ng RNA using the

857 Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche) after treatment with DNase

858 I (18068015, Thermo Fisher Scientific) following the manufacturer's instructions using random

859 hexamer method. The following primer sets for mouse sequences were used: Tap1_F 5'-

860 GGACTTGCCTTGTTCCGAGAG-3', Tap1_R 5'-GCTGCCACATAACTGATAGCGA-3',

861 Psmb8_F 5'-ATGGCGTTACTGGATCTGTGC-3', Psmb8_R 5'-

862 CGCGGAGAAACTGTAGTGTCC-3', Nlrc5_F 5'-CCTGCGTCCCAGTCATTC-3', Nlrc5_R 5'-

863 CTGCTGGTCAGTGATGGAGA-3', Erap1_F 5'-TAATGGAGACTCATTCCCTTGGA-3', Erap1_R

864 5'-AAAGTCAGAGTGCTGAGGTTT G-3', H2-K1_F 5'-GCTGGTGAAGCAGAGAGACTCAG-3',

865 H2-K1_R 5'-GGTGACTTTATCTTC AGGTCTGCT-3', H2-D1_F 5'-

866 AGTGGTGCTGCAGAGCATTACAA-3', H2-D1_R 5'-GGTGAC TTCACCTTTAGATCTGGG-3',

867 B2m_F 5'-TTCTGGTGCTTGTCTCACTGA-3', B2m_R 5'-CAG TATGTTCGGCTTCCCATTC-3',

868 Cdkn1a_F 5'-CGGTGTCAGAGTCTAGGGGA-3', Cdkn1a_R ATC ACCAGGATTGGACATGG-3',

869 Trp53_F 5'-CTAGCATTCAGGCCCTCATC-3', Trp53_R 5'-TCCGACTGTGACTCCTCCAT-3',

870 Csf3_F 5'-ATGGCTCAACTTTCTGCCCAG-3', Csf3_R 5'- CTGACAGTGACCAGGGGAAC-3',

871 Socs3_F 5'-ATGGTCACCCACAGCAAGTTT-3', Socs3_R 5'-TCCAGTAGAATCCGCTCTCCT-3',

872 Ptpn2_F 5'-ATGTCGGCAACCATCGAGC-3', Ptpn2_R 5'- TGTTTCGGTTTCTGTTTTCTGGA-3',

873 Irf1_F 5'- ATGCCAATCACTCGAATGCG-3', Irf1_R 5'-TTGTATCGGCCTGTGTGAATG-3',

35

874   Ccl5_F 5'-CTGCTGCTTTGCCTACCTCT-3', Ccl5_R 5'- CGAGTGACAAACACGACTGC-3',

875   Il18-F 5'-CAGGCCTGACATCTTCTGCAA-3', Il18-R 5'- TCTGACATGGCAGCCATTGT-3',

876   Hprt_F 5′-TCAGTCAACGGGGGACATAAA-3′, Hprt_R 5′-GGGGCTGTACTGCTTAACCAG-3′,

877   Rplp0_F 5′-GCTCCAAGCAGATGCAGCA-3′, Rplp0_R 5′-CCGGATGTGAGGCAGCAG-3′,

878   Quantitative PCR with reverse transcription (qRT–PCR) was carried out in triplicate (10 cDNA

879   ng per reaction) using SYBR Green PCR Master Mix (Applied Biosystems) on the ViiA 7 Real-

880   Time PCR System (Life technologies). Hprt, Rplp0 (also known as 36b4) served as endogenous

881   normalization controls.

882

883   **Tumor measurement by ultrasound and bioluminescence imaging**

884   High-contrast ultrasound imaging was performed on a Vevo 2100 System with a MS250 13- to

885   24-MHz scanhead (VisualSonics) to stage and quantify liver tumor burden. Tumor volume was

886   analyzed using Vevo LAB software. Bioluminescence imaging was used to track luciferase

887   expression in orthotopically injected liver tumor cells expressing a Luc-GFP reporter as well as

888   primary HTVI tumor harboring luciferase construct (vector described above). Mice were injected

889   IP with luciferin (5 mg/mouse; Gold Technologies) and then imaged on a Xenogen IVIS

890   Spectrum imager (PerkinElmer) 10 minutes later. Quantification of luciferase signaling was

891   analyzed using Living Image software (Caliper Life Sciences).

892

893   **Flow cytometry and sample preparation**

894   For *in vivo* sample preparation, orthotopically injected liver tumors were isolated by removing

895   the adjacent normal tissue, and allocated for 10% formalin fixation, OCT frozen blocks, snap

896   frozen tissue, and flow cytometry analysis. To prepare single cell suspensions for flow cytometry

897   analysis, liver tumor was mechanically disrupted to a single cell suspension using a 150 µm

898   metal mesh and glass pestle in ice-cold 3% FBS/HBSS and passed through a 70 µm strainer.

899   The liver homogenate was spun down at 400 g for 5 minutes at 4°C, and the pellet was

900   resuspended in 15ml 3% FCS/HBSS, 500ul (500U) heparin, and 8ml Percoll (GE), mixed by

901   inversion, and spun at 500 g for 10 min at 4°C. After removal of supernatant, cells were

902   resuspended in PBS supplemented with 2% FBS. Samples were blocked with anti-CD16/32

903   (1:200, FC block, #553142) (BD Pharmigen) for 20 minutes and then incubated with the

904   following antibodies for 30 minutes on ice: CD3 (1:200, 17A2, #612803, RRID:AB_2870130),

905   CD19 (1:200, 1D3, #563235, RRID:AB_2738085), CD4 (1:800, RM4-5, #563151,

906   RRID:AB_2687549), Ly6G (1:200, 1A8, #563005, RRID:AB_2737946), CD44 (1:200, IM7,

907   #560568, RRID:AB_1727481), CD11b (1:800, M1/70, #563553, RRID:AB_2738276) (BD

908   Biosciences); MHC-I (1:100, H-2k^b; AF6-88.5.5.3, #17-5958-82, RRID:AB_1311280), CD119

909   (1:100, 2E2, #13-1191-82, RRID:AB_2572773), Armenian Hamster IgG isotype (1:100,

910   eBio299Arm, #13488881, RRID:AB_470094) (Thermo Fisher); CD45 (1:400, 30-F11M,

911   #103128, RRID:AB_493715), Gr-1 (1:200, RB6-8C5, #108406, RRID:AB_313371), F4/80

912   (1:100, BM8, #123116, RRID:AB_893481), CD8 (1:400, 53-6.7, #100721, RRID:AB_312760),

913   Ly6C (1:200, HK1.4, #128026, RRID:AB_10640120), CD11c (1:200, N418, #117335,

914   RRID:AB_11219204), CD69 (1:200, H1.2F3, #104522, RRID:AB_2260065), CD106 (1:100,

915   MVCAM.A, #105717, RRID:AB_1877142), CD62L (1:200, MEL-14, #104435,

916   RRID:AB_10900082), PD-1 (1:100, 29F.1A12, #135215, RRID:AB_10696422) (Biolegend);

917   IFNGR2 (1:100, REA381, #130-105-670, RRID:AB_2652258) (Miltenyi Biotec); Streptavidin

918   (1:200, #20-4317-U100), TIGIT (1:100, 1G9, #20-1421-U025, RRID:AB_2621591), NK1.1

919   (1:100, PK136, #65-5941-U100, RRID:AB_2621910) (Tonbo); human antibody HLA-A,B,C

920   (1:100, W6/32, #17-9983-42, RRID:AB_10733389) (Thermo Fisher). To distinguish live/dead

921   cells, DAPI and Ghost dye violet 510 (1:1000, #13-0870-T100) (Tonbo) were used depending

922   on whether the cells are fixed. For fixed cells, cells were stained in PBS prior to antibody

923   staining. Flow cytometry was performed on an LSRFortessa or Guava flow cytometer (Luminex

924   Corporation), and data were analyzed using FlowJo (TreeStar).

925 **Neutralizing antibody and liposomal clodronate studies**

926 To determine the specific immune cell dependency of senescence surveillance, depleting

927 antibodies or drugs were administrated to the mice one day after doxycycline withdrawal. For

928 NK cell depletion, mice were injected intraperitoneally (IP) with an α-NK1.1 antibody (250 μg;

929 PK136, BioXcell) twice per week. For T cell depletion, mice were injected IP with either an α-

930 CD4 (200 μg; GK1.5, BioXcell) or α-CD8 antibody (200 μg; 2.43, BioXcell) twice per week.

931 Depletion of NK, CD4+, and CD8+ T cells was confirmed by flow cytometric analysis of liver

932 tumor tissue. For neutrophil/myeloid-derived suppressive cells depletion, mice were injected

933 intraperitoneally with an α-Gr-1 (200 μg; RB6-8C5, BioXcell) twice per week. For control, isotype

934 control antibody (200 μg; LTF-2, BioXcell) was IP twice per week. For macrophage depletion,

935 mice were injected intravenously (IV) with clodronate liposomes (50 mg/kg of mouse weight;

936 ClodronateLiposomes.com) twice per week. PBS was used as a control.

937

938 **Immunofluorescence and immunohistochemistry**

939 Tissues were fixed overnight in 10% neutral buffered formalin (Richard-Allan Scientific),

940 embedded in paraffin and cut into 5 μm sections. Sections were deparaffinized and rehydrated

941 with a histoclear/alcohol series and subjected to antigen retrieval by boiling in citrate antigen

942 retrieval buffer (Vector). Slides were then blocked in PBS/0.1% Triton X-100 containing 1% BSA.

943 Primary antibodies were incubated overnight at 4°C in blocking buffer. The following primary

944 antibodies were used: GFP (ab13970, Abcam, 1:500, RRID:AB_300798), Ki67 (#550609, BD

945 Biosciences, 1:200, RRID:AB_393778), CD8 (#14-0808-82, eBioscience, 1:200,

946 RRID:AB_2572861), CD45 (#70257, Cell Signaling Technology, 1:100, RRID:AB_2799780),

947 F4/80 (#70076, Cell Signaling Technology, 1:200), p21 (#556431, BD Biosciences, 1:200,

948 RRID:AB_396415). For immunohistochemistry, Vector ImmPress HRP kits and ImmPact DAB

949 (Vector Laboratories) were used for secondary detection. For immunofluorescence, the

950   following secondary antibodies were used: goat anti-chicken AF488 (A11039, Invitrogen, 1:500,

951   RRID:AB_2534096), donkey anti-rabbit AF594 (A21207, Invitrogen, 1:500, RRID:AB_141637),

952   goat anti-rabbit AF594 (A11037, Invitrogen, 1:500, RRID:AB_2534095), donkey anti-rabbit

953   AF647 (A31573, Invitrogen, 1:500, RRID:AB_2536183). All secondary antibodies were diluted in

954   blocking buffer and incubated for 1 h at room temperature. Subsequently, slides were washed

955   and nuclei were counterstained with PBS containing DAPI (1 µg/ml), and mounted under cover

956   slips with ProLong Gold (Life Technologies). Images were acquired with a Zeiss AxioImager

957   microscope using Axiovision software.

958

959   Triple immunofluorescence staining of PD-L1, CD68 and GFP of 2-3 µm sections was

960   performed using a Leica Bond RX platform (Leica Biosystems) with ER2 buffer (AR9640, Leica

961   Biosystems) for epitope retrieval. The following primary antibodies were used: PD-L1 (D5V3B,

962   #64988, Cell Signaling Technology, 1:150, 30min, RRID:AB_2799672), CD68 (orb47985,

963   Biorbyt, 1:1000, 30 min), GFP (TP401, Amsbio, 1:1000, overnight, after Opal protocol,

964   RRID:AB_10890443). Antibodies were detected using Opal 4-Color Automation IHC Kit

965   (NEL8720001KT, Akoya) with Opal 520 Reagent for PD-L1, Opal 570 Reagent for CD68, and

966   BrightVision Poly-HRP-Anti Rabbit antibody (DPVR-110HRP, Immunologic). GFP was

967   visualized using Donkey anti-Rabbit IgG (H+L) Alexa Fluor™ 647 (A31573, Thermo Fisher,

968   1:500, 1h, RRID:AB_2536183). Nuclei were stained with DAPI (D9542, Sigma). Specificity of

969   staining was confirmed with polyclonal rabbit IgG (Abcam, ab37415, RRID:AB_2631996).

970   Fluorescence images were acquired with a NanoZoomer-2.0 HT C9600 digital scanner

971   (Hamamatsu) and visualized with QuPath software (75) using the same settings.

972

973   **Generation of IFN-γ sensing (IGS) reporter**

974   In order to generate the IFN-γ sensing reporter from our study, we have adapted the construct

975   design from the previously described paper (57). In brief, we have crafted a 5x Interferon

976   Gamma-activated sequence (GAS) inserted in front of a mini promoter (minimal TATA-box

977   promoter with low basal activity) followed by ZsGreen1 reporter. Right after the reporter

978   sequence, this lentiviral construct also contains RFP driven by the PGK promoter to have

979   constitutive RFP expression for cell visualization. The cells were transduced with virus and

980   sorted through flow cytometry with high RFP level for stable expression of the construct in the

981   cells.

982

983   **Single-cell RNA-seq (scRNAseq) analyses**

984   *Data pre-processing and QC*

985   All scRNAseq data were processed into count matrices using 10x Genomics CellRanger 6.0.0

986   with default parameters using reference mouse genome GRCm38/mm10 augmented with

987   BioLegend TotalSeqB hashtag oligonucleotide barcode sequences for demultiplexing cellular

988   compartments in downstream analysis. Count matrices were processed to remove empty and

989   low quality droplets by removing (in order): transcripts with more than 10 million or fewer than

990   100 total reads, droplets with library size greater than 150,000 or lower than 300 total read

991   count, and droplets with fewer than 15 distinct expressed transcripts.

992

993   Potential doublets were removed using Solo version 1.2 (76) by training one doublet

994   classification model per sample using default parameters for Solo and removing droplets using

995   a threshold of 0.5. Subsequently, dead / dying cells were filtered by removing droplets with high

996   mitochondrial RNA content (greater than 20% of transcript counts mapped to MT genes) or high

997   ribosomal transcript count (greater than 15% of total transcript counts mapped to ribosomal

998   genes). After pre-processing, poor quality samples with either low numbers of recovered cells or

999   low number of distinct transcripts recovered were removed.

1000

1001   *scRNA-seq normalization and dimensionality reduction*

1002   Filtered count matrices for each sample were combined into a single dataset, normalized to

1003   counts per million (CPM) and log-transformed. Unwanted variation due to total transcript counts

1004   and percentage of mitochondrial reads per cell were regressed out prior to scaling each

1005   transcript to zero mean and unit variance. Principal Component Analysis (PCA) was then

1006   performed on a restricted subset of 5000 highly variable genes using the method described in

1007   (77) and implemented in Scanpy version 1.8.2. The top 50 principal components were kept to

1008   create a UMAP projection (78) using k=10 nearest neighbors to obtain a non-linear 2D

1009   embedding for downstream visualization. Leiden clustering (79) was implemented using Scanpy

1010   version 1.8.2.

1011

1012   The entropy was computed per collection date for each Leiden cluster and revealed 4/42

1013   clusters with more than 100 cells had low batch entropy (< 0.5). Harmony integration was

1014   therefore applied for batch correction (80) using 40 PCs. Reclustered cells verified that post-

1015   integration all Leiden clusters with more than 100 cells had batch entropy > 0.5.

1016

1017   *scRNA-seq compartment demultiplexing and cell-type assignment*

1018   Cellular compartments were identified from post-integration Leiden clusters using HashSolo (76).

1019   To specifically identify immune cells, we first used hashtag oligonucleotide barcodes matching

1020   FACS-sorted CD45+ populations and all cells clustering with this compartment were included in

1021   the majority label. We manually validated the compartment calls using canonical markers (i.e.

1022   *Ptprc* / CD45 positive; *Vim*, *Col1a2* and *Krt8* negative, for immune cells). A final count of 13,236

1023   (6664 from SEN tumors and 6572 from PRO tumors) CD45 cells and 17,782 genes across 5

1024   samples were recovered and further analyzed using the workflow below.

1025

1026   To define immune subtype clusters, PCA, UMAP projection (with 10 PCs and 10 neighbors),

1027   and Leiden clustering at low resolution (k=10, resolution =0.5) were repeated in immune

1028   compartment cells. Major immune subtypes were annotated by examining top differentially

1029   expressed genes (DEGs) in conjunction with the following marker genes: T-cells and NK cells

1030   (*Cd3e, Nkg7*), B-cells (*Cd19*), plasma cells (*Jchain*), granulocytes (*Cxcr2, Csf3r*), dendritic cells

1031   (*Clec9a*), monocytes / macrophages (*Cd68, Mafb, Csf1r*), basophils (*Il3ra, Cxcr2*).

1032

1033   Differentially expressed genes were computed by contrasting each Leiden cluster against all

1034   other immune cells using the "rank_gene_groups" function implemented in Scanpy version 1.8.2

1035   using the Wilcoxon rank sum test with Benjamini-Hochberg correction.

1036

1037   This annotation process was repeated on the T/NK-cell subset to characterize the following

1038   phenotypes by marker gene expression: naïve CD4 T-Cells (*Cd4+ Foxp3- Cd69-*), activated

1039   CD4 T-Cells (*Cd4+ Cd69+*), CD4+ Tregs (*Cd4+ Foxp3+*), naïve CD8 T-Cells (*Cd8+ Cd69-*

1040   *Lag3-*), senescent-enriched CD8 T-Cells **(***Cd8+ Cd69+*), proliferating-enriched CD8 T-Cells

1041   (*Cd8+ Lag3+*), NKT Cells (*Klrb1c+*).

1042

1043   *scRNA-seq analysis of T/NK cells*

1044   Differential abundance analysis using Milo was performed specifically on T cell subsets (35)

1045   using a neighborhood size of n=50. Differentially abundant T-cell subtypes were identified by

1046   applying the Simes method for multiple hypothesis correction within a subtype cluster or

1047   Benjamini-Hochberg FDR correction across subtypes (setting FDR threshold at 0.2).

1048   Enrichment of a subset of *Cd8+ Cd69+* T-cells upon p53 reactivation was denoted "Senescent-

1049   enriched CD8 T-cells" and depletion of a subset of Cd8+ Cd69+ T-cells was denoted "Growing-

1050   enriched CD8 T-cells". For visualization purposes, significantly differentially enriched / depleted

1051   neighborhoods by SpatialFDR were visualized as implemented in the MiloPy Python package at

1052   FDR threshold of 0.2.

1053

1054   Differential gene expression analysis within CD8+ T-cell compartment was performed using the

1055   "rank_gene_groups" function as implemented in Python package Scanpy version 1.8.2.

1056   Geneset enrichment analysis using GSEA prerank as implemented in Python package GSEApy

1057   version 0.12.1. was performed using genes ranked by logFC. Genes were denoted as

1058   differentially expressed using an adjusted p-value cut-off of 0.05 and any human genesets

1059   tested were mapped to corresponding mouse orthologs using Ensembl annotations.

1060

1061   *scRNA-seq analysis of monocyte / macrophages*

1062   Similarly the T/NK-cell analysis, unbiased differential abundance analysis was performed using

1063   Milo as described above, but with a larger neighborhood size of 500 to account for the increase

1064   in cell number in the monocyte / macrophage cluster as well as aggregation at the level of

1065   Leiden clusters, not marker-defined subtypes.

1066

1067   Two Leiden clusters, one significantly enriched and one depleted upon p53 reactivation, were

1068   isolated for differential expression analysis and geneset enrichment as described in the

1069   preceding section and significantly enriched / depleted neighborhoods were visualized using the

1070   same SpatialFDR cut-off of 0.2.

1071

1072   **Statistical analyses**

1073   Statistical analyses were performed as described in the figure legend for each experiment.

1074   Group size was determined on the basis of the results of preliminary experiments, and no

1075   statistical method was used to predetermine sample size. The indicated sample size (n)

1076 represents biological replicates. All samples that met proper experimental conditions were

1077 included in the analysis. In particular, we have observed that in the orthotopic transplantation

1078 setting, the undesired lung metastasis (lung weight > 300 mg) occurred due to the technical

1079 limitation of liver injection. The lung metastasis may affect the tumor regression phenotype upon

1080 p53 restoration, and the mice were thus excluded from the analysis. Survival was measured

1081 using the Kaplan–Meier method. Statistical significance was determined by Student t test, log-

1082 rank test, Mann–Whitney test, Fisher exact test, and Pearson correlation using Prism 6

1083 Software (GraphPad Software) as indicated. Significance was set at $P < 0.05$.

1084

1085 **Figure Preparation**

1086 Figures were prepared using BioRender.com for scientific illustrations and Illustrator CC 2020

1087 (Adobe).

1088

1089 **Data Availability**

1090 RNA-seq data generated in this study are available in the Gene Expression Omnibus (GEO)

1091 database under the super-series GSE203140. The mass spectrometry proteomics data have

1092 been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the

1093 dataset identifier PXD034465.

1094

1095

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1097 **Authors' Contributions**

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45

46

REFERENCES

1. Di Micco R, Krizhanovsky V, Baker D, d'Adda di Fagagna F. Cellular senescence in ageing: from mechanisms to therapeutic opportunities. Nat Rev Mol Cell Biol **2021**;22(2):75-95 doi 10.1038/s41580-020-00314-w.
2. Munoz-Espin D, Serrano M. Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol **2014**;15(7):482-96 doi 10.1038/nrm3823.
3. Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR*, et al.* An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Dev Cell **2014**;31(6):722-33 doi 10.1016/j.devcel.2014.11.012.
4. Xu M, Pirtskhalava T, Farr JN, Weigand BM, Palmer AK, Weivoda MM*, et al.* Senolytics improve physical function and increase lifespan in old age. Nat Med **2018**;24(8):1246-56 doi 10.1038/s41591-018-0092-9.
5. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell **1997**;88(5):593-602 doi 10.1016/s0092-8674(00)81902-9.
6. Ewald JA, Desotelle JA, Wilding G, Jarrard DF. Therapy-induced senescence in cancer. Journal of the National Cancer Institute **2010**;102(20):1536-46 doi 10.1093/jnci/djq364.
7. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol **2010**;5:99-118 doi 10.1146/annurev-pathol-121808-102144.
8. Demaria M, O'Leary MN, Chang J, Shao L, Liu S, Alimirah F*, et al.* Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. Cancer Discov **2017**;7(2):165-76 doi 10.1158/2159-8290.CD-16-0241.
9. Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J*, et al.* Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS biology **2008**;6(12):2853-68 doi 10.1371/journal.pbio.0060301.
10. Tasdemir N, Banito A, Roe JS, Alonso-Curbelo D, Camiolo M, Tschaharganeh DF*, et al.* BRD4 Connects Enhancer Remodeling to Senescence Immune Surveillance. Cancer Discov **2016**;6(6):612-29 doi 10.1158/2159-8290.CD-16-0217.
11. Chien Y, Scuoppo C, Wang X, Fang X, Balgley B, Bolden JE*, et al.* Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. Genes Dev **2011**;25(20):2125-36 doi 10.1101/gad.17276711.
12. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ*, et al.* Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell **2008**;133(6):1019-31 doi 10.1016/j.cell.2008.03.039.
13. Basisty N, Kale A, Jeon OH, Kuehnemann C, Payne T, Rao C*, et al.* A proteomic atlas of senescence-associated secretomes for aging biomarker development. PLoS biology **2020**;18(1):e3000599 doi 10.1371/journal.pbio.3000599.
14. Hernandez-Segura A, de Jong TV, Melov S, Guryev V, Campisi J, Demaria M. Unmasking Transcriptional Heterogeneity in Senescent Cells. Curr Biol **2017**;27(17):2652-60 e4 doi 10.1016/j.cub.2017.07.033.
15. Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V*, et al.* Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature **2007**;445(7128):656-60 doi 10.1038/nature05529.
16. Kang TW, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D*, et al.* Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature **2011**;479(7374):547-51 doi 10.1038/nature10599.

47

1185   17.   Ovadya Y, Landsberger T, Leins H, Vadai E, Gal H, Biran A, *et al.* Impaired immune
1186   surveillance accelerates accumulation of senescent cells and aging. Nat Commun
1187   **2018**;9(1):5435 doi 10.1038/s41467-018-07825-3.
1188   18.   Lujambio A. To clear, or not to clear (senescent cells)? That is the question. Bioessays
1189   **2016**;38 Suppl 1:S56-64 doi 10.1002/bies.201670910.
1190   19.   Di Mitri D, Toso A, Chen JJ, Sarti M, Pinton S, Jost TR, *et al.* Tumour-infiltrating Gr-1+
1191   myeloid cells antagonize senescence in cancer. Nature **2014**;515(7525):134-7 doi
1192   10.1038/nature13638.
1193   20.   Ruscetti M, Leibold J, Bott MJ, Fennell M, Kulick A, Salgado NR, *et al.* NK cell-mediated
1194   cytotoxicity contributes to tumor control by a cytostatic drug combination. Science
1195   **2018**;362(6421):1416-22 doi 10.1126/science.aas9090.
1196   21.   Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, *et al.*
1197   Hepatocellular carcinoma. Nat Rev Dis Primers **2021**;7(1):6 doi 10.1038/s41572-020-
1198   00240-3.
1199   22.   Chiang DY, Villanueva A, Hoshida Y, Peix J, Newell P, Minguez B, *et al.* Focal gains of
1200   VEGFA and molecular classification of hepatocellular carcinoma. Cancer Res
1201   **2008**;68(16):6779-88 doi 10.1158/0008-5472.CAN-08-0742.
1202   23.   Llovet JM, Castet F, Heikenwalder M, Maini MK, Mazzaferro V, Pinato DJ, *et al.*
1203   Immunotherapies for hepatocellular carcinoma. Nat Rev Clin Oncol **2022**;19(3):151-72
1204   doi 10.1038/s41571-021-00573-2.
1205   24.   Sangro B, Sarobe P, Hervas-Stubbs S, Melero I. Advances in immunotherapy for
1206   hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol **2021**;18(8):525-43 doi
1207   10.1038/s41575-021-00438-0.
1208   25.   Levine AJ. Targeting the P53 Protein for Cancer Therapies: The Translational Impact of
1209   P53 Research. Cancer Res **2022**;82(3):362-4 doi 10.1158/0008-5472.CAN-21-2709.
1210   26.   Xiao Y, Chen J, Zhou H, Zeng X, Ruan Z, Pu Z, *et al.* Combining p53 mRNA
1211   nanotherapy with immune checkpoint blockade reprograms the immune
1212   microenvironment for effective cancer therapy. Nat Commun **2022**;13(1):758 doi
1213   10.1038/s41467-022-28279-8.
1214   27.   Demir O, Barros EP, Offutt TL, Rosenfeld M, Amaro RE. An integrated view of p53
1215   dynamics, function, and reactivation. Curr Opin Struct Biol **2021**;67:187-94 doi
1216   10.1016/j.sbi.2020.11.005.
1217   28.   Suda T, Liu D. Hydrodynamic gene delivery: its principles and applications. Mol Ther
1218   **2007**;15(12):2063-9 doi 10.1038/sj.mt.6300314.
1219   29.   Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, *et al.* Integrative
1220   transcriptome analysis reveals common molecular subclasses of human hepatocellular
1221   carcinoma. Cancer Res **2009**;69(18):7385-92 doi 10.1158/0008-5472.CAN-09-1089.
1222   30.   Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory
1223   CD8 T cell differentiation. Cell **2002**;111(6):837-51 doi 10.1016/s0092-8674(02)01139-x.
1224   31.   Bruix J, Cheng AL, Meinhardt G, Nakajima K, De Sanctis Y, Llovet J. Prognostic factors
1225   and predictors of sorafenib benefit in patients with hepatocellular carcinoma: Analysis of
1226   two phase III studies. J Hepatol **2017**;67(5):999-1008 doi 10.1016/j.jhep.2017.06.026.
1227   32.   Adrover JM, Aroca-Crevillen A, Crainiciuc G, Ostos F, Rojas-Vega Y, Rubio-Ponce A, *et*
1228   *al.* Programmed 'disarming' of the neutrophil proteome reduces the magnitude of
1229   inflammation. Nat Immunol **2020**;21(2):135-44 doi 10.1038/s41590-019-0571-2.
1230   33.   Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjallman AH, Ballmer-Hofer K,
1231   Schwendener RA. Clodronate-liposome-mediated depletion of tumour-associated
1232   macrophages: a new and highly effective antiangiogenic therapy approach. Br J Cancer
1233   **2006**;95(3):272-81 doi 10.1038/sj.bjc.6603240.
1234   34.   De Simone G, Andreata F, Bleriot C, Fumagalli V, Laura C, Garcia-Manteiga JM, *et al.*
1235   Identification of a Kupffer cell subset capable of reverting the T cell dysfunction induced

48

1236   by hepatocellular priming. Immunity **2021**;54(9):2089-100 e8 doi
1237   10.1016/j.immuni.2021.05.005.

1238 35.   Dann E, Henderson NC, Teichmann SA, Morgan MD, Marioni JC. Differential
1239   abundance testing on single-cell data using k-nearest neighbor graphs. Nat Biotechnol
1240   **2022**;40(2):245-53 doi 10.1038/s41587-021-01033-z.

1241 36.   Kim HD, Park S, Jeong S, Lee YJ, Lee H, Kim CG*, et al.* 4-1BB Delineates Distinct
1242   Activation Status of Exhausted Tumor-Infiltrating CD8(+) T Cells in Hepatocellular
1243   Carcinoma. Hepatology **2020**;71(3):955-71 doi 10.1002/hep.30881.

1244 37.   Li Y, Wang Z, Jiang W, Zeng H, Liu Z, Lin Z*, et al.* Tumor-infiltrating TNFRSF9(+) CD8(+)
1245   T cells define different subsets of clear cell renal cell carcinoma with prognosis and
1246   immunotherapeutic response. Oncoimmunology **2020**;9(1):1838141 doi
1247   10.1080/2162402X.2020.1838141.

1248 38.   Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC*, et al.*
1249   Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in
1250   human cancer. Immunity **2013**;39(4):782-95 doi 10.1016/j.immuni.2013.10.003.

1251 39.   Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C*, et al.* Activated monocytes in
1252   peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease
1253   progression through PD-L1. J Exp Med **2009**;206(6):1327-37 doi 10.1084/jem.20082173.

1254 40.   Lu LG, Zhou ZL, Wang XY, Liu BY, Lu JY, Liu S*, et al.* PD-L1 blockade liberates intrinsic
1255   antitumourigenic properties of glycolytic macrophages in hepatocellular carcinoma. Gut
1256   **2022** doi 10.1136/gutjnl-2021-326350.

1257 41.   Milanovic M, Fan DNY, Belenki D, Dabritz JHM, Zhao Z, Yu Y*, et al.* Senescence-
1258   associated reprogramming promotes cancer stemness. Nature **2018**;553(7686):96-100
1259   doi 10.1038/nature25167.

1260 42.   Yousef H, Czupalla CJ, Lee D, Chen MB, Burke AN, Zera KA*, et al.* Aged blood impairs
1261   hippocampal neural precursor activity and activates microglia via brain endothelial cell
1262   VCAM1. Nat Med **2019**;25(6):988-1000 doi 10.1038/s41591-019-0440-4.

1263 43.   Rapisarda V, Borghesan M, Miguela V, Encheva V, Snijders AP, Lujambio A*, et al.*
1264   Integrin Beta 3 Regulates Cellular Senescence by Activating the TGF-beta Pathway.
1265   Cell Rep **2017**;18(10):2480-93 doi 10.1016/j.celrep.2017.02.012.

1266 44.   Jochems F, Thijssen B, De Conti G, Jansen R, Pogacar Z, Groot K*, et al.* The Cancer
1267   SENESCopedia: A delineation of cancer cell senescence. Cell Rep **2021**;36(4):109441
1268   doi 10.1016/j.celrep.2021.109441.

1269 45.   Perna F, Berman SH, Soni RK, Mansilla-Soto J, Eyquem J, Hamieh M*, et al.* Integrating
1270   Proteomics and Transcriptomics for Systematic Combinatorial Chimeric Antigen
1271   Receptor Therapy of AML. Cancer Cell **2017**;32(4):506-19 e5 doi
1272   10.1016/j.ccell.2017.09.004.

1273 46.   Hoare M, Ito Y, Kang TW, Weekes MP, Matheson NJ, Patten DA*, et al.* NOTCH1
1274   mediates a switch between two distinct secretomes during senescence. Nat Cell Biol
1275   **2016**;18(9):979-92 doi 10.1038/ncb3397.

1276 47.   Castro F, Cardoso AP, Goncalves RM, Serre K, Oliveira MJ. Interferon-Gamma at the
1277   Crossroads of Tumor Immune Surveillance or Evasion. Front Immunol **2018**;9:847 doi
1278   10.3389/fimmu.2018.00847.

1279 48.   Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev
1280   Immunol **2005**;5(5):375-86 doi 10.1038/nri1604.

1281 49.   Manguso RT, Pope HW, Zimmer MD, Brown FD, Yates KB, Miller BC*, et al.* In vivo
1282   CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. Nature
1283   **2017**;547(7664):413-8 doi 10.1038/nature23270.

1284 50.   Song MM, Shuai K. The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not
1285   SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. J Biol
1286   Chem **1998**;273(52):35056-62 doi 10.1074/jbc.273.52.35056.

1287   51.   Kleppe M, Soulier J, Asnafi V, Mentens N, Hornakova T, Knoops L*, et al.* PTPN2
1288   negatively regulates oncogenic JAK1 in T-cell acute lymphoblastic leukemia. Blood
1289   **2011**;117(26):7090-8 doi 10.1182/blood-2010-10-314286.
1290   52.   Zhou F. Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen
1291   processing and presentation. Int Rev Immunol **2009**;28(3-4):239-60 doi
1292   10.1080/08830180902978120.
1293   53.   McCarthy MK, Weinberg JB. The immunoproteasome and viral infection: a complex
1294   regulator of inflammation. Front Microbiol **2015**;6:21 doi 10.3389/fmicb.2015.00021.
1295   54.   Yoshihama S, Vijayan S, Sidiq T, Kobayashi KS. NLRC5/CITA: A Key Player in Cancer
1296   Immune Surveillance. Trends Cancer **2017**;3(1):28-38 doi 10.1016/j.trecan.2016.12.003.
1297   55.   Kalaora S, Lee JS, Barnea E, Levy R, Greenberg P, Alon M*, et al.* Immunoproteasome
1298   expression is associated with better prognosis and response to checkpoint therapies in
1299   melanoma. Nat Commun **2020**;11(1):896 doi 10.1038/s41467-020-14639-9.
1300   56.   Efeyan A, Ortega-Molina A, Velasco-Miguel S, Herranz D, Vassilev LT, Serrano M.
1301   Induction of p53-dependent senescence by the MDM2 antagonist nutlin-3a in mouse
1302   cells of fibroblast origin. Cancer Res **2007**;67(15):7350-7 doi 10.1158/0008-5472.CAN-
1303   07-0200.
1304   57.   Hoekstra ME, Bornes L, Dijkgraaf FE, Philips D, Pardieck IN, Toebes M*, et al.* Long-
1305   distance modulation of bystander tumor cells by CD8(+) T cell-secreted IFNgamma. Nat
1306   Cancer **2020**;1(3):291-301 doi 10.1038/s43018-020-0036-4.
1307   58.   Ruscetti M, Morris JPt, Mezzadra R, Russell J, Leibold J, Romesser PB*, et al.*
1308   Senescence-Induced Vascular Remodeling Creates Therapeutic Vulnerabilities in
1309   Pancreas Cancer. Cell **2020**;181(2):424-41 e21 doi 10.1016/j.cell.2020.03.008.
1310   59.   Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced in vivo growth and resistance to
1311   rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity
1312   **1994**;1(6):447-56 doi 10.1016/1074-7613(94)90087-6.
1313   60.   Gao J, Shi LZ, Zhao H, Chen J, Xiong L, He Q*, et al.* Loss of IFN-gamma Pathway
1314   Genes in Tumor Cells as a Mechanism of Resistance to Anti-CTLA-4 Therapy. Cell
1315   **2016**;167(2):397-404 e9 doi 10.1016/j.cell.2016.08.069.
1316   61.   Paffenholz SV, Salvagno C, Ho YJ, Limjoco M, Baslan T, Tian S*, et al.* Senescence
1317   induction dictates response to chemo- and immunotherapy in preclinical models of
1318   ovarian cancer. Proc Natl Acad Sci U S A **2022**;119(5) doi 10.1073/pnas.2117754119.
1319   62.   Shao DD, Xue W, Krall EB, Bhutkar A, Piccioni F, Wang X*, et al.* KRAS and YAP1
1320   converge to regulate EMT and tumor survival. Cell **2014**;158(1):171-84 doi
1321   10.1016/j.cell.2014.06.004.
1322   63.   Zhu C, Kim K, Wang X, Bartolome A, Salomao M, Dongiovanni P*, et al.* Hepatocyte
1323   Notch activation induces liver fibrosis in nonalcoholic steatohepatitis. Sci Transl Med
1324   **2018**;10(468) doi 10.1126/scitranslmed.aat0344.
1325   64.   Susaki EA, Tainaka K, Perrin D, Yukinaga H, Kuno A, Ueda HR. Advanced CUBIC
1326   protocols for whole-brain and whole-body clearing and imaging. Nat Protoc
1327   **2015**;10(11):1709-27 doi 10.1038/nprot.2015.085.
1328   65.   Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence
1329   data. Bioinformatics **2014**;30(15):2114-20 doi 10.1093/bioinformatics/btu170.
1330   66.   Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S*, et al.* STAR: ultrafast
1331   universal RNA-seq aligner. Bioinformatics **2013**;29(1):15-21 doi
1332   10.1093/bioinformatics/bts635.
1333   67.   Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput
1334   sequencing data. Bioinformatics **2015**;31(2):166-9 doi 10.1093/bioinformatics/btu638.
1335   68.   Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
1336   RNA-seq data with DESeq2. Genome Biol **2014**;15(12):550 doi 10.1186/s13059-014-
1337   0550-8.

1338 69. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV*, et al.* Enrichr: interactive and
1339   collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics
1340   **2013**;14:128 doi 10.1186/1471-2105-14-128.
1341 70. Foroutan M, Bhuva DD, Lyu R, Horan K, Cursons J, Davis MJ. Single sample scoring of
1342   molecular phenotypes. BMC Bioinformatics **2018**;19(1):404 doi 10.1186/s12859-018-
1343   2435-4.
1344 71. Binder JX, Pletscher-Frankild S, Tsafou K, Stolte C, O'Donoghue SI, Schneider R*, et al.*
1345   COMPARTMENTS: unification and visualization of protein subcellular localization
1346   evidence. Database (Oxford) **2014**;2014:bau012 doi 10.1093/database/bau012.
1347 72. Cancer Genome Atlas Research Network. Electronic address wbe, Cancer Genome
1348   Atlas Research N. Comprehensive and Integrative Genomic Characterization of
1349   Hepatocellular Carcinoma. Cell **2017**;169(7):1327-41 e23 doi 10.1016/j.cell.2017.05.046.
1350 73. Colaprico A, Silva TC, Olsen C, Garofano L, Cava C, Garolini D*, et al.* TCGAbiolinks: an
1351   R/Bioconductor package for integrative analysis of TCGA data. Nucleic Acids Res
1352   **2016**;44(8):e71 doi 10.1093/nar/gkv1507.
1353 74. Hanzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray
1354   and RNA-seq data. BMC Bioinformatics **2013**;14:7 doi 10.1186/1471-2105-14-7.
1355 75. Bankhead P, Loughrey MB, Fernandez JA, Dombrowski Y, McArt DG, Dunne PD*, et al.*
1356   QuPath: Open source software for digital pathology image analysis. Sci Rep
1357   **2017**;7(1):16878 doi 10.1038/s41598-017-17204-5.
1358 76. Bernstein NJ, Fong NL, Lam I, Roy MA, Hendrickson DG, Kelley DR. Solo: Doublet
1359   Identification in Single-Cell RNA-Seq via Semi-Supervised Deep Learning. Cell Syst
1360   **2020**;11(1):95-101 e5 doi 10.1016/j.cels.2020.05.010.
1361 77. Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell
1362   gene expression data. Nat Biotechnol **2015**;33(5):495-502 doi 10.1038/nbt.3192.
1363 78. McInnes L, Healy J, Melville J. UMAP: Uniform Manifold Approximation and Projection
1364   for Dimension Reduction. arXiv **2018**:doi: 10.48550/ARXIV.1802.03426.
1365 79. Traag VA, Waltman L, van Eck NJ. From Louvain to Leiden: guaranteeing well-
1366   connected communities. Sci Rep **2019**;9(1):5233 doi 10.1038/s41598-019-41695-z.
1367 80. Korsunsky I, Millard N, Fan J, Slowikowski K, Zhang F, Wei K*, et al.* Fast, sensitive and
1368   accurate integration of single-cell data with Harmony. Nat Methods **2019**;16(12):1289-96
1369   doi 10.1038/s41592-019-0619-0.
1370
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1374 **MAIN FIGURE LEGENDS**

1375 **Figure 1. A p53-restorable tumor model to study senescence immune surveillance**

1376 A, Generation of the p53-restorable, NRAS-driven mouse liver cancer model using the sleeping

1377 beauty transposon system delivered through hydrodynamic tail vein injection (HTVI). (Created

1378 with BioRender.com.)

1379 B, Representative ultrasonogram of HTVI and orthotopic injection liver cancer models at

1380 indicated time after p53 restoration.

1381 C, Survival analysis of mice in the HTVI model.

1382 D, Representative hematoxylin and eosin (H&E), immunofluorescence (IF) and senescence-

1383 associated β-Gal (SA-β-Gal) staining of p53-suppressed (p53 Off) and -restored (p53 On for 14

1384 days) tumor sections generated from the HTVI model. Scale bar, 50 μm.

1385 E to G, Orthotopic injection of GFP-luciferase vector-transduced NSP tumor cells into the livers

1386 of immunocompetent and –deficient mouse strains.

1387 E, Tumor size change measured by ultrasound upon p53 restoration. R2G2, Rag2-Il2rg double

1388 knockout mouse. Data are presented as mean ± s.e.m. N ≥ 9 for each strain.

1389 F, Representative macroscopic pictures at 21 days of p53 On or end-point p53 Off tumor.

1390 G, Representative immunohistochemistry (IHC) staining of GFP-labeled tumor cells at day 21

1391 upon p53 restoration. Scale bar, 100 μm.

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1393 **Figure 2. Senescence triggers an immune evasion-to-immune recognition tumor switch**

1394 A, Representative images of CD45 and GFP staining marking immune cells and tumor cells,

1395 respectively in p53-suppressed and p53-restored tumor (7 days after p53 restoration). Right

1396 panel is the quantification of the area of CD45+ staining calculated from 3 random fields per

1397 mouse. Each dot represents a mouse.

1398 B, Flow cytometry analysis of global immune landscape in orthotopic NSP liver tumor model.

1399 Immunophenotyping of senescent tumors is performed 9 days after Dox withdrawal, a time point

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1400 when the senescent state is fully established yet, preceding the massive tumor regression. G-

1401 MDSC, granulocytic myeloid-derived suppressor cells. M-MDSC, monocytic myeloid-derived

1402 suppressor cells. Data is pooled from 2 independent experiments with n=7 in the proliferating

1403 group and n=9 in the senescent group. Note that, as absolute number of CD45+ cells increases

1404 in senescent NSP tumor lesions (A), so do the total numbers of the indicated cell types.

1405 C, Flow cytometry analysis of CD8 T cells. Data is pooled from 2 independent experiments with

1406 n=11 in the proliferating and n=10 in the senescent groups. Experiments were performed at 9

1407 days after Dox withdrawal.

1408 D, Representative tissue clearing images of the orthotopic NSP liver tumors. T cells, neutrophils

1409 and vasculature are labeled by CD3, MPO and CD31 staining, respectively. Samples were

1410 collected at 9 days after Dox withdrawal.

1411 E, Tumor size change measured by ultrasound upon p53 restoration in mice after depleting

1412 specific immune cell types using antibodies or drugs.

1413 F, Left panel, UMAP plot of CD8+ T cells isolated from p53-suppressed proliferating (PRO) and

1414 p53-reactivated senescent (SEN) tumors. Right panel, GSEA of a T cell exhaustion marker

1415 genes in CD8+ T cells from proliferating (p53-suppressed) vs senescent (p53-reactivated)

1416 tumors.

1417 G, UMAP plot of the expression of selected genes (*Cd8a*, *Cd44*, *Tnfrsf9*, *Cd69*, *Tox, Fasl*)

1418 between CD8+ T cells isolated from senescent (p53-reactivated) and proliferating (p53-

1419 suppressed) tumors.

1420 H, Representative immunofluorescence images of CD8 T cells and F4/80 positive macrophages

1421 staining in the orthotopic NSP liver tumor. Tumor samples were collected at 9 days after Dox

1422 withdrawal.

1423 Data is presented as mean ± s.e.m. Scale bar, 100 μm. A two-tailed student t-test is used. *p <

1424 0.05; **p < 0.01.

1425

1426 **Figure 3. Senescence remodels tissue sensing programs and cell-surfaceome landscape**

1427 A, GSEA (Reactome) of RNA-Seq data from proliferating (PRO, p53 Off) vs. senescent (SEN,

1428 p53 On for 8 days) NSP liver tumor cells *in vitro.*

1429 B, Subcellular localization of DEGs (p < 0.05; fold change > 2) all detected genes (TPM > 1)

1430 from RNA-seq.

1431 C, Gene Ontology (GO) analysis of DEGs encoding plasma membrane proteins upregulated in

1432 senescent cells.

1433 D, Transcriptomic analysis of all differential expressed genes (DEGs, proliferating vs. senescent)

1434 in the presence or absence JQ1 treatment. C1 cluster (in red) contains the senescence-specific

1435 genes sensitive to JQ1 and C4 cluster (in blue) contains the proliferation-specific genes

1436 sensitive to JQ1.

1437 E, Meta-analysis of RNA-seq datasets from SENESCOPEDIA by performing subcellular

1438 localization of DEGs (same as Fig. 2D) and Fisher exact test to examine the relative enrichment

1439 of up- and downregulated EC/PM-DEGs deviated from the random distribution. See also

1440 Supplementary Fig. S7E and S7F.

1441 F, Mass spectrometry (MS) analysis of plasma membrane-enriched proteome in proliferating

1442 and senescent cells. Protein level is normalized to mean expression of the protein of all samples.

1443 Controls are the samples without biotin labeling serving as background. Red and blue boxes

1444 represent proteins enriched in senescent and proliferating cells respectively. N=6 for both

1445 senescent and proliferating experimental group, and N=3 and 4 respectively for their control.

1446 G, Distribution of up- and downregulated Genecard-annotated plasma membrane (PM) proteins

1447 profiled by MS.

1448 H, Volcano plot of Genecard-annotated plasma membrane proteins profiled by MS.

1449 EC, extracellular; PM, plasma membrane.

1450

1451 **Figure 4. Senescent cells are primed to sense and amplify IFN-γ signaling**

54

1452   A and B, IFNGR1 level on proliferating and senescent cells profiled by mass spectrometry and

1453   validated by flow cytometry (B). AU, arbitrary unit. Data is presented as mean ± s.e.m. n = 6 for

1454   both proliferating and senescent group.

1455   C, Transcriptomic analysis of selected genes regulating IFN-γ signaling from RNA-seq data of 3

1456   independent p53-restorable cell lines (NSP, NSM2, NSP5) restoring p53 along with NSP cells

1457   treated with two other senescence triggers. T+P, trametinib plus palbociclib.

1458   D, mRNA expression of selected genes involved in IFN-γ signaling in human cell lines triggered

1459   to senesce. Treatment: Ali, alisertib; Eto, etoposide; number indicates the length of treatment

1460   (days). Data is obtained from the public dataset SENESCOPEDIA (44).

1461   E, Top panel, immunoblot analysis of NSP cells under different senescent triggers, in presence

1462   or absence of IFN-γ (1 ng/ml). Bottom panel, quantification of the intensity of signal from

1463   immunoblot. p-Stat1, phospho-Stat1 (Tyr701).

1464
1465   **Figure 5. Senescence and extracellular IFN-γ cooperate to upregulate antigen processing**

1466   **and presentation machinery**

1467   A and B, mRNA expression of genes in proliferating and senescent NSP cells *in vitro* in the

1468   presence or absence of IFN-γ (50 pg/ml) treatment. mRNA level is normalized to the mean

1469   expression of the gene in all samples. A, differential expressed genes encoding SASP factors in

1470   our model. B, IFN-γ response genes from Hallmark signature database.

1471   C, RT-qPCR of selected antigen presentation pathway genes in proliferating and senescent

1472   cells treated with low (50 pg/ml) or high (1 ng/ml) concentration of IFN-γ. Samples are from 2

1473   biological replicates.

1474   D, MHC-I level of proliferating and senescent cells treated with IFN-γ for 24 hours measured by

1475   flow cytometer.

1476   MFI, median fluorescence intensity. Data is presented as mean ± s.e.m.

1477

Figure 6. Senescence enhances IFN-γ-mediated heterotypic signaling from activated immune cells to tumor cells

A, Graphic illustration of IFN-γ sensing (IGS) reporter. (Created with BioRender.com.)

B, Left panel, representative flow cytometry plots measuring ZsGreen1 signals in proliferating and senescent NSP cells treated with 1 ng/ml IFN-γ. Right panel, quantification of the percentage of ZsGreen1 positive cells upon IFN-γ treatment.

C and D, Representative 3D imaging of tissue cleared tumors from the orthotopically injected liver NSP cell line expressing IGS reporter (C). Quantification of 3 randomly selected fields from the liver tumor of each mouse (D). N=5 and N=3 for the proliferating and senescent group (9 days after p53 restoration) respectively.

E, Top panel, cytometric bead array (CBA) assay for IFN-γ level from *in vivo* tumor tissue lysate samples (7 days after p53 restoration). Bottom panel, Transcripts of indicated genes from RNA-seq of in vivo bulk samples of tumors generated by HTVI (PRO, p53 Off; SEN, p53 restoration for 12 days). TPM, Transcripts Per Kilobase Million. Noted *Ifna/b* cluster contains 14 *Ifna* subtypes and 1 *Ifnb* gene.

F and G, Expression of Ifng in tumor infiltrating immune cells profiled by scRNA-seq in NSP transplantable model (as in Figure 2, sample collected at day 8 after p53 restoration).

H, UMAP plot of the expression of Havcr2 (encoding TIM-3) and Ifng in CD8 T cells harvested from proliferating (P) and senescent (S) tumor lesion. Top panel is replicated from Figure 2F (left) to indicate cells corresponding to each condition.

I, Quantification of ZsGreen1 intensity of NSP tumor cells in the OT-I T cells and SIINFEKL-expressing tumor cells co-culture experiment (E/T ratio 5:1) after 20 h of co-culture. Signal measured by flow cytometry. T+P, trametinib plus palbociclib. See experimental details in supplementary Fig. S12E.

MFI, median fluorescence intensity. Data is presented as mean ± s.e.m. Two-tailed student t-test is used. *p < 0.05. Scale bar, 100 μm.

1504

1505 **Figure 7. IFN-γ signaling in senescent tumor cells is necessary for immune surveillance**

1506 A, *Ifngr1* knockout (KO) of both proliferating and senescent NSP cells validated by flow

1507 cytometry.

1508 B, Tumor regression phenotype of *Ifngr1* KO or control sgRNA-transfected tumor cells

1509 orthotopically injected into Bl/6N mice upon p53 restoration. A control sgRNA targeting a gene

1510 desert located on Chr8 (Ctrl KO) serves as a control.

1511 C, Tumor regression phenotype of parental NSP tumor cells orthotopically injected into WT or

1512 *Ifng* KO mice upon p53 restoration.

1513 D, Representative macroscopic images of tumor collected at day 21 after p53 restoration from

1514 (C).

1515 E, Flow cytometry analysis of CD45 abundance in tumor from indicated groups.

1516 F, Representative immunofluorescence in p53-suppressed (proliferating) and p53-restored

1517 (senescent, 7 days after p53 restoration) tumor from the indicated host.

1518 NSP tumor cells were transduced with GFP-expressing vector for visualization. Scale bar, 50

1519 μm

1520 Data is presented as mean ± s.e.m. Two-tailed student t-test is used. *p < 0.05; **p < 0.01; ***p

1521 < 0.001.

1522
1523

Fig. 1. A p53-restorable tumor model to study senescence immune surveillance
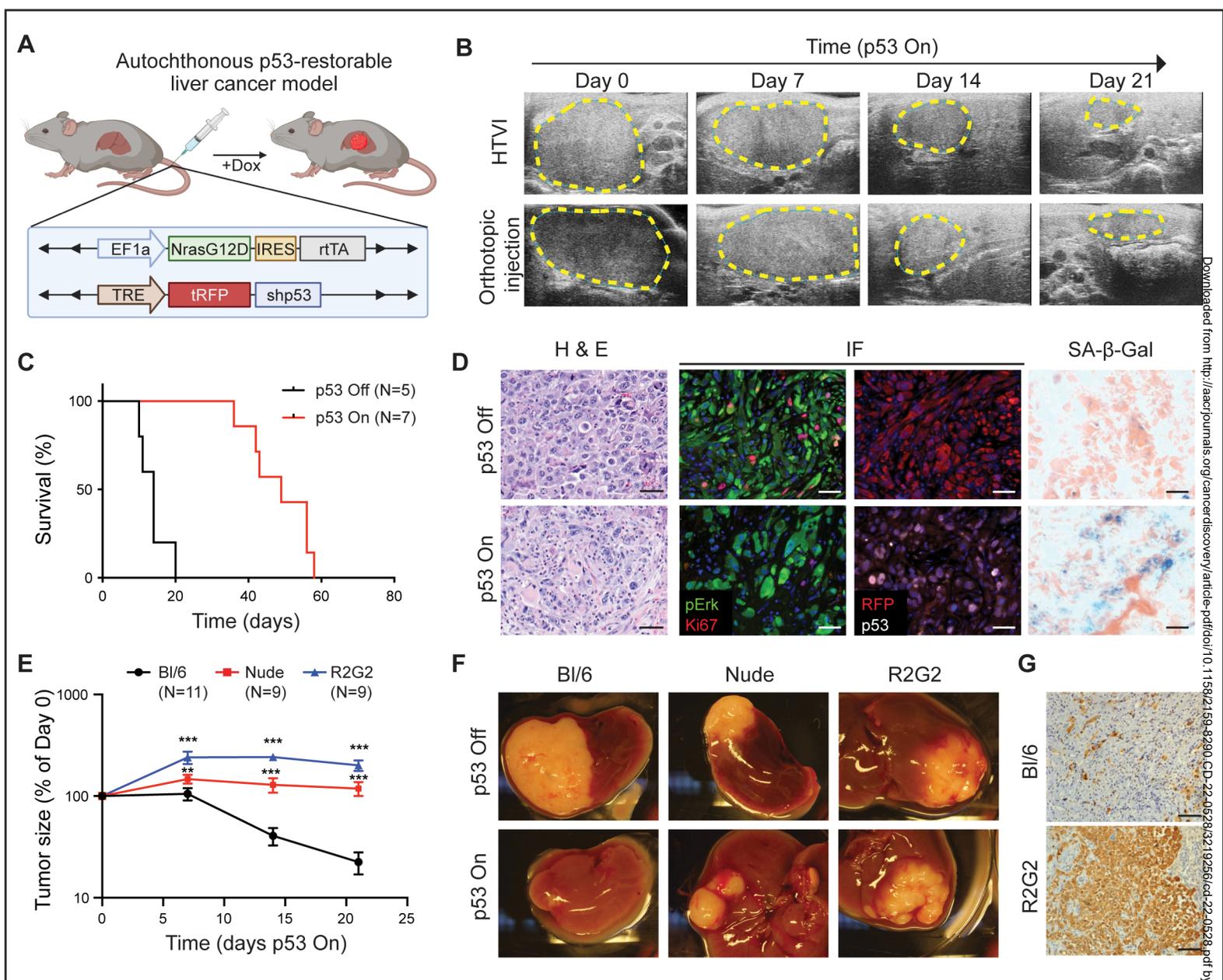
Figure 2. Senescence triggers an immune evasion-to-immune recognition tumor switch

Figure 3. Senescence remodels tissue sensing programs and cell-surfaceome landscape

A RNA-seq: p53-On (SEN) vs p53-Off (PRO)

SEN - UP
SEN - DN

NES score (SEN vs. PRO)

Immune System
Innate Immune System
Vesicle-Mediated Transport
Membrane Trafficking
Neutrophil Degranulation
Transport To The Golgi
Mitotic Prometaphase
M Phase
RNA processing
Cell Cycle, Mitotic
Metabolism Of RNA
Cell Cycle

B Differentially expressed genes (subcellular localization)

SEN - UP
N=1265
40% | 14% | 25% | 21%

SEN - DN
N=1346
35% | 7% | 15% | 43%

All detected
N=12894
46% | 6% | 15% | 33%

Extracellular (EC) - SASP
Plasma Membrane (PM) - Cell-surface
Nucleus
Rest

C Plasma Membrane genes - SEN-UP
(GO_Biological Process)

-log(p-val)

Regulation of cell migration
Regulation of peptidyl-tyrosine phosphorylation
TM receptor protein tyrosine kinase signaling pathway
Cell-matrix adhesion
Positive regulation of signal transduction
Integrin-mediated signaling pathway

E Pan-senescence analysis

SEN - UP
SEN - DN

# of datasets

EC | PM | EC | PM

Significant (p <0.01)
Not significant

G Cell surfaceome dynamics
(PM-annotated)

N=233
47% | 20% | 33%

SEN-UP
SEN-DN
NC

D JQ1-sensitive gene programs

SEN-specific
PRO-specific

JQ-1
PRO -
SEN -
PRO +
SEN +

C1   C2   C3   C4
N=771   N=665   N=588   N=801

Cell surface-enriched Mass Spectrometry (MS)

C1
C2
C3
C4

DEGs (% in cluster)

EC
PM

F

PRO Ctrl | SEN Ctrl | PRO | SEN

Cell surface-enriched Mass Spectrometry (MS)

EGFR
DBN1
VCAM1
IFNGR1
BMPR2
ITGB4

PODXL
LRP2
CDHR2
ERBB2
CPEB4
SVIL

Protein log2FC

H

-log(p-val)

CDH1
SLC4A7
F3
ABCA7
CD44
IFNGR1
VCAM1
COPB1

Protein abundance
log2FC (SEN/PRO)

Figure 4. Senescent cells are primed to sense and amplify IFN-γ signaling

Figure 5. Senescence and extracellular IFN-γ cooperate to upregulate antigen processing and presentation machinery
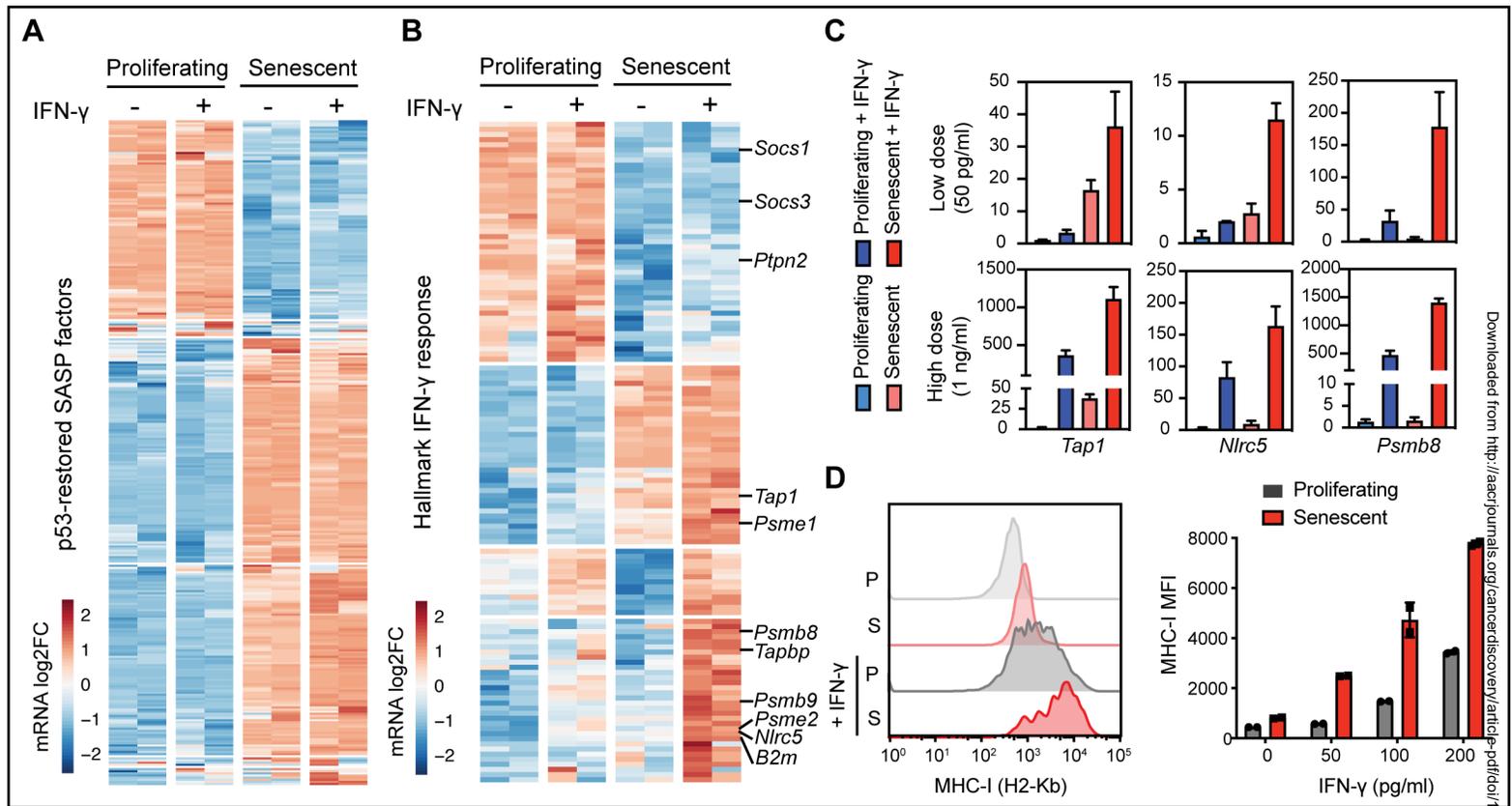
Figure 6. Senescence enhances IFN-γ-mediated heterotypic signaling from activated immune cells to tumor cells

Figure 7. IFN-γ signaling in senescent tumor cells is necessary for immune surveillance