

1 **A combined PD-1/C5a blockade synergistically protects against lung cancer**
2 **growth and metastasis**

3 Daniel Ajona^{1,2,3,4}, Sergio Ortiz-Espinosa¹, Haritz Moreno^{1,2}, Teresa Lozano^{2,5}, María J
4 Pajares^{1,2,3,6}, Jackeline Agorreta^{1,2,3,6}, Cristina Bértolo¹, Juan J Lasarte^{2,5}, Silvestre Vicent^{1,2,6},
5 Axel Vater⁷, Fernando Lecanda^{1,2,3}, Luis M Montuenga^{1,2,3,6}, Ruben Pio^{1,2,3,4}

6
7 **Affiliations of authors:** ¹University of Navarra, Center for Applied Medical Research
8 (CIMA), Program in Solid Tumors and Biomarkers, Pamplona, Spain. ²Navarra's Health
9 Research Institute (IdiSNA), Pamplona, Spain. ³Centro de Investigación Biomédica en Red de
10 Cáncer (CIBERONC). ⁴University of Navarra, School of Sciences, Department of
11 Biochemistry and Genetics, Pamplona, Spain. ⁵University of Navarra, CIMA, Program in
12 Immunology and Immunotherapy, Pamplona, Spain. ⁶University of Navarra, School of
13 Medicine, Department of Histology and Pathology, Pamplona, Spain. ⁷Aptarion biotech,
14 Berlin, Germany.

15 **Running head:** Anti-PD-1 and anti-C5a combined immunotherapy in lung cancer

16 **Keywords:** PD-1, C5a, immunotherapy, combined therapy, complement system, lung cancer

17 **Grant support:** This work was supported by Foundation for Applied Medical Research
18 (FIMA), Red Temática de Investigación Cooperativa en Cáncer (RD12/0036/0040),
19 CIBERONC CB16/12/00443, Fondo de Investigación Sanitaria-Fondo Europeo de Desarrollo
20 Regional (FEDER, PI13/00806, PI14/01686), Spanish Ministry of Economy and
21 Competitiveness (SAF2013-46423-R and SAF2015-71606-R), and European Commission
22 (618312 KRASmiR FP7-PEOPLE- 2013-CIG). S.V. is a fellow of the Ramón y Cajal
23 Program (MICINN, RYC-2011- 09042). F.L. is funded by “la Caixa” Foundation and Caja
24 Navarra Foundation.

25

1 **Corresponding author:** Ruben Pio. Program in Solid Tumors and Biomarkers. CIMA
2 Building, Pio XII 55, 31008 Pamplona, Spain. E-mail: rpio@unav.es. Tel: +34 948194700.
3 Fax: +34 948194714.

4 **Disclosure of potential conflicts of interest:** Axel Vater is founder of APTARION biotech,
5 which holds intellectual property rights to certain anti-C5a L-aptamers, including AON-D21.
6 The other authors declare no conflict of interest.

7 **Statement of significance:**

8 Using a variety of preclinical models of lung cancer, we demonstrate that the blockade of C5a
9 results in a substantial improvement in the efficacy of anti-PD-1 antibodies against lung
10 cancer growth and metastasis. This study provides the preclinical rationale for the combined
11 blockade of PD-1/PD-L1 and C5a to restore antitumor immune responses, inhibit tumor cell
12 growth, and improve lung cancer patient outcomes.

1 **ABSTRACT**

2 Disruption of the programmed cell death protein 1 (PD-1) pathway with immune checkpoint
3 inhibitors represents a major breakthrough in the treatment of non-small cell lung cancer. We
4 hypothesized that a combined inhibition of C5a/C5aR1 and PD-1 signaling may have an
5 antitumor synergistic effect. RMP1-14 antibody was used to block PD-1 and an L-aptamer to
6 inhibit signaling of complement C5a with its receptors. Using syngeneic models of lung
7 cancer we demonstrate that the combination of C5a and PD-1 blockade markedly reduces
8 tumor growth and metastasis, and leads to prolonged survival. This effect is accompanied by a
9 negative association between the frequency of CD8 T cells and myeloid-derived suppressor
10 cells (MDSCs) within the tumors, which may result in a more complete reversal of CD8 T-
11 cell exhaustion. Our study provides support for the clinical evaluation of anti-PD-1 and anti-
12 C5a drugs as a novel combination therapeutic strategy for lung cancer.

1 INTRODUCTION

2 Immunotherapy based on checkpoint inhibitors has emerged as a potent tool for the treatment
3 of lung cancer, the leading cause of cancer death worldwide. Among the numerous
4 immunotherapeutic strategies, monoclonal antibodies that inhibit the interaction between
5 programmed death-1 (PD-1) and its ligands have shown the most compelling clinical results
6 in lung cancer. Monoclonal antibodies raised against PD-1 have been approved by the FDA
7 for patients with metastatic non-small cell lung cancer (1). By blocking PD-1, these drugs
8 remove the inhibition of T-cell activation and restore antitumor immune responses. However,
9 PD-1 inhibition is not capable of reversing all resistance mechanisms and a proportion of
10 patients do not respond adequately to anti-PD-1 immunotherapies. Consequently,
11 combination therapies, that block more than one immunomodulatory pathway, have been
12 proposed to further enhance the antitumor efficacy of anti-PD-1 individual treatments (2).

13 A hallmark mechanism of synergy in immunotherapy is the reactivation of effector T cells
14 together with the elimination of immunosuppressive cells, such as myeloid-derived
15 suppressor cells (MDSCs) or regulatory T cells (Tregs). In this regard, PD-1/PD-L1 blockade
16 is not able to reduce the T cell suppressive activity of the tumor microenvironment, which can
17 be caused by the accumulation of MDSCs (3) or Tregs (4). Therefore, a successful reversal of
18 the tumor immunosuppressive microenvironment represents a window of opportunity to
19 overcome tumor resistance in cancer immunotherapy.

20 Anaphylatoxin C5a, an active proteolytic fragment released after activation of the
21 complement system, contributes to lung cancer progression by promoting an
22 immunosuppressive microenvironment in which MDSCs are involved (5,6). All pathways of
23 complement, an essential part of innate immunity, converge in the cleavage and activation of
24 the central components C3 and C5, which leads to the release of anaphylatoxins C3a and C5a.

25 Although C5a is generally known to be a chemoattractant for pro-inflammatory leukocytes,

1 several studies have demonstrated a tumor-promoting role for this molecule and the idea of
2 blocking complement for the treatment of cancer is gaining recognition (7). In mouse models
3 of cancer, complement deficiencies and pharmacological blockade of complement-related
4 mediators, including C5a, have been associated with impaired tumor growth (8). C5a is also
5 able to modulate antitumor immunity by altering T cell responses in premetastatic niches (9).
6 In the case of lung cancer, the expression of some immunosuppressive molecules is
7 significantly reduced after blockade of the C5a receptor-1 (C5aR1) (5). Besides, C5aR1
8 blockade diminishes the percentage and activity of MDSCs in cancer models, including lung
9 cancer (5,8,10). By studying C3 knockout mice, it has been recently shown that complement
10 inhibits antitumor immunity through a PD-1 independent pathway (11). All these studies
11 reveal the important role played by C5a/C5aR1 signaling in tumor immunity, and point to this
12 pathway as a potential therapeutic target in the context of checkpoint inhibition.

13 In this study we have evaluated the therapeutic efficacy of the combined administration of
14 anti-PD-1 and anti-C5a drugs in a variety of lung cancer models. From these experiments, we
15 conclude that C5a blockade results in a more permissive environment for immune-mediated
16 tumor rejection and, consequently, its combined administration with anti-PD-1 antibodies
17 synergistically impairs lung cancer growth and metastasis.

1 RESULTS

2 C5aR1 genetic deficiency and C5a pharmacological blockade decreases lung tumor 3 growth

4 To explore the role of C5a/C5aR1 signaling in lung cancer initiation and progression, we
5 crossed C5aR1 deficient mice (C5aR1^{+/-}) to Kras^{LSL-G12D/+} mice that develop lung
6 adenocarcinomas by inducing the expression of oncogenic Kras upon intratracheal inoculation
7 of Cre recombinase. Deletion of C5aR1 had a noticeable effect on mutant Kras-induced lung
8 carcinogenesis (Fig. 1A). Thus, Kras^{LSL-G12D/+};C5aR1^{-/-} mice developed significantly smaller
9 tumors compared to C5aR1 wild-type littermates, while no differences were observed in the
10 total number of tumors (Fig. 1B). Kras^{LSL-G12D/+};C5aR1^{-/-} mice showed a reduced percentage
11 of myeloid-derived suppressor cells (MDSCs) in their spleens (Fig. 1C). No differences were
12 observed in the frequencies of CD4 T cells, CD8 T cells or Tregs (data not shown).
13 Immunohistochemical analysis of the tumors showed an increase in CD3 cells from Kras^{LSL-}
14 ^{G12D/+};C5aR1^{-/-} mice close to statistical significance (Fig. 1D). No differences were found in
15 proliferation (Ki67), apoptosis (cleaved caspase-3) or angiogenesis (CD31) (data not shown).
16 Using an independent mouse cohort, we observed that survival of Kras^{LSL-G12D/+};C5aR1^{-/-} mice
17 was significantly longer than that of Kras^{LSL-G12D/+};C5aR1^{+/+} littermates (Fig. 1E).
18 We next investigated the effect of the pharmacological blockade of C5a in a syngeneic mouse
19 model based on the subcutaneous growth of 393P cells, a mouse model of Kras-driven lung
20 adenocarcinoma (12). We used AON-D21 (formerly NOX-D21), an L-aptamer which tightly
21 binds to both mouse and human C5a and efficiently inhibits the interaction with its receptors
22 (Supplementary Results, Supplementary Fig. S1 and Supplementary Table S1 show
23 information about the pharmacology and other characteristics of this molecule). AON-D21
24 treatment led to a partial reduction of tumor growth when compared to the effect of revAON-
25 D21, a non-functional control L-aptamer (Fig. 1F). Analysis of splenocyte subpopulations

1 revealed a reduction in the percentage of MDSCs (Fig. 1G), with no significant differences in
2 CD4 T cells, CD8 T cells or Tregs (data not shown). These genetic and pharmacological
3 results demonstrate the influence of the C5a/C5aR1 pathway on lung tumor growth and the
4 potential value of blocking C5a for the treatment of lung cancer.

5

6 **C5a inhibition synergizes with PD-1 blockade to prevent lung cancer growth and** 7 **metastasis**

8 We postulated that an anti-C5a treatment may enhance the capacity of PD-1 blockade to
9 control lung tumor growth. In the subcutaneous 393P model, the combination of the anti-C5a
10 L-aptamer AON-D21 (every other day) with the anti-PD-1 monoclonal antibody RMP1-14
11 (days 7, 10 and 14) resulted in a significant reduction of tumor growth, as compared to the
12 effect of each treatment alone (Fig. 2A and Supplementary Fig. S2A). By day 22, tumor
13 volumes in mice treated with the combined therapy were lower than those in control mice,
14 mice treated with the anti-PD-1 antibody or treated with the anti-C5a aptamer. Remarkably,
15 by day 41, all the mice (n=9) in the combination group showed complete tumor rejection. In
16 contrast, at day 48, only one mouse in the control group, two in the group treated with AON-
17 D21 and four in the group treated with anti-PD-1 showed tumor rejection. Moreover, all these
18 mice resisted a tumor rechallenge performed after an untreated period of eighty days,
19 suggesting the development of an efficient antitumor long-term memory response
20 (Supplementary Fig. S2B). We also tested the effect of the combination in a different
21 syngeneic mouse model based on the subcutaneous implantation of Lewis lung
22 adenocarcinoma (LLC) cells (Fig. 2B and Supplementary Fig. S2C). Anti-PD-1 treatment on
23 days 7, 10 and 14 administered in combination with AON-D21 (every other day) resulted in
24 an attenuation of tumor growth that became significant by day 14. Although complete
25 rejections were not observed in this model, a survival analysis confirmed the benefit of the

1 combined treatment (Fig. 2C). The combination treatment was also able to increase the
2 survival of mice following injection of LLC cells into the tail vein (Fig. 2D), suggesting that
3 the treatment inhibited lung cancer metastasis. Lastly, C5a blockade synergized with PD-1
4 blockade to prevent multiorgan metastases after intracardiac injection of highly aggressive
5 Kras-mutated lung adenocarcinoma Lacun3 cells (Fig. 2E). A survival analysis performed in
6 an independent experiment in which anti-PD-1 treatment was ceased on day 10 confirmed the
7 benefit of the combined treatment (Fig. 2F). In conclusion, the combined immunotherapy
8 based on C5a and PD-1 blockade showed synergistic effects on both lung cancer growth and
9 metastatic progression.

10

11 **The antitumor activity of the combined treatment is mediated by CD8 T cells**

12 To identify changes induced in the tumor microenvironment by C5a and/or PD-1 blockade,
13 we analyzed the immune cells present in 393P tumor-bearing mice. By day 14, statistically
14 significant differences in tumor size were observed between groups, and were maintained
15 until the end of the experiment (Fig. 3A). Animals were sacrificed at day 26 before tumors
16 were completely rejected by the combined treatment. At this point, the combined anti-
17 C5a/PD-1 treatment resulted in a significant elevation of the percentage of CD8 T cells in the
18 spleens of the treated animals, as compared with the control mice; while no changes were
19 observed in other immune subpopulations (Supplementary Fig. S3). In tumors, the effect on
20 the frequency of CD8 T cells was stronger (Fig. 3B and Supplementary Fig. S4). In addition,
21 the combination treatment led to a decrease in the frequency of MDSC leukocyte
22 subpopulation in tumors, as compared to the control group (Fig. 3B). None of the treatments
23 significantly modified the frequency of CD4 T cells, NK cells or Tregs in tumors, except for a
24 reduction of NK cells in tumors treated with the dual blockade (Supplementary Fig. S5).
25 Further analyses revealed that in tumors treated with the dual therapy there was a negative

1 correlation between CD8 T cells and MDSCs, as well as an increase in CD45-positive cells
2 per mg of tumor (Fig. 3B). In support of the relevance of MDSCs in the model, anti-PD-1
3 treatment in combination with MDSC depletion had a therapeutic effect similar to that
4 observed with the anti-PD-1/anti-C5a treatment (Fig. 3C). In relation to the involvement of
5 potential effector cells, a selective depletion of CD8 T cells completely abrogated the
6 antitumor efficacy of the combination therapy against 393P cells (Fig. 3D). CD4 T cells and
7 NK cells seemed to be dispensable, although in the case of NK cells a moderate growth
8 increase was observed at the last days of the experiment, suggesting that NK cell depletion
9 may partially hamper the antitumor effect of the therapy. Tumor-infiltrating immune cells
10 were also analyzed in mice bearing LLC tumors treated with anti-C5a and/or anti-PD-1
11 agents. Results were similar to those previously obtained with 393P tumors, although in this
12 case significant differences were not always found (Supplementary Fig. S6 and
13 Supplementary Fig. S7A and B).

14 We also analyzed the mRNA expression in the tumors of a battery of immune-related
15 mediators. In 393P tumors, we found significant differences in the expression of three
16 molecules: IL2, LAG3 and CCL17 (Fig. 3E and Supplementary Table S2). In the LLC model,
17 mRNA expression of the T-cell activating cytokine IL2 was consistently elevated after the
18 combination treatment, albeit without reaching statistical significance (Supplementary Fig.
19 S7C). Results for LAG3 and CCL17 were not consistent with those obtained in the 393P
20 model (data not shown). From all these experiments, we conclude that the anti-PD-1/anti-C5a
21 combination synergistically impairs tumor growth by the action of CD8 T cells in association
22 with an elevation of IL2.

23

24 **The combined anti-C5a/anti-PD-1 therapy is effective against established tumors and is**
25 **associated with an activation of CD8 T cells**

1 To better evaluate the potential of the combined therapy in a model that more closely
2 resembles the clinical setting, we treated established 393P tumors during a defined period of
3 time. We observed regressions in 7 out of 8 mice treated with the combination of anti-C5a
4 (days 9, 10 and every other day until day 24) and anti-PD-1 (days 11, 14 and 18) drugs (Fig.
5 4A). The combined treatment resulted in a significant improvement in survival, despite the
6 fact that after treatment termination most tumors started growing again (Fig. 4B). In an
7 independent experiment, we analyzed the expression of surface activation markers in tumor-
8 infiltrating CD8 T cells. In agreement with previous results, we observed an increase in the
9 percentage of tumor CD8 T cells (Fig. 4C). Besides, these cells showed a marked reduction in
10 the exhaustion markers PD-1, GITR and LAG-3 (Fig. 4C). PD-1 was already downregulated
11 in those tumors treated with anti-PD-1 alone; however, the expression of the two other
12 markers was reduced only upon the administration of the combination therapy. We also found
13 a trend of an increase in IL2 protein expression within the tumors (Fig. 4D). In conclusion,
14 the combination of anti-C5a/anti-PD-1 drugs shows a significant therapeutic efficacy on
15 tumor-bearing mice. This activity seems to be associated with an increase in the expression of
16 IL2 and an attenuated CD8 T-cell dysfunction as compared to the anti-PD-1 monotherapy.

1 **DISCUSSION**

2 Numerous studies are underway to identify synergistic combinations of checkpoint inhibitors
3 with chemotherapy, radiotherapy, targeted therapy or other immunotherapy strategies (2).
4 Many of these combinations are built on PD-1/PD-L1 inhibition, for which the presence of
5 immunosuppressive pathways within the tumor microenvironment represents a major hurdle.
6 In this study we provide the framework for the clinical evaluation of an innovative
7 combination strategy, in which anti-PD-1 antibodies are administered together with drugs that
8 inhibit C5a signaling pathways. Overall, our study demonstrates that C5a blockade greatly
9 synergizes with anti-PD-1 inhibition in preclinical models of local and metastatic lung cancer
10 growth. The rationale behind this approach is that the activation and expansion of effector T
11 cells can be optimized by targeting immunosuppressive cells in the tumor microenvironment.
12 Previous studies performed in diverse mouse models have led to the proposal that C5a/C5aR1
13 inhibition may represent a novel therapeutic target for cancer (13). Among the different
14 potential mechanisms, C5a/C5aR1 blockade could partially reverse the immunosuppressive
15 tumor microenvironment linked to the activity of MDSCs (5,8,10). Nonetheless, in the
16 preclinical lung cancer models of our study the anti-C5a L-aptamer AON-D21 used as
17 monotherapy only showed modest antitumor effects. In contrast, its administration in
18 combination with an anti-PD-1 antibody greatly influenced the antitumor immune response in
19 a way that resulted in a remarkable antitumor activity, both in primary lung tumors and
20 metastatic sites.

21 The most plausible explanation for the synergism between anti-C5a and anti-PD-1 drugs is
22 that C5aR1 signaling mediates mechanisms that hamper the anti-tumor activity of anti-PD-1
23 antibodies. In agreement with this postulate, our data show that the combined treatment leads
24 to a lower frequency of MDSCs and a higher frequency of CD8 T cells associated with a
25 decreased expression of exhaustion markers, which suggests a more complete restoration of

1 CD8 T-cell effector functions. The combination treatment was also associated with an
2 increase in the levels of IL2, a pleiotropic cytokine with a critical role in multiple aspects of
3 CD8 T-cell activation (14). The essential role played by CD8 T cells was reinforced by the
4 complete abrogation of the immunotherapeutic effect after depletion of these cells. Previous
5 studies had already suggested a role of complement in the activity of tumor infiltrating CD8 T
6 cells. Induction of a tumor-specific CD8 T-cell response was improved upon transient
7 inhibition of the complement system (15), and a simultaneous blockade of C3aR and C5aR1
8 inhibited the development of breast tumors by enhancing the effector capacity of CD8 T cells
9 (11). Interestingly, in the same study, a combined inhibition of C3aR/C5aR1 and PD-1
10 enhanced antitumor effects (11). Considering these studies, a direct effect of C5a blockade on
11 CD8 T cells is also a plausible mechanism in our models, which may lead to CD8 T-cell
12 expansion. Contrastingly, in other pathological conditions, C5aR1 signaling seemed to be
13 essential for an optimal generation of CD8 T-cell responses (16). In regards to the potential
14 role played by MDSCs, their contribution to the antitumor activity of the combined therapy is
15 supported by the reduction in the percentage of these cells in some of the studied models, its
16 negative correlation with CD8 T cells, and the biological relevance of these cells in these
17 models. Nevertheless, we cannot completely exclude the possibility that the reduced
18 frequency of MDSCs was a bystander finding in our experiments, since C5a inhibition, alone
19 or in combination with PD-1 inhibition, did not always lead to significant changes. In-depth
20 mechanistic studies are still required to properly assess the roles played by MDSCs and CD8
21 T cells, without excluding the involvement of other immune cells, such as NK cells or
22 monocytes. Interestingly, a recent study has demonstrated that C5a promotes
23 immunosuppressive responses by contributing to the expression of PD-L1 on monocytes (17).
24 A comparison of the two subcutaneous tumor growth models used in our study suggests a
25 more effective antitumor immune response in the Kras-driven model of lung adenocarcinoma.

1 Consequently, the impact of Kras mutations on the antitumor efficacy of the combination
2 should be addressed, as well as the potential influence of other genetic determinants leading to
3 neoantigen generation. Further mechanistic studies would also provide guidance for the most
4 efficient administration schedule for immune stimulation, as well as for the potential benefit
5 of blocking other complement mediators, such as C3aR (11). The impact of the alternative
6 receptor for C5a, C5aR2 (C5L2), which functions are still poorly understood, should also be
7 investigated. Finally, the extension of this combination to other tumor types merits further
8 evaluation. Its applicability to models of lung squamous cell carcinoma, the second most
9 common subtype of lung cancer for which anti-PD-1 therapies have also shown clinical utility
10 (1), deserves particular consideration.

11 A major concern in the application of immunotherapy combinations is the potentiation of
12 adverse effects as a result of an excessive immune activation (2). It is premature to speculate
13 about the safety of the combination presented in this study, but clinical data from the use of
14 the C5-blocking antibody eculizumab show that safety issues associated with complement
15 inhibition are mainly related to the blockade of C5b-mediated bacterial lysis (18). AON-D21
16 selectively blocks C5a and does not interfere with C5b biology despite binding to intact C5
17 (19,20). Such a selective blockade of C5a/C5aR1 was safe and generally well tolerated in
18 clinical trials (21,22). The substance class of L-aptamers (Spiegelmers) was also generally
19 safe and well tolerated in clinical phase I and IIa studies (23).

20 In conclusion, our work supports the notion that the efficacy of anti-PD-1 therapies is limited
21 by their inability to fully reprogram the tumor microenvironment, which maintains other
22 immunosuppressive mediators. In this context, C5a/C5aR1 blockade overcomes some of the
23 resistance mechanisms, markedly improving antitumor immune responses. These findings
24 provide support for the clinical evaluation of anti-PD-1 and anti-C5a drugs as a combination
25 therapy for lung cancer.

1 **METHODS**

2 *Cell lines*

3 393P cells, derived from Kras^{LA1/+p53R172HΔG} mice, were a generous gift from Dr. JM
4 Kurie (The University of Texas, MD Anderson Cancer Center, Houston, TX). Lewis lung
5 carcinoma (LLC) cells were obtained from the American Type Culture Collection. The
6 Lacun3 cell line was previously established by our group from a chemically induced lung
7 adenocarcinoma carcinoma and stably transfected with the luciferase gene (24). Non-
8 transduced Lacun3 cells were used for the survival experiment. Cells were grown in RPMI
9 1640 supplemented with 2 mM glutamine, 10% Fetalclone (Thermo), 100 U/ml penicillin and
10 100 mg/ml streptomycin (Invitrogen). All cell lines were routinely tested for Mycoplasma.
11 Cell line authentication was not routinely performed.

12 *AON-D21 L-aptamer*

13 AON-D21, a follow-up of previously described NOX-D19 and NOX-D20 (19,20), is a novel
14 PEG-modified L-aptamer (Spiegelmer). revAON-D21, a Spiegelmer of the reverse AON-D21
15 sequence was used as negative control. Both aptamers were synthesized at NOXXON Pharma
16 (Berlin, Germany). Methods for the evaluation of AON-D21 affinity, pharmacokinetics and
17 inhibitory capacity are described in the Supplementary Methods section.

18 *Mouse models and therapeutic protocols*

19 All animal experiments were conducted in accordance with the protocols approved by the
20 Institutional Animal Care Committee. Kras^{LSL-G12D/+};C5aR1^{+/-} animals were intercrossed to
21 generate Kras^{LSL-G12D/+};C5aR1^{+/+} or Kras^{LSL-G12D/+};C5aR1^{-/-} offspring. Kras^{LSL-G12D/+} mice
22 harbor a conditionally activatable allele of oncogenic mutant Kras. Intratracheal inoculation
23 with adenoviral Cre (AdCre) led to expression of mutant Kras. Animals were followed for a
24 period of four months. Murine 393P cells or LLC cells (1.5x10⁶) were resuspended in 50 μl of
25 PBS and injected subcutaneously in the right flanks of eight weeks old Sv/129 or C57BL/6J

1 mice, respectively. One day before and on the day of cell injection mice were treated with
2 AON-D21 or revAON-D21 (10 mg/kg in saline, IP), and treatment was continued every other
3 day until the end of the experiment. For combination therapy, tumor-bearing mice were
4 treated with anti-PD-1 blocking antibody (RMP1-14, BioXcell) at days 7, 10 and 14 after cell
5 inoculation (100 μ g per mouse in PBS, IP). Depletion of CD8⁺, CD4⁺ or NK⁺ cells was
6 achieved by intraperitoneal injection of 100 μ g of antimouse CD8 α (clone 2.43; BioXcell),
7 CD4 (clone GK1.5; BioXcel) or NK1.1 (clone PK136; BioXcell), respectively, at days 6, 11,
8 14, 18, 21 and 28 after cell inoculation. Irrelevant IgG (BioXcell) was administered as
9 control. MDSCs were depleted by intraperitoneal injection of 200 μ g of antimouse anti-Gr-1
10 (Ly6G/C; clone RB6-8C5; BioXcell) on the day of cell injection and every other day until the
11 end of the experiment. Tumors were measured periodically and volumes were calculated by
12 the formula $(L \times W^2)/2$, where L is the length and W is the width. Animals were euthanized
13 when tumor diameters reached 17 mm or when they appeared under distress. The metastasis
14 model based on LLC cells was performed by intravenous injection of 1×10^6 cells into the tail
15 vein of C57BL/6J mice. The multiorgan metastatic potential of luciferase-transfected Lacun3
16 cells was evaluated after inoculation of 2×10^5 cells into the left cardiac ventricle of BALB/c
17 mice as previously described (24,25). In these two metastasis models, treatments were
18 performed as described above, except for the injections of anti-PD-1 blocking antibody,
19 which were performed at days 3, 7 and 10 after cell inoculation. Finally, the experiments
20 performed with established tumors (Fig. 4) were carried out as follows: subcutaneously
21 inoculated 393P cells (1.5×10^6) were allowed to grow for 9 days, mice were randomized and
22 treatment started with AON-D21 (10 mg/kg, IP) at days 9 and 10 and every other day until
23 day 24. Anti-PD-1 blocking antibody (100 μ g per mouse) was administrated at days 11, 14
24 and 18 after cell inoculation.

25 ***Flow cytometry analysis***

1 Tumors and spleens from tumor-bearing mice were mechanically disaggregated as previously
2 described (5). Erythrocytes were lysed in a buffer containing 155 mM NH₄Cl and 10 mM
3 KHCO₃. Single-cell suspensions were treated with Fc block (2.4G2; BD Pharmingen) and
4 then stained with a labeled primary antibody against mouse CD45 (30-F11; Biolegend), CD8a
5 (53-6.7; BD Pharmingen), NK1.1 (PK136; Biolegend), CD11b (M1/70; Biolegend), Ly6C
6 (AL21; BD Pharmingen), Ly6G (1A8; Biolegend), LAG-3 (C9B7W; Biolegend), PD-1
7 (29F.1A12; Biolegend) and GITR (YGITR 765; Biolegend) diluted in FACS buffer (PBS,
8 0.1% NaN₃, 1% BSA). Staining of CD4 T cells and Tregs was performed using a kit from
9 eBioscience according to the manufacturer's instructions. As an example, the gating strategy
10 for MDSCs and CD8 T cells is shown in Supplementary Fig. S8. When indicated, dead cells
11 were excluded using Zombie NIR Fixable Viability Kit (Biolegend), and the absolute number
12 of CD8 T cells per milligram of tumor was determined using Cytognos Perfect Microspheres
13 (Cytognos). Cells were acquired using a BD Biosciences FACSCalibur flow cytometer,
14 except for the analysis of LAG-3, GITR, and PD-1 expression on CD8 T cells, which was
15 performed on a FACSCANTO II flow cytometer. Data were analyzed using BD CellQuest
16 Pro (BD Biosciences) and FlowJo software (Tree Star).

17 ***Immunohistochemistry***

18 Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections.
19 After antigen retrieval with EDTA buffer (CD3 and Ki67) or citrate buffer (cleaved caspase-3
20 and CD31), sections were incubated with the primary antibodies (anti-CD3, 1:300, Thermo
21 Scientific; anti-Ki67, 1:100, Neomarkers; anti-cleaved caspase-3, 1:100, Cell Signaling; anti-
22 CD31, 1:20, Dianova) overnight at 4°C, and revealed with the EnVision HRP System (Dako)
23 and diaminobenzidine. Quantification of staining was performed either automatically (Ki67,
24 cleaved caspase-3 and CD31) or manually (CD3).

25 ***Expression of immune molecules within the tumors***

1 Portions of $\sim 0.1 \text{ cm}^3$ were cut from the edge of tumors, frozen in dry ice, and maintained at -
2 80°C until extraction. mRNA expression was evaluated by real-time PCR as previously
3 described (5). For protein analysis, frozen tumors were lysed in an extraction buffer (100 mM
4 Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium
5 deoxycholate, pH 7.4) containing a cocktail of protease inhibitors. Extracts were analyzed
6 using the OptEIA mouse IL2 ELISA kit (BD Biosciences).

7 ***Statistical analyses***

8 For dot plots, individual results and median per group are shown. For tumor volumes, the
9 mean \pm SEM is depicted. Comparisons between two groups were performed using the Mann-
10 Whitney U-test. Comparisons between treatment strategies and between immune-regulator
11 levels were performed using the Kruskal-Wallis test with the Mann-Whitney U-test as the
12 post hoc test. Survival curves were generated using the Kaplan-Meier method, and differences
13 were analyzed with the log rank test. For these analyses, survival times were defined as the
14 number of days from the inoculation of the cells until the mice were euthanized or expired
15 naturally. Spearman's rank correlation test was performed to analyze association.
16 Significances in tumor volumes and survival tests were always calculated versus the control
17 group. $P < 0.05$ was considered significant. Statistical analyses were performed using Prism
18 software (GraphPad).

1 **AUTHORS' CONTRIBUTIONS**

2 **Conception and design:** DA, LMM and RP

3 **Acquisition of data:** DA, SOE, HM, TL, MJP, JA, CV, AV, SV, FL, RP

4 **Analysis and interpretation of data:** All authors

5 **Writing, review, and/or revision of the manuscript:** All authors

1 **ACKNOWLEDGEMENTS**

2 We are grateful to Ignacio Melero for critically reviewing the manuscript. We thank Carolina
3 Zandueta, Cristina Sainz and Amaya Lavín for technical assistance, Jonathan M. Kurie (The
4 University of Texas, MD Anderson Cancer Center, Houston, TX) for the generous gift of
5 393P cells, and Diego Alignani (Cytometry Platform, CIMA), Noelia Casares and Sandra
6 Hervas-Stubbs (Program in Immunology and Immunotherapy, CIMA) for their help with flow
7 cytometry. The authors would also like to thank the following individuals at Noxxon Pharma:
8 Lucas Bethge (chemical synthesis of AON-D21, revAON-D21 and hybridization probe for
9 bioanalysis), Christian Maasch (AON-D21 affinity determination and bioanalysis from mouse
10 pharmacokinetics by surface plasmon resonance experiments), Klaus Buchner and Dirk
11 Zboralski (cell-based potency assays) and Kai Hoehlig for helpful discussions.

1 **REFERENCES**

- 2 1. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WEE, Poddubskaya E, et al.
3 Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N*
4 *Engl J Med* 2015;373:123-35.
- 5 2. Melero I, Berman DM, Aznar MA, Korman AJ, Pérez Gracia JL, Haanen J. Evolving
6 synergistic combinations of targeted immunotherapies to combat cancer. *Nat Rev*
7 *Cancer* 2015;15:457-72.
- 8 3. Youn J-I, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived
9 suppressor cells in tumor-bearing mice. *J Immunol* 2008;181:5791-802.
- 10 4. Mkrtychyan M, Najjar YG, Raulfs EC, Abdalla MY, Samara R, Rotem-Yehudar R, et
11 al. Anti-PD-1 synergizes with cyclophosphamide to induce potent anti-tumor vaccine
12 effects through novel mechanisms. *Eur J Immunol* 2011;41:2977-86.
- 13 5. Corrales L, Ajona D, Rafail S, Lasarte JJ, Riezu-Boj JI, Lambris JD, et al.
14 Anaphylatoxin C5a creates a favorable microenvironment for lung cancer progression.
15 *J Immunol* 2012;189:4674-83.
- 16 6. Berraondo P, Minute L, Ajona D, Corrales L, Melero I, Pio R. Innate immune
17 mediators in cancer: between defense and resistance. *Immunol Rev* 2016;274:290-306.
- 18 7. Pio R, Ajona D, Lambris JD. Complement inhibition in cancer therapy. *Semin*
19 *Immunol* 2013;25:54-64.
- 20 8. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A,
21 Gerard C, et al. Modulation of the antitumor immune response by complement. *Nat*
22 *Immunol* 2008;9:1225-35.
- 23 9. Sharma SK, Chintala NK, Vadrevu SK, Patel J, Karbowniczek M, Markiewski MM.
24 Pulmonary alveolar macrophages contribute to the premetastatic niche by suppressing
25 antitumor T cell responses in the lungs. *J Immunol* 2015;194:5529-38.

- 1 10. Vadrevu SK, Chintala NK, Sharma SK, Sharma P, Cleveland C, Riediger L, et al.
2 Complement C5a receptor facilitates cancer metastasis by altering T cell responses in
3 the metastatic niche. *Cancer Res* 2014;74:3454-65.
- 4 11. Wang Y, Sun S-N, Liu Q, Yu Y-Y, Guo J, Wang K, et al. Autocrine complement
5 inhibits IL10-dependent T-cell mediated antitumor immunity to promote tumor
6 progression. *Cancer Discov* 2016;6:1022-35.
- 7 12. Gibbons DL, Lin W, Creighton CJ, Rizvi ZH, Gregory PA, Goodall GJ, et al.
8 Contextual extracellular cues promote tumor cell EMT and metastasis by regulating
9 miR-200 family expression. *Genes Dev* 2009;23:2140-51.
- 10 13. Pio R, Corrales L, Lambris JD. The role of complement in tumor growth. *Adv Exp*
11 *Med Biol* 2014;772:229-62.
- 12 14. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the
13 immune system. *Nat Rev Immunol* 2012;12:180-90.
- 14 15. Janelle V, Langlois M-P, Tarrab E, Lapierre P, Poliquin L, Lamarre A. Transient
15 complement inhibition promotes a tumor-specific immune response through the
16 implication of natural killer cells. *Cancer Immunol Res* 2014;2:200-6.
- 17 16. Kim AHJ, Dimitriou ID, Holland MCH, Mastellos D, Mueller YM, Altman JD, et al.
18 Complement C5a receptor is essential for the optimal generation of antiviral CD8+ T
19 cell responses. *J Immunol* 2004;173:2524-9.
- 20 17. An L-L, Gorman J V, Stephens G, Swerdlow B, Warrenner P, Bonnell J, et al.
21 Complement C5a induces PD-L1 expression and acts in synergy with LPS through
22 Erk1/2 and JNK signaling pathways. *Sci Rep* 2016;6:33346.
- 23 18. Ward PA, Guo R-F, Riedemann NC. Manipulation of the complement system for
24 benefit in sepsis. *Crit Care Res Pract* 2012;2012:427607.
- 25 19. Yatime L, Maasch C, Hoehlig K, Klussmann S, Andersen GR, Vater A. Structural

- 1 basis for the targeting of complement anaphylatoxin C5a using a mixed L-RNA/L-
2 DNA aptamer. *Nat Commun* 2015;6:6481.
- 3 20. Hoehlig K, Maasch C, Shushakova N, Buchner K, Huber-Lang M, Purschke WG, et al.
4 A novel C5a-neutralizing mirror-image (L-)aptamer prevents organ failure and
5 improves survival in experimental sepsis. *Mol Ther* 2013;21:2236-46.
- 6 21. Sun S, Zhao G, Liu C, Fan W, Zhou X, Zeng L, et al. Treatment with anti-C5a
7 antibody improves the outcome of H7N9 virus infection in African green monkeys.
8 *Clin Infect Dis* 2015;60:586-95.
- 9 22. Jayne DR, Bruchfeld A, Schaier M, Ciechanowski K, Harper L, Jadoul M, et al.
10 OP0227 oral C5a receptor antagonist CCX168 phase 2 clinical trial in ANCA-
11 associated renal vasculitis. *Ann Reum Dis* 2014;73:148.
- 12 23. Vater A, Klussmann S. Turning mirror-image oligonucleotides into drugs: the
13 evolution of Spiegelmer(®) therapeutics. *Drug Discov Today* 2015;20:147-55.
- 14 24. Bleau A-M, Freire J, Pajares MJ, Zudaire I, Anton I, Nistal-Villán E, et al. New
15 syngeneic inflammatory-related lung cancer metastatic model harboring double
16 KRAS/WWOX alterations. *Int J Cancer* 2014;135:2516-27.
- 17 25. Antón I, Molina E, Luis-Ravelo D, Zandueta C, Valencia K, Ormazabal C, et al.
18 Receptor of activated protein C promotes metastasis and correlates with clinical
19 outcome in lung adenocarcinoma. *Am J Respir Crit Care Med* 2012;186:96-105.
20

1 **FIGURE LEGENDS**

2 **Figure 1.** C5aR1 genetic deficiency and C5a pharmacological blockade decreases lung tumor
3 burden. **A**, Representative H&E-stained lung sections from $Kras^{LSL-G12D/+}$ (n=24) and $Kras^{LSL-}$
4 $G12D/+;C5aR1^{-/-}$ mice (n=18) treated with AdCre and allowed to develop tumors for four
5 months. **B**, Quantification of number of tumors per mouse and tumor areas. Data correspond
6 to two independent experiments. **C**, Flow cytometric analysis of splenic CD11bLy6C
7 MDSCs. **D**, Immunohistochemical evaluation of CD3-positive cells in the lung tumors (n=12
8 per group) with two representative images. Scale bar, 30 μ m. **E**, Kaplan-Meier survival
9 curves of $Kras^{LSL-G12D/+}$ (n=14) and $Kras^{LSL-G12D/+;C5aR1^{-/-}$ (n=13) mice from an independent
10 experiment. **F**, Tumor growth of 393P cells injected subcutaneously into syngeneic Sv/129
11 mice treated with the C5a antagonist AON-D21 (n=8) or the negative control revAON-D21
12 (n=7). **G**, Flow cytometric analysis of splenic MDSCs from 393P tumor-bearing mice on day
13 40. * $P < 0.05$; ** $P < 0.01$.

14 **Figure 2.** Combined blockade of PD-1 and C5a decreases lung tumor burden and metastatic
15 progression. **A**, Tumor growth of 393P cells implanted subcutaneously in syngeneic Sv/129
16 mice treated with anti-C5a (every other day), anti-PD-1 (days 7, 10, 14), or both (9 mice per
17 group). **B**, Tumor growth of LLC cells injected subcutaneously in syngeneic C57BL/6J mice
18 treated as above (8 mice per group), and Kaplan-Meier survival curves of these mice (**C**). **D**,
19 Kaplan-Meier survival curves after intravenous injection of LLC tumors into the tail vein of
20 mice treated as indicated. **E**, Bioluminescence quantitation after intracardiac inoculation of
21 syngeneic Lacun3 cells in BALB/c mice treated with anti-C5a (every other day), anti-PD-1
22 (days 3, 7 and 10) or both (6-7 mice per group). Light was measured at day 0, at day 7 and at
23 the end of the experiment (day 14). A representative mouse from each treatment group (at
24 days 7 and 14) are shown. Dot plot shows the relative change of bioluminescence between
25 days 0 and 14. At day 7, no significant changes were still observed between groups (data not

1 shown). **F**, Kaplan-Meier survival curves from an independent experiment of mice inoculated
2 in the left cardiac ventricle with naïve Lacun3 cells non-transduced with luciferase and treated
3 as indicated (9-10 mice per group). In all the experiments of this figure revAON-D21 was
4 used in the control group. * $P < 0.05$; *** $P < 0.001$.

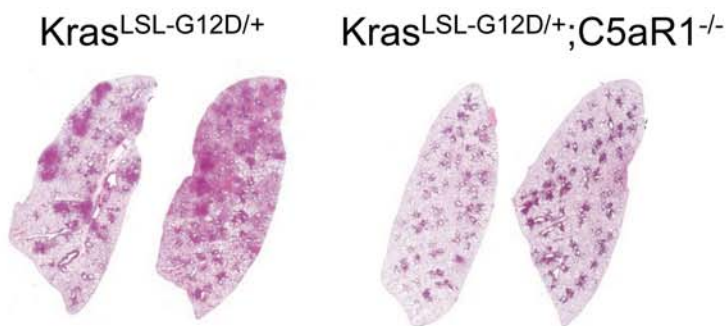
5 **Figure 3.** The antitumor activity observed after the C5a and PD-1 blockade is mediated by
6 CD8 T cells. **A**, Tumor growth of 393P cells in mice treated with AON-D21, anti-PD-1, or
7 their combination for endpoint flow cytometry analysis (n=8 per group). RevAON-D21 was
8 used as control. **B**, Flow cytometric analysis of CD8 T cells, MDSCs and CD45-positive cells
9 (n=4 per group) in tumors harvested at day 26. The correlation between CD8 T cells and
10 MDSCs in the combined group is also shown. Spearman's rank correlation test was performed
11 to analyze the association. Data on CD45-positive cells per mg of tumor were generated in an
12 independent experiment. **C**, Tumor growth of 393P cells in mice treated as indicated with and
13 without MDSC depletion with anti-Gr-1. RevAON-D21 was used as negative control and anti-
14 C5a/anti-PD-1 as positive control. Five mice were used per group. Statistically significant
15 differences in tumor volume at the end of the experiment are shown. **D**, Tumor growth of
16 393P cells in mice treated with revAON-D21 (control), the anti-C5a/anti-PD-1 combination,
17 and the combination in the presence of depleting antibodies against CD8, CD4 or NK cells.
18 An additional control group consisting of mice treated with revAON-D21 and an irrelevant
19 IgG was used, but not shown for the sake of clarity since no differences were observed
20 between control groups. Five mice were used per group. Statistically significant differences in
21 tumor volume at the end of the experiment are shown. **E**, IL2 mRNA levels in 393P tumors
22 determined by real-time PCR and expressed as relative to β -actin mRNA. * $P < 0.05$;
23 ** $P < 0.01$; *** $P < 0.001$.

24 **Figure 4.** Combined C5a and PD-1 blockade is able to control the growth of established
25 tumors in association with an activation of CD8 T cells. **A**, Subcutaneously injected 393P

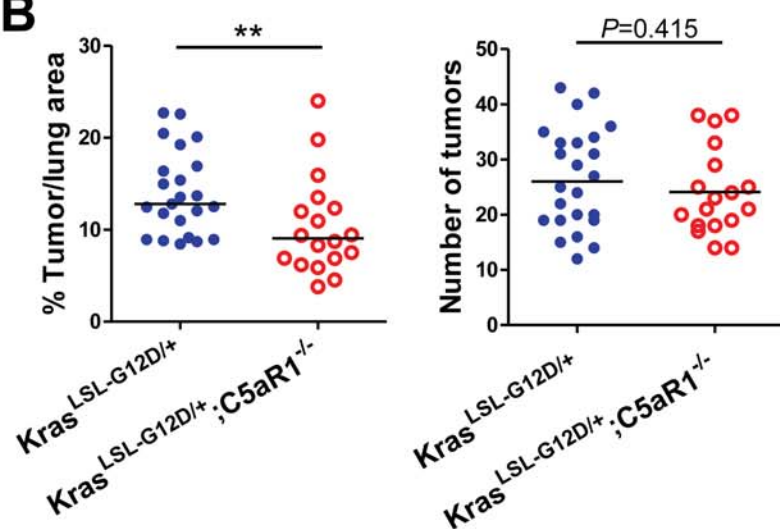
1 cells were allowed to grow for 9 days and mice were randomized into four groups (eight-nine
2 mice per group). Mice were treated with anti-C5a (days 9, 10 and every other day until day
3 24), with anti-PD-1 (days 11, 14 and 18) or both. Mice in the control group were treated with
4 revAON-D21. The waterfall plot shows the responses to treatment as measured between
5 treatment initiation (day 9) and one day after treatment termination (day 25). **B**, Kaplan-Meier
6 survival curves of these mice. The experiment was terminated when all mice with growing
7 tumors died. The statistical significance corresponds to the comparison between the control
8 group and the combined treatment. **C**, Flow cytometric analysis of CD8 T cells and
9 exhaustion markers PD-1, GITR and LAG-3 (expressed as mean fluorescence intensity on
10 CD8 T cells) in 393P tumors grown in mice treated as above in an independent experiment
11 terminated at day 19. For this analysis, dead cells were excluded using Zombie NIR Fixable
12 Viability Kit. Four animals were used per group. Of note, one of the mice treated with the
13 combination anti-C5a/anti-PD-1 showed a complete remission of the tumor and,
14 consequently, could not be analyzed (which deterred us to perform a statistical analysis of the
15 data). **D**, IL2 protein levels in these tumors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

FIGURE 1

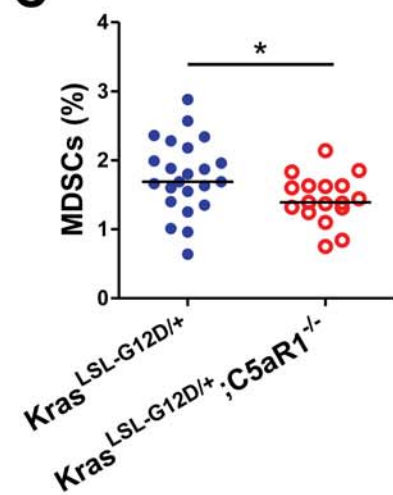
A



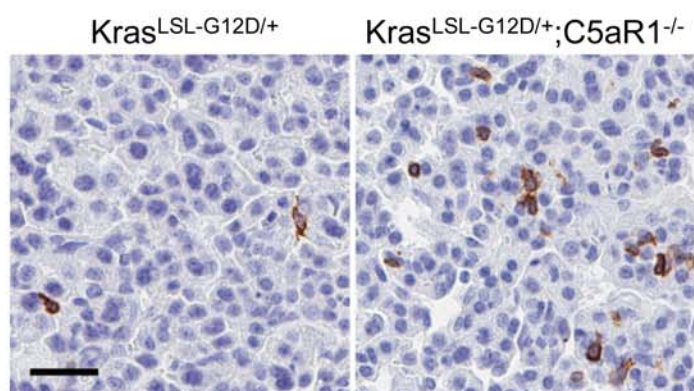
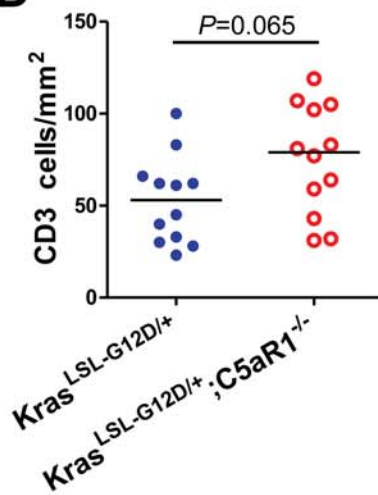
B



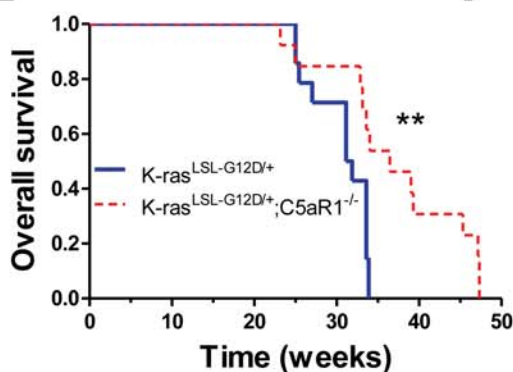
C



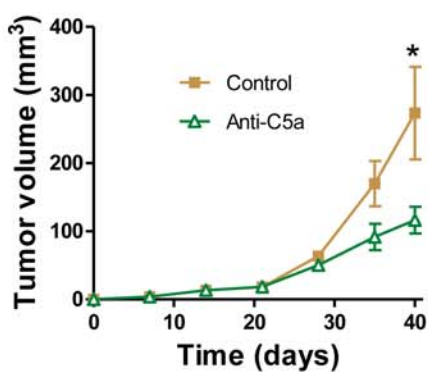
D



E



F



G

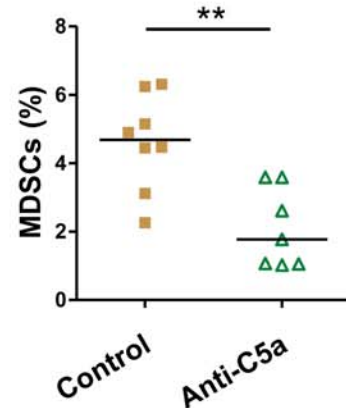


FIGURE 2

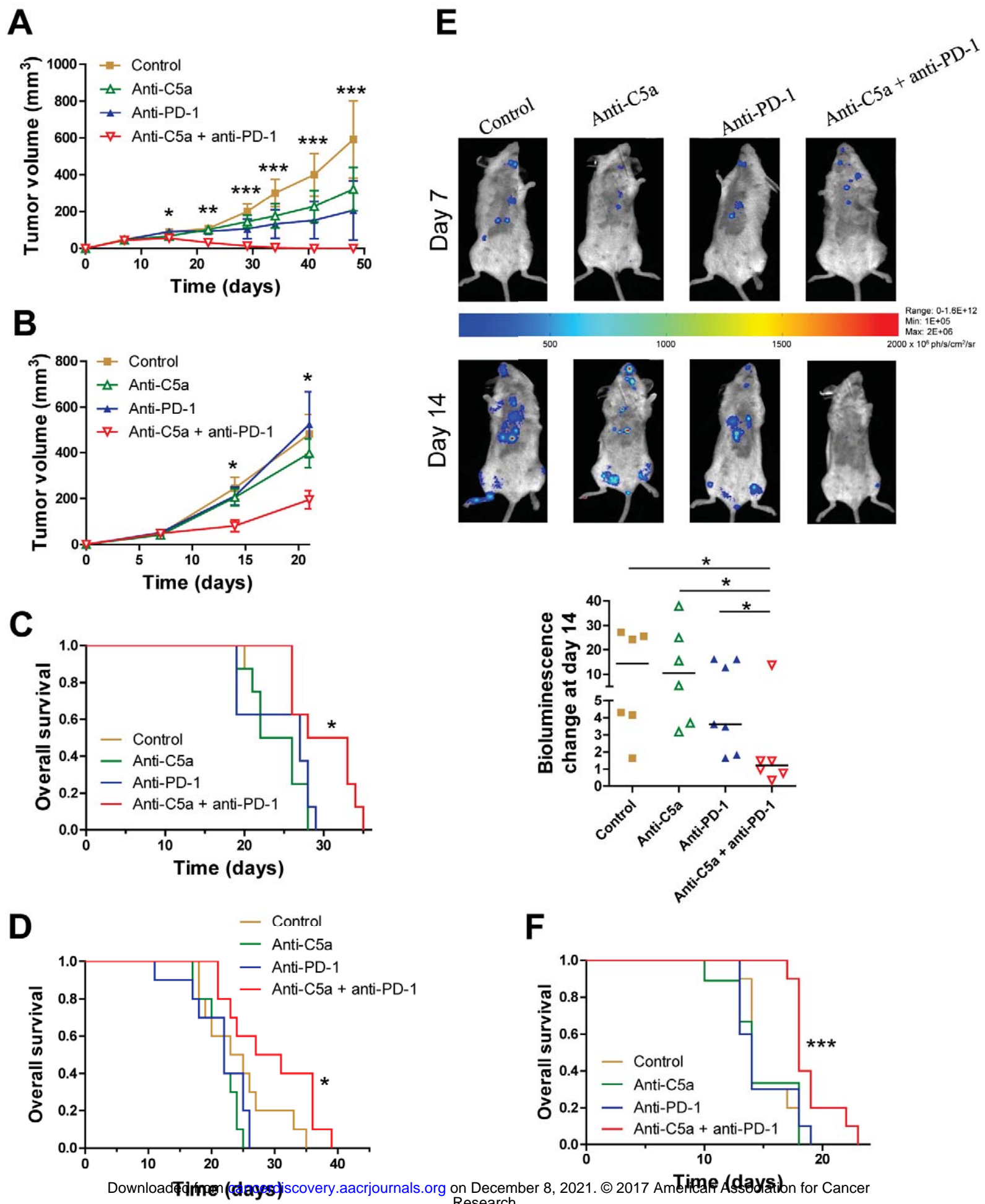


FIGURE 3

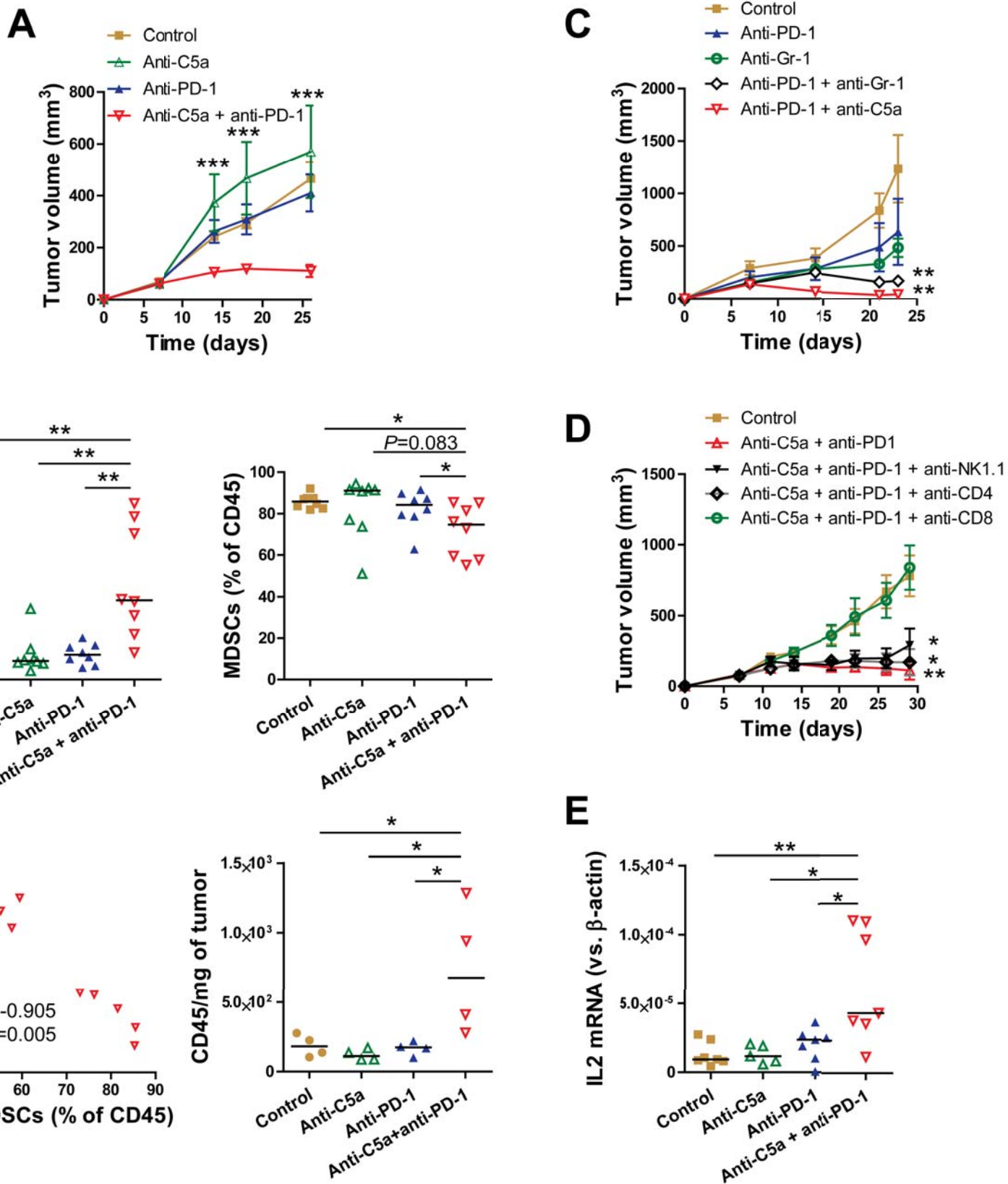
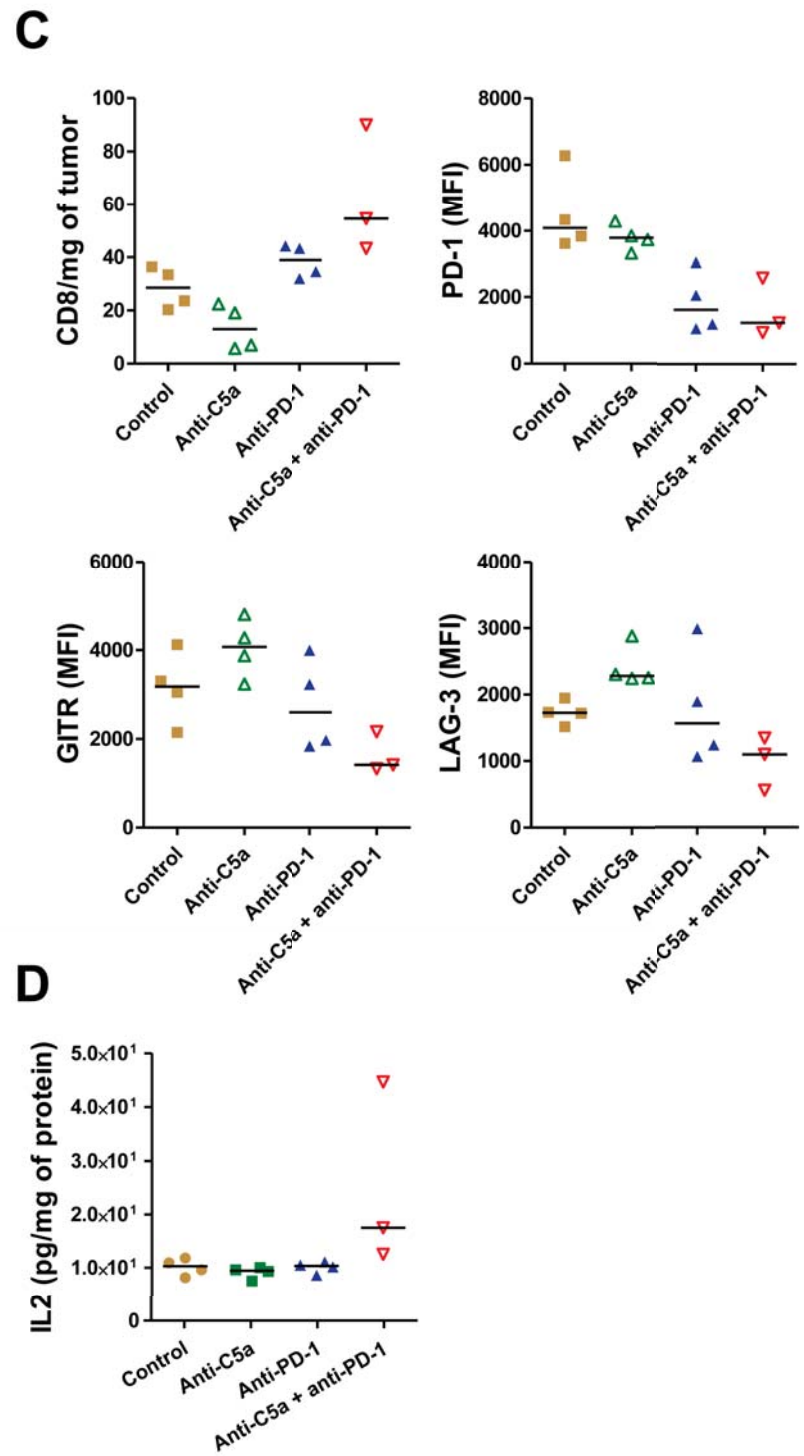
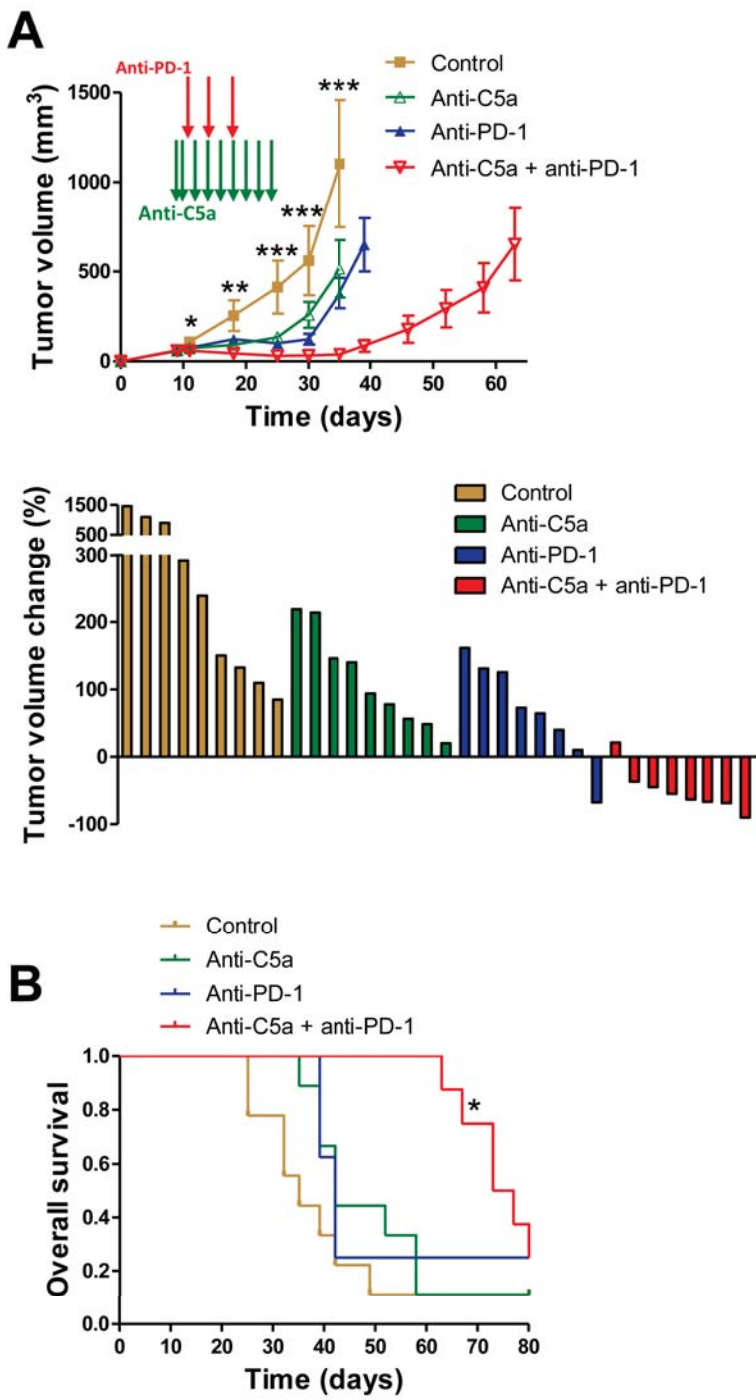


FIGURE 4



CANCER DISCOVERY

A Combined PD-1/C5a Blockade Synergistically Protects Against Lung Cancer Growth and Metastasis

Daniel Ajona, Sergio Ortiz-Espinosa, Haritz Moreno, et al.

Cancer Discov Published OnlineFirst March 13, 2017.

Updated version	Access the most recent version of this article at: doi: 10.1158/2159-8290.CD-16-1184
Supplementary Material	Access the most recent supplemental material at: http://cancerdiscovery.aacrjournals.org/content/suppl/2017/03/11/2159-8290.CD-16-1184.DC1
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link <http://cancerdiscovery.aacrjournals.org/content/early/2017/03/11/2159-8290.CD-16-1184>. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.