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14. ABSTRACT

This pilot proposal stems from our discovery that novel small molecule that interact with the retinoblastoma (Rb) protein domain C, AP-3-84, can induce myeloid-preferential apoptosis. Our data indicate that treatment with AP-3-84 can lead to myeloid cell depletion (in *ex vivo* cultures of human myeloid cells derived from ovarian tumors, as well as in a murine *in vivo* model). We will test the hypothesis that treatment with Rb modulators AP-3-84 will result in ovarian tumor regression and/or improved survival by increasing myeloid cell death and increasing anti-tumor cell-mediated responses. Specific aims will address: (1) To establish the impact of exposure to AP-3-84 on gene expression, viability and function of myeloid and lymphoid cell subsets isolated from fresh human ovarian cancer tissue. (2) A-To define the efficacy of treatment with AP-3-84 in achieving tissue and tumor myeloid cell depletion (TAM, MSDCs, rDCs) at different points of ovarian tumor progression in murine models and its effect on overall survival. B-in the same model, to assess the changes in anti-tumor cellular immunity induced by AP-3-84 treatment. The proposed studies seeks to establish that the Rb protein can serve as a new molecular target in immunosuppressive myeloid cells in ovarian cancer models, and that its blockage results in myeloid cell death within the tumor microenvironment, leading to a decrease in local immunosuppression, and enhanced T-cell control.

15. SUBJECT TERMS
Ovarian Cancer, Rb/Retinoblastoma Protein, Apoptosis, Cell Death, Tumor Microenvironment (TME), Tumor Associated Macrophages (TAM), myeloid cells, CDK4/CDK6

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1. INTRODUCTION:

Ovarian cancer is the most lethal gynecologic cancer in the Western world and has an incidence of approximately 22,200 women/year, 60% of the patients being diagnosed after the disease has spread outside of the ovaries, which is associated with a dismal 5-year survival rate of 28.9%. 5-year survival rates have improved little in the last 30 years. Poor clinical prognosis is associated with an increase in tumor infiltration of myeloid cells including tumor-associated macrophages (TAMs). In this study, we seek to develop the first small molecule therapeutics to reduce TAMs *in vivo* by targeting the retinoblastoma protein (Rb), a transcription factor crucial for myeloid cell phenotype, function and survival, yet without dampening T-cell from mediating subsequent tumor control. We hypothesize treatment with Rb modulators AP-3-84 will result in ovarian tumor regression and improved survival by increasing myeloid cell death particularly TAMs and increasing anti-tumor T-cell cellular responses. First, we establish the impact of AP-3-84 treatment on viability and function of purified myeloid and lymphoid cells isolated from human or murine ovarian cancer tissue. With this aim, we also will examine on the gene expression difference of cells which demonstrate different sensitivity to the compound. Secondly, we will define efficacy of treatment with AP-3-84 in achieving tissue and tumor myeloid cell depletion and the impact of myeloid cell depletion on T-cell cell activation, priming, infiltration, and consequently in altering tumor burden and animal survival. Third, we will identify the molecular mechanism of action (MOA) of AP-3-84.

2. KEYWORDS:

Ovarian Cancer, Rb/Retinoblastoma Protein, Apoptosis, Cell Death, Tumor Microenvironment (TME), Tumor Associated Macrophages (TAM), myeloid cells, CDK4/CDK6

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1. The major goal for Aim 1 within the first 12 months (*Major Tasks and Substasks progress report/completion in first 12 months highlighted in yellow below*) is to determine the activity of AP-3-84 in myeloid and lymphoid cells *ex vivo* isolated from human ovarian cancer tissues.

NOTE TIMELINES ARE NOW EXTENDED TO 30 MONTHS BY A NO COST EXTENSION AS A RESULT OF COVID-19 SHUTDOWN IN MONTHS 13-18 OF CURRENT AWARD

Major Task 1:70% Completed: Direct apoptosis effects on myeloid cells within human ovarian tumors	**Target Month**
Subtask 1: Local IRB Approval; **100% Completed by 3 months as proposed.**	1-3
Subtask 2: Establishing direct apoptosis effects on myeloid cells within human ovarian tumors; **100% Completion as of 16 months**	3-13

Subtask 3: Establishing direct apoptosis effects on myeloid cells within human ovarian tumors (13-24 samples). **20% Completion as of 24 months**	13-27
Milestone Achieved	28

Major Task 2 – 66% Completed: Gene expression/protein change in myeloid and lymphoid cells after Rb inhibition by compound AP-3-84 as compared to carrier control.	
Subtask 1: Establishing gene expression change in myeloid and lymphoid cells after Rb modulation by compound AP-3-84 as compared to carrier control. (1 sample); **100% Completed**	10
Subtask 2: Completion of 4 added samples **50% Completion as of 24 months**	18-29
Subtask 3: Bioinformatic Analysis **50% Completion as of 24 months**	19-27
Milestone(s) Achieved:	30

Aim 2. The major goal for Aim 2 is to define the efficacy of AP-3-84 on tumor myeloid cell depletion at different points of ovarian cancer progression *in vivo* and impact on tumor burden and survival of the animal model.

NOTE TIMELINES ARE NOW EXTENDED TO 30 MONTHS BY A NO COST EXTENSION AS A RESULT OF COVID-19 SHUTDOWN IN MONTHS 13-18 OF CURRENT AWARD

Major Task 1 – 100% Completed: Differences between AP-3-84 therapy and carrier control in decreasing ovarian tumor burden.	
IACUC Approval: **100% Completed**	1-3
Subtask 1: First Experiment; **100% Completed**	3
Subtask 2: Second Experiment;**100% Completed**	10
Milestone Achieved: **100% Completed**	10

Major Task 2 – 100% Completed (ahead of schedule): Survival after treatment with Rb modulator and change in the distribution of myeloid and T-cell subsets in vivo.	
Subtask 1: Collection of data (time points); **100% Completed**	10
Milestone(s) Data analysis achieved:	10 (Originally 18)

Major Task 3 – 100% Completed: Direct tissue measurements after Rb modulator to assess if acute depletion of myeloid cells in the tumor microenvironment is matched with increased T-cell infiltrates.	**Months**
Subtask 1: Collection of data (time points): **100% Completed**	6-10
Milestone(s) Data analysis achieved **100% Completed**	13

Major Task 4 – 75% Completed: After survival following treatment with Rb modulator therapy determine if mice can prevent tumor recurrence and/or T-cells protect against additional tumor challenge?	
Subtask 1: Per survival outcome of Aim 2-Major Task 1/First Experiment; **100% Completed**	6-9
Subtask 2: Subtask 1: Per survival outcome of Aim 2-Major Task 1/Second Experiment **50% Completed**	18-27
Milestone(s) Achieved:	28
Major Task 5 – 30% Completed: Final results and manuscript Preparation	
Final data analysis and manuscript preparation 3**0% Completed**	18-27
Milestone completed	30

What was accomplished under these goals?

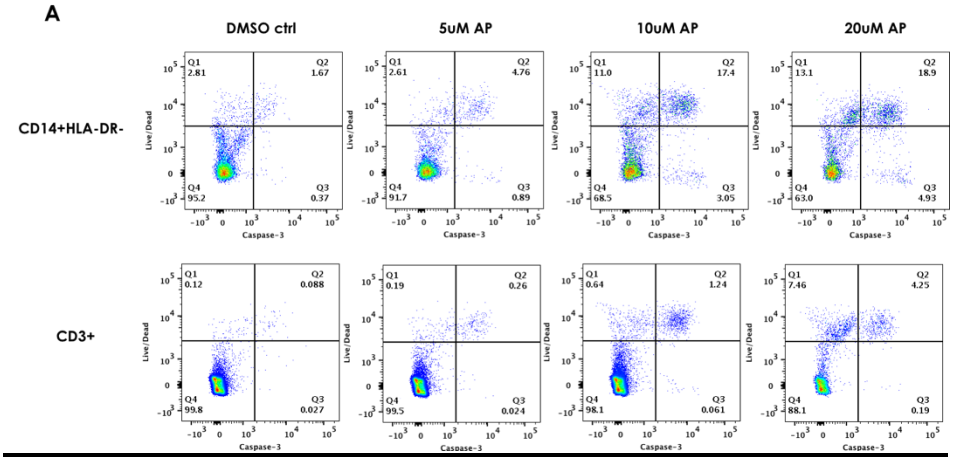
For AIM 1 (refer above to Major Tasks and Subtasks):

NOTE TIMELINES ARE NOW EXTENDED TO 30 MONTHS BY A NO COST EXTENSION AS A RESULT OF COVID-19 SHUTDOWN IN MONTHS 13-17 OF CURRENT AWARD

Completion of Regulatory Approvals. An IRB protocol collaborating with Christiana Care Health System was established and approved in July 2019. Clinical samples started to be received in January 2020. (Major Task 1, Subtask 1).

1) ***Establishing direct apoptosis effects on myeloid cells within human ovarian tumors***

Analysis of blood monocytes (CD14+HLA-DR-) and ascites macrophages (CD163+CD68+) cells following analysis by flowcytometry were detected to be sensitive to induced apoptosis by exposure to AP-3-84 whereas CD3+ T cells had a lower apoptosis response. Data documenting this observation and intended to be supported by added samples in year 2 is shown in Figure 1.



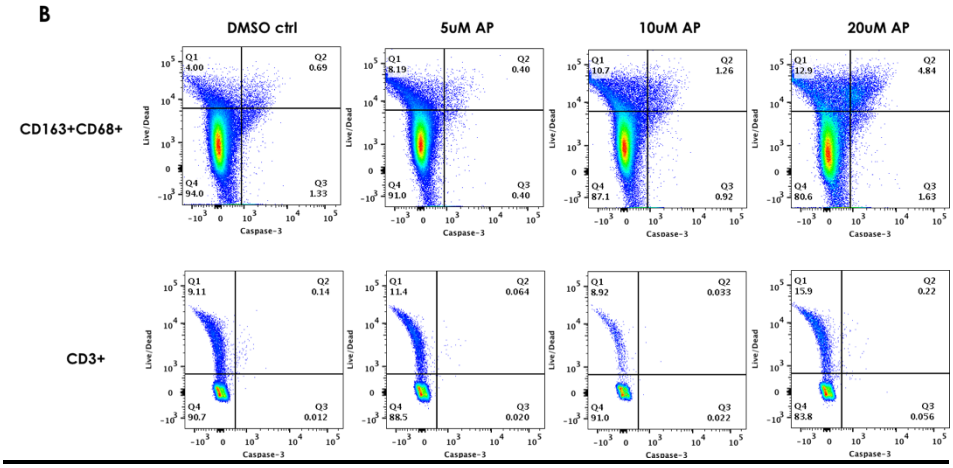


Figure 1. AP-3-84 preferentially induces apoptosis in monocytic myeloid suppressor cells and macrophages. (**A**) PBMCs isolated from ovarian cancer patient were cultured in complete RPMI media treated with or without AP-3-84 for around 24h. Surface staining and intracellular staining were performed to assess apoptosis. (**B**) Ascites cells enriched from ovarian cancer patient were cultured in complete RPMI media treated with or without AP-3-84 for around 24h. Surface staining and intracellular staining were performed to assess apoptosis.

For AIM 2 (refer above to Major Tasks and Subtasks):

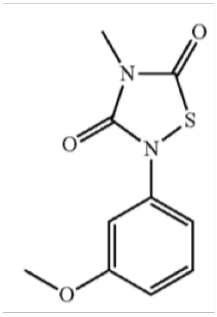
NOTE TIMELINES ARE NOW EXTENDED TO 30 MONTHS BY A NO COST EXTENSION AS A RESULT OF COVID-19 SHUTDOWN IN MONTHS 13-18 OF CURRENT AWARD

1) ***Small molecule AP-3-84 induces apoptosis in murine myeloid cells from ovarian tumor-bearing mice ex vivo*** Compound AP-3-84 (Figure 2A) was discovered as an antagonist to E7 interactions with Rb through a proposed mechanism of displacing E7 from the Rb B-box domain and restoring the binding capacity of Rb with E2F1 to achieve proliferation inhibition. The binding biochemical property is shown in Figure 2B. However, the E7-negative cells also showed effect when exposed to the compound, an apoptosis inducing effect rather than proliferation inhibition. Both tumor cells and 3% thioglycolate induced macrophages demonstrated apoptosis effect as shown in Figure 2C. This apoptosis effect was also found in ascites cells collected from mice bearing stage III ovarian cancer as shown in Figure 2D. In addition, ascites macrophages were noted to be more sensitive to the compound than the associated tumor cells. **We have reproduced this result in year 2.**

(Next Page) Figure 2. AP-3-84 induction of myeloid cell apoptosis in tumor-bearing mice.
(A) Chemical structure of AP-3-84. (**B**) IC50 values for AP-3-84 in the onco-viral protein present system. (**C**) Mouse triple negative breast cancer cells A7C11 were cultured with AP-3-84 treatment for 4h. Mouse 3% Thioglycollate Induced macrophages were cultured with AP-3-84 treatment overnight. Apoptosis was assessed through flow cytometry. (**D**) Ascites cells from stage III ID8 mouse ovarian

cancer model were treated with AP-3-84 for 36h. Apoptosis was induced in both tumor and macrophages.

A



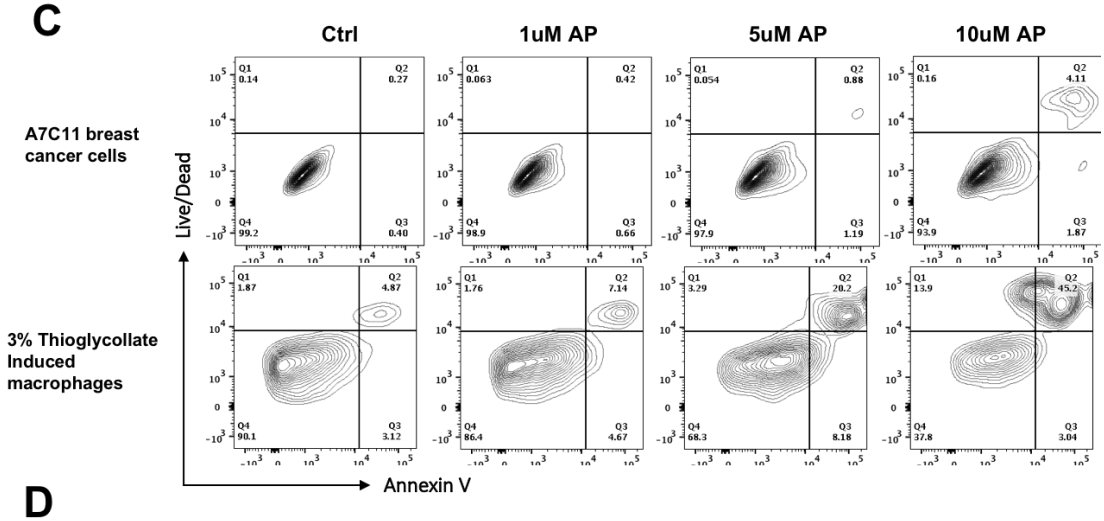
B

TABLE 1

IC50 values for AP-3-84 inhibiting HPV-E7-mediated disruption of pRb/E2F and disrupting pRb/viral Oncoprotein complexes

	16E7 (500nM)	1AE7 (500nM)	E1A (100nM)	K_D (uM)
pRb/E2F	0.34 ± 1.9	3.5 ± 1.7	3.2 ± 2.7	0.815 ± 0.070
pRb	0.29 ± 1.7	4.0 ± 2.5	2.8 ± 2.1	

C



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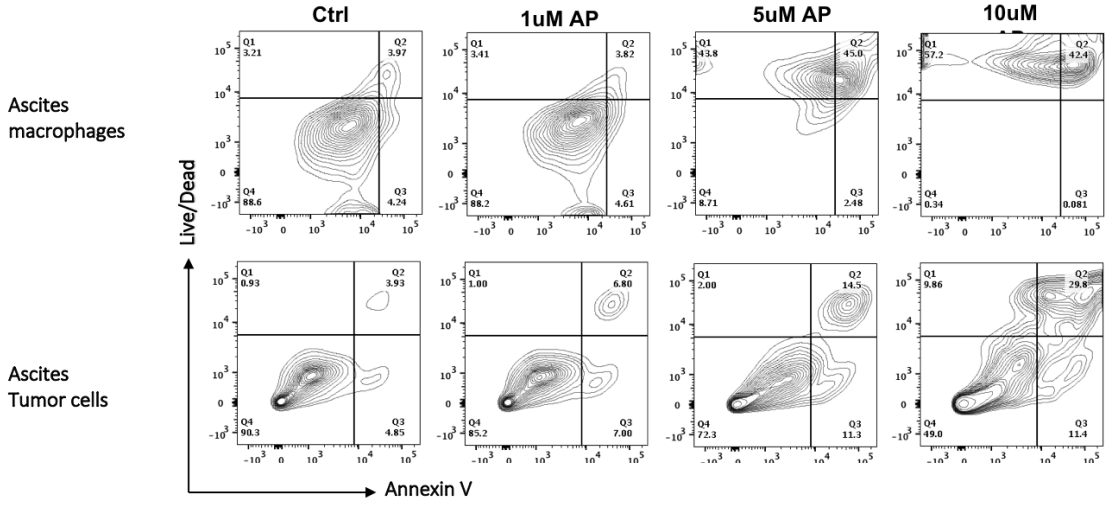


2) ***<u>AP-3-84 activity is not redundant with CDK4/CDK6 inhibitor Palbociclib and is a potentially safe drug candidate.</u>***

Palbociclib is a CDK4/CDK6 inhibitor and targets the same pathway that interacts with Rb. We established the mechanism of action (MOA) of AP-3-84 and palbociclib is different in that palbociclib inhibit cell proliferation whereas AP-3-84 induced apoptosis (Figure 3A). Another difference is that AP-3-84 showed less direct cancer cell cytotoxicity effect than palbociclib (Figure 3B). Moreover, we found the two compounds target different cell populations. AP-3-84 was found to be sensitive in inducing apoptosis of spleen monocytes whereas palbociclib induced apoptosis in neutrophils though such apoptosis effects were not observed in cancer cells (Figure 3C). In line with this finding, AP-3-84 demonstrated apoptosis inducing effect on ascites macrophages from mouse bearing ovarian cancer, whereas palbociclib did not (Figure 3D). SafetyScreen 44 assay (Cerep44), evaluating off-target interactions by AP-3-84 was completed (Figure 3E). **We have reproduced this result in year 2.**

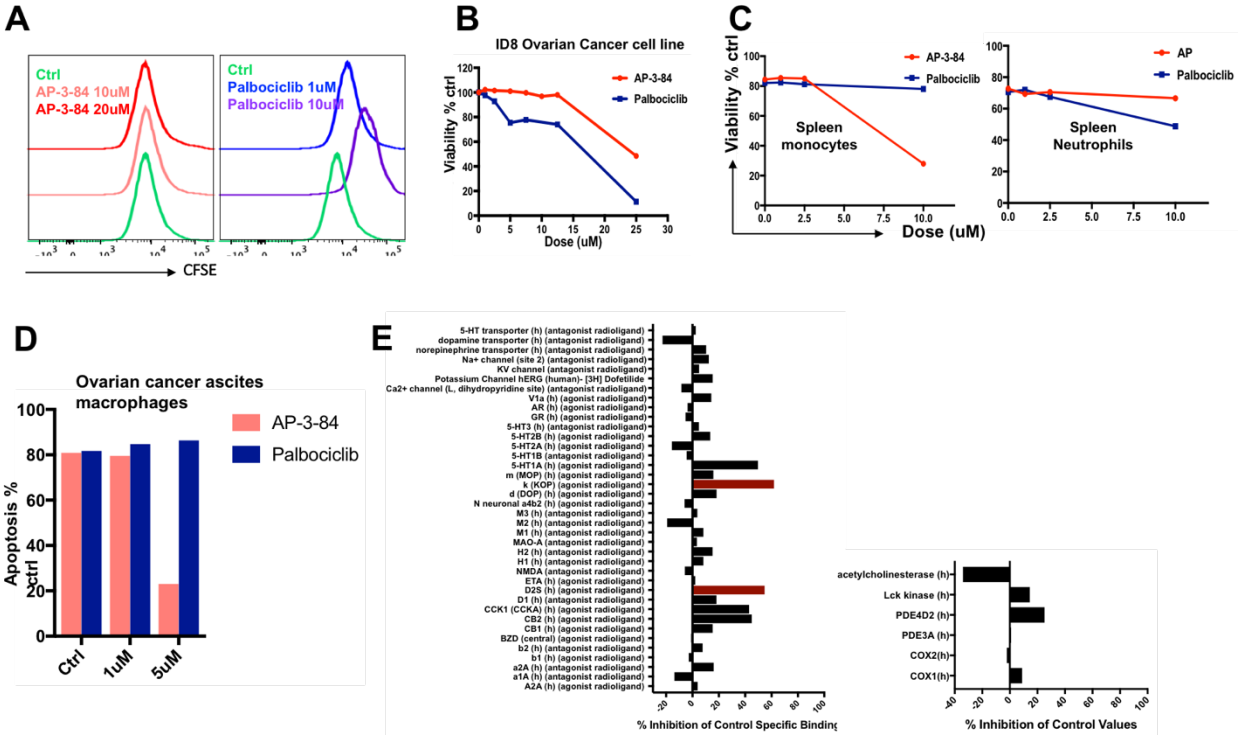


Figure 3. AP-3-84 is distinct from Palbociclib mechanism of action and has a favorable Cerep44 profile. (A) CFSE proliferation inhibition assay with AP-3-84 or palbociclib on Mouse triple negative breast cancer cells A7C11 cells. (B) Cancer cell cytotoxicity assay by MTS on mouse ovarian cancer ID8 cells treated with AP-3-84 or palbociclib for 72h. (C) splenocytes isolated from 6-8 weeks-old mouse were cultured with AP-3-84 or palbociclib treatment for 4h. Apoptosis was assessed by flow cytometry. (D) mouse ascites cells from ID8 ovarian cancer-bearing mouse were cultured in complete RPMI 1640 media with AP-3-84 or palbociclib treatment for 72h. Apoptosis was assessed by flow cytometry. (E) SafetyScreen 44 assay (Cerep44) greater than 90% inhibition was found and only two receptors were found to be inhibited more than 50%.

3) ***<u>Macrophages targeted by AP-3-84 in tumor microenvironment (TME) of stage III mouse ovarian cancer model are confirmed as immunosuppressive.</u>***

To recapitulate human metastatic ovarian cancer, we established a stage III mouse ovarian cancer model by injecting 2M ID8 mouse ovarian cancer cells to the peritoneal cavity (Figure 4A). This model generates ascites as advanced human ovarian cancer does. The onset and progression of ascites was shown to be corelated with poor prognosis. We propose, as reported by other researchers, studying the ascites is of utmost importance for advanced mouse ovarian cancer models. Immunosuppressive markers such as PD-L1 and Arginase were abundantly detected in the ascites macrophages (Figure 4A). immunosuppressive cytokines IL-10 and TNF-a was found to be secreted by macrophages (Figure 4A). Ascites macrophages increased as tumor progressed (Figure 4B). Consistently, macrophage amount negatively correlated with tumor burden and positively correlated with inhibitory T cells (Figure 4C and 4D). Results confirmed ascites macrophages are immunosuppressive in ovarian cancer progression, providing the rationale of targeting macrophage depletion. **We have reproduced this result in year 2.**

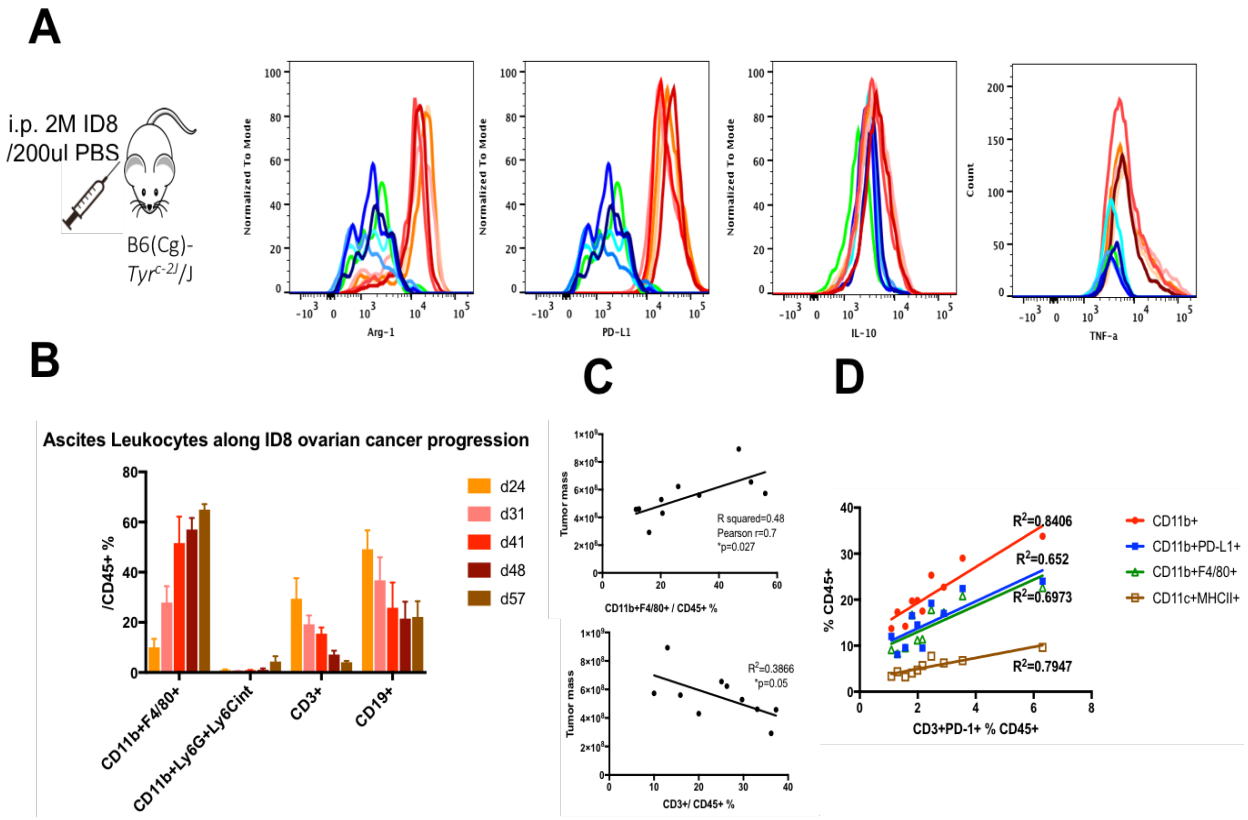
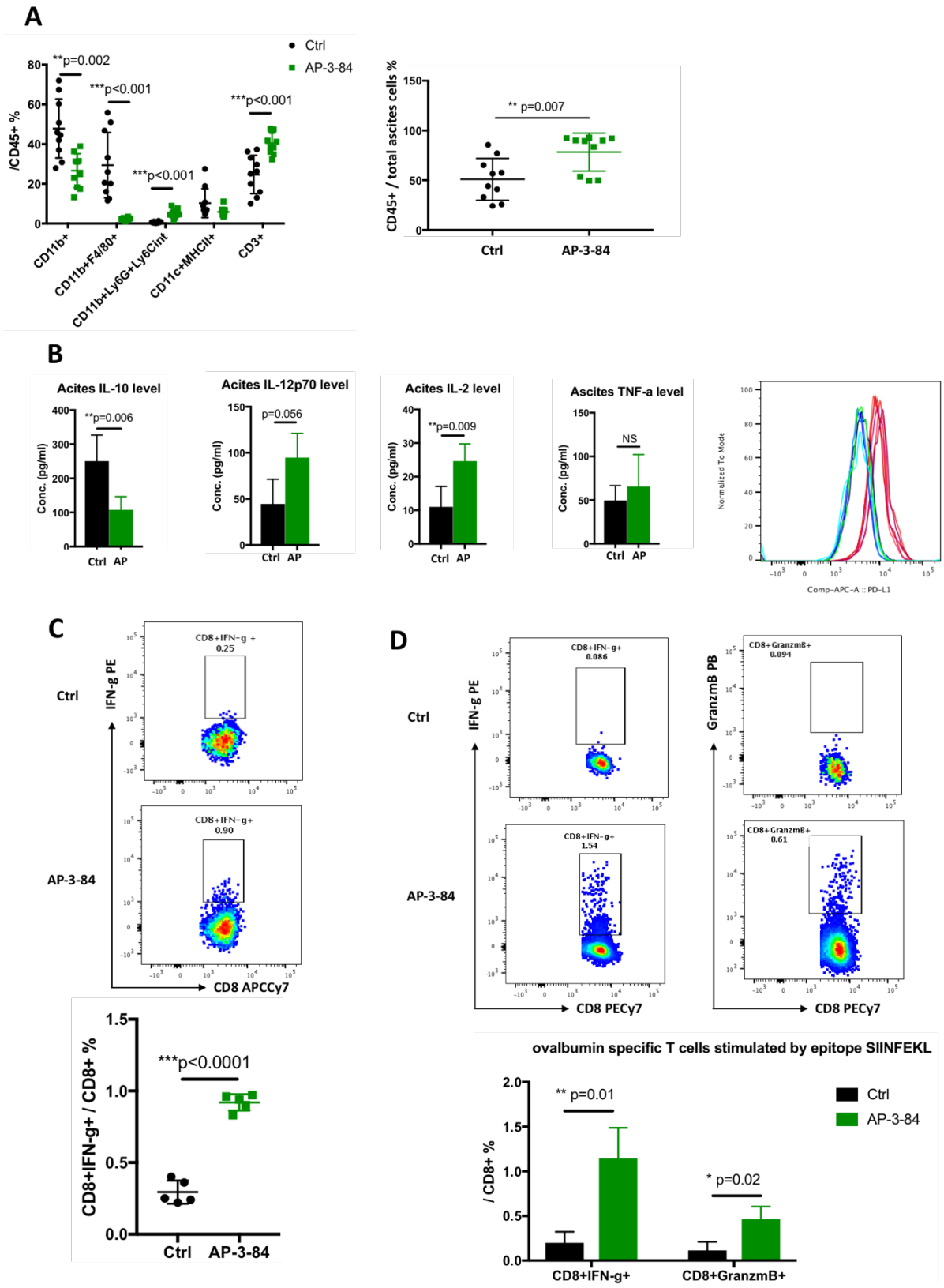


Figure 4. Increase in Immunosuppressive macrophages upon ovarian tumor progression. (**A**) Stage III ID8 mouse ovarian cancer model and immunosuppressive markers and cytokines of ascites macrophages. (B) Ascites macrophages declined along tumor progression. (C) ascites macrophages positively corelates with tumor burden whereas T cells the opposite. (D) ascites macrophages or myeloid cells negatively corelates with inhibitory T cells.

4) ***AP-3-84 depletes macrophages from the tumor microenvironment (TME) and changes the inflammatory profile of TME in Ovarian Cancer***

AP-3-84 or vehicle control was administered at 10mg/kg of mouse weight consecutively for 7 days starting at day 26 after tumor inoculation. We found the AP-3-84 treatment significantly decreased frequency of macrophages in the ascites of tumor-bearing mice. Overall myeloid cells (CD45+11b+ cells) were significantly decreased. Frequency of CD3+ T cells was increased whereas CD11c+MHCII+ DCs were not detected to change (Figure 5A). Through counting cells from ascites of both control and treatment group, we found a significant increase of CD3+ T cell (CD4+ and CD8+) infiltrates in addition to the frequency increase of CD3+ T cells (data not shown). Immunosuppressive cytokines and markers were decreased after the treatment (Figure 5B). Frequency of CD8+IFN-g+ clones were found to be significantly increased in the treatment group (Figure 5C). To assess the tumor antigen specific immunity, we also established the ID8-ova mouse ovarian cancer model by injecting 2M ID8 cells transfected with ovalbumin to the peritoneal cavity. At terminal point of the study, spleen from both groups were harvested and splenocytes were cultured ex vivo with ovalbumin epitope SIINFEKL and Golgistop. We found CD8+ cytotoxic effector clones CD8+GranzmB+ and CD8+IFN-g+ were significantly increased in the treatment group (Figure 5D). To summarize, we found Rb modulator AP-3-84 depleted immunosuppressive macrophages in the TME of stage III ovarian cancer, causing an enhanced T cell infiltration, changed the immunosuppressive TME to a more immunostimulatory TME, and promoted T cell mediated anti-cancer immunity locally and systematically. **We have reproduced depletion of myeloid cells in year 2.**

(Next page) Figure 5. AP-3-84 depleted immunosuppressive macrophages in the TME of stage III ovarian cancer resulting in enhanced anti-tumor T cell infiltration and changed the immunosuppressive TME to a more immunostimulatory TME. (**A)** Ascites was collected at day 36 from Stage III ID8 mouse ovarian cancer model for profile of immune cell subsets by staining with surface markers followed by flow cytometry. (B) Ascites supernatant was harvested after centrifugation. Cytokine level from each mice group was examined with V-PLEX Proinflammatory Panel 1 Mouse Kit from MSD (Meso Scale Diagnostics). (C) Ascites was collected from stage III mouse ovarian cancer model after 7X treatment at day 34. After red blood cell lysis, ascites cells were cultured in complete RPMI 1640 media supplemented with Golgistop for 6h. Frequency of cytotoxic IFN-g+ CD8 effector clones was determined. (D) Mice bearing ID8-ova tumor cells were treated with vehicle control (5% DMSO/5% Tween-80) or AP-3-84 at 10mg/kg of mice weight. The treatment was continued daily for 7 days. Mice were euthanized at terminal point and splenocytes were collected and cultured 4h with GolgiStop and 1ug/ml Ovalbumin epitope SIINFEKL. Ovalbumin antigen specific CD8+IFN-g+ and CD8+GranzmB+ effector clones were assessed after surface and intracellular staining followed by flow cytometry detection.

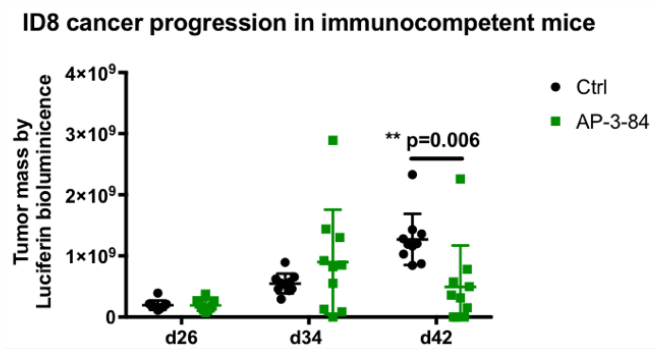

A
Ctrl
AP-3-84
**p=0.002
***p<0.001
***p<0.001
***p<0.001
/CD45+ %
CD11b+
CD11b+F4/80+
CD11b+Ly6G+Ly6Cint
CD11c+MHCII+
CD3+
** p=0.007
CD45+ / total ascites cells %
Ctrl
AP-3-84
B
Acites IL-10 level
**p=0.006
Conc. (pg/ml)
Ctrl AP
Acites IL-12p70 level
p=0.056
Conc. (pg/ml)
Ctrl AP
Acites IL-2 level
**p=0.009
Conc. (pg/ml)
Ctrl AP
Ascites TNF-a level
NS
Conc. (pg/ml)
Ctrl AP
Normalized To Mode
Comp-APC-A :: PD-L1
C
Ctrl
AP-3-84
CD8+IFN-g + 0.25
CD8+IFN-g+ 0.90
IFN-g PE
CD8 APCCy7
***p<0.0001
CD8+IFN-g+ / CD8+ %
Ctrl
AP-3-84
D
Ctrl
AP-3-84
CD8+IFN-g+ 0.086
CD8+IFN-g+ 1.54
IFN-g PE
CD8 PECy7
CD8+GranzmB+ 0.094
CD8+GranzmB+ 0.61
GranzmB PB
CD8 PECy7
ovalbumin specific T cells stimulated by epitope SIINFEKL
** p=0.01
* p=0.02
/ CD8+ %
Ctrl
AP-3-84
CD8+IFN-g+
CD8+GranzmB+

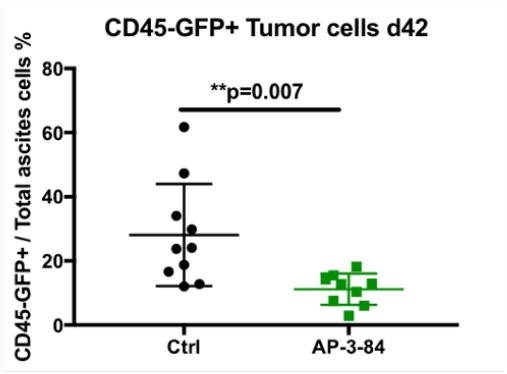
5) ***AP-3-84 treatment significantly delayed ovarian cancer progression in immunocompetent mice but not in immunodeficient NSG mice***

The Stage III ID8 ovarian cancer model was used to assess the in vivo effect of AP-3-84 which was administered to mice at 10mg/kg of mice weight daily for 7 days consecutively. By the end of the treatment at day 42, we did not see an immediate tumor burden change between the control and treatment group (Figure 5A). However, one week after the treatment completion, there was a significant tumor burden drop comparing control and treatment groups which was a dramatic visual difference seen from the IVIS images (Figure 5A). We also examined the amount of tumor cells in the ascites through flow cytometry because the tumor cells are GFP transfected and auto-fluorescent. It was found the CD45-GFP+ cells in treatment group were significantly reduced (Figure 5A). Thus we conclude that AP-3-84 significantly delayed ovarian cancer progression. To investigate whether this in vivo anti-tumor effect was mediated through the enhanced anti-tumor immunity locally and systematically. We established the same model using NSG mice and treated the mice using the same regimen as that for the immunocompetent mice. Interestingly, there was no tumor burden change comparing the control and treated mice (Figure 5B). Therefore, AP-3-84 delayed tumor progression through the improved immunostimulatory immune system rendering it a promising immunotherapeutic candidate by targeting Rb, a novel immuno-target. **We have reproduced this result in year 2.**

(Next Page) Figure 5 AP-3-84 decreases ovarian tumor burden in intact but not immunodeficient NSG mice. (**A**) Stage III ID8 mouse ovarian cancer model was established by injection of 2M ID8 luciferin transfected cells in PBS to mouse peritoneal cavity at day 0. Mice were treated with vehicle control (5% DMSO/5% Tween-80) or AP-3-84 at 10mg/kg of mice weight daily for 7 days (n=10). At day 34 and day 42, tumor burden from all groups of mice was assessed. Visual difference demonstrated by IVIS images are representative ones of 5 mice (one cage) from control or treated group respectively. At day 42, ascites cells were harvested for assessment of GFP+CD45- tumor cells in both groups by flow cytometry. (**B**) Stage III ID8 mouse ovarian cancer model was established by injection of 2M ID8 luciferin transfected cells in PBS to NSG mouse peritoneal cavity at day 0. At day 24, mice were treated with vehicle control (5% DMSO/5% Tween-80) or AP-3-84 at 10mg/kg of mice weight daily for 7 days (n=10). At day 31 and day 36, tumor burden from all groups of mice was assessed.

A


ID8 cancer progression in immunocompetent mice
Tumor mass by Luciferin bioluminicence
4×10^9
3×10^9
2×10^9
1×10^9
0
** p=0.006
Ctrl
AP-3-84
d26
d34
d42


CD45-GFP+ Tumor cells d42
CD45-GFP+ / Total ascites cells %
80
60
40
20
0
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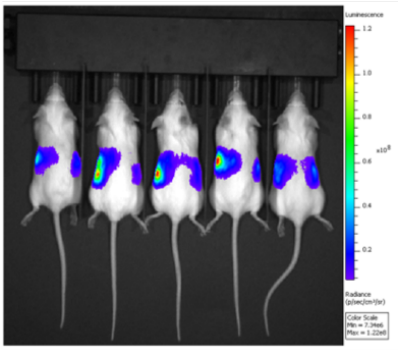
AP-3-84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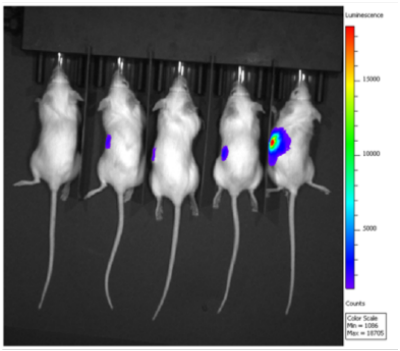
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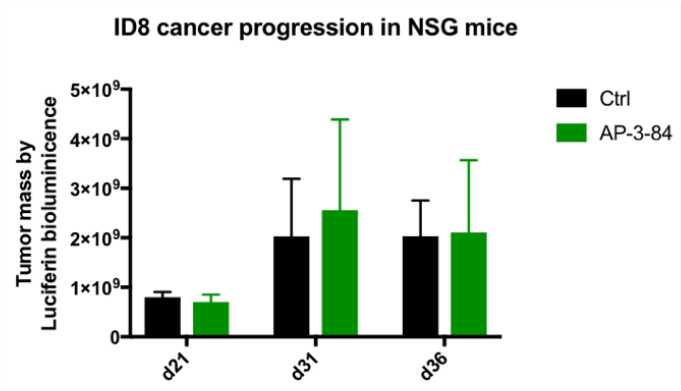
ID8 cancer progression in NSG mice
Tumor mass by Luciferin bioluminicence
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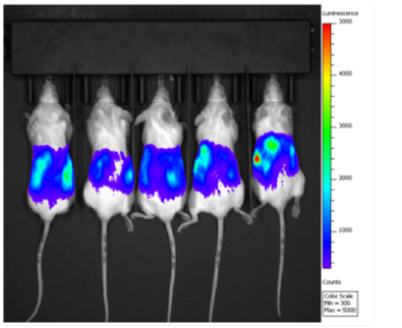
AP-3-84
d21
d31
d36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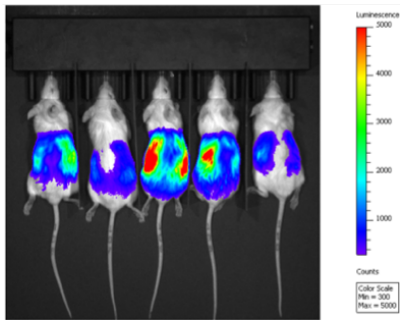

AP-3-84

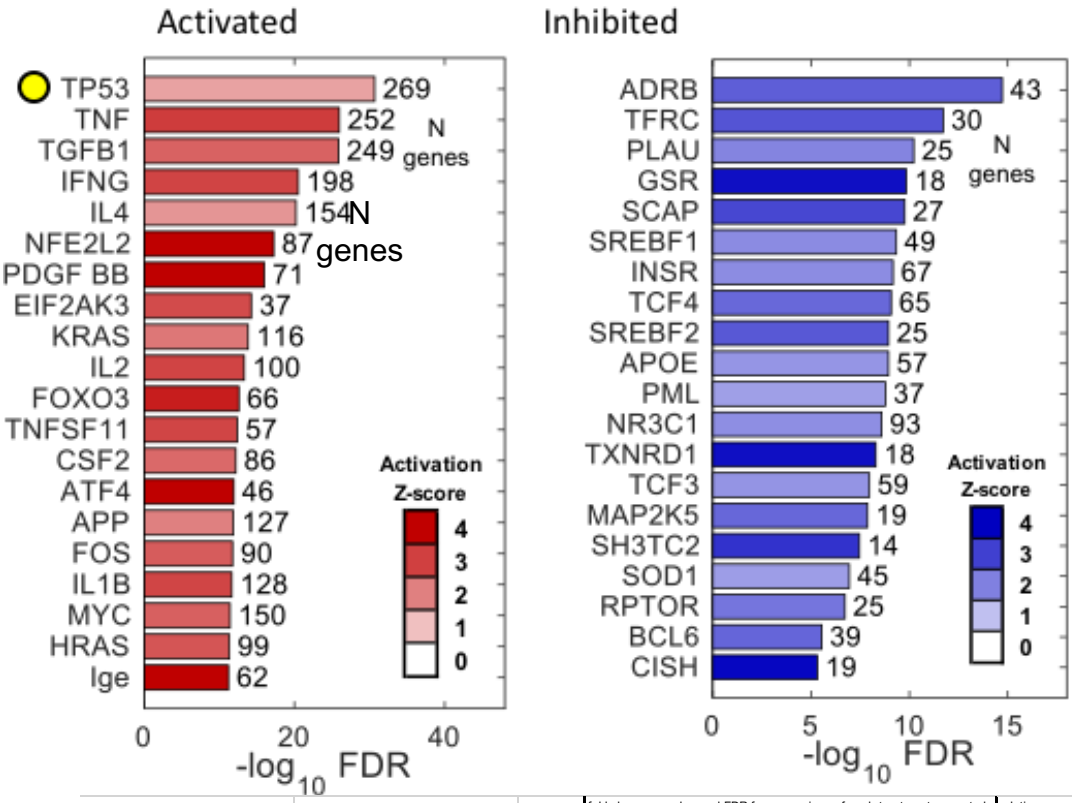
6) ***AP-3-84 ex vivo treatment of thioglycolate stimulated murine macrophages increase gene transcription associated with p53 activation.*** To determine the modulation of myeloid cells when exposed to AP-3-84, thioglycolate-stimulated macrophages were cultured ex-vivo and treated with AP-3-84 before harvesting mRNA at 4 and 18 hours. Apoptosis induction was independently confirmed at 18 hrs. as shown in Figure 1. Bioinformatic analysis of gene expression data evidenced the top regulator of gene expression at 4 hours to be gene activation mediated by p53 activation (Panel A and B). Results show a preferential induction of p53 as the top regulator cell network induced by AP-3-84.

(next page) Figure 6. AP-3-84 modulates myeloid gene transcription with top activated gene regulator as p53 activation. Mouse 3% Thioglycollate Induced macrophages were cultured with AP-3-84 treatment and mRNA isolated at 4 and 18 hours after exposure. Panel A shows top changes for stimulated and inhibited gene regulators to stress the predominance of p53 activated gene regulation as top activated gene expression. Panel B lists top p53 activated genes as measured at 4 and 18 hours.

7) ***AP-3-84 ex vivo treatment of thioglycolate stimulated murine macrophages increase p53 protein levels as well as increase p53 protein in myeloid cells from human ovarian patients.*** To confirm relationship between gene induction and protein expression in ovarian tumor-bearing mice murine and ovarian myeloid cells from ovarian patients, we measured p53 expression by immunofluorescence, western and flowcytometry. Panel A shows p53 expression in thioglycolate-stimulated macrophages cultured ex-vivo with AP-3-84 by microscopy while Panel B shows data by Western blot analysis including measures for MDM2 and Bax. Panel C extends data analysis to ascites myeloid cells from ovarian cancer patients (collected post surgery) measuring induction of p53 expression by flowcytometry after exposure to AP-3-84.

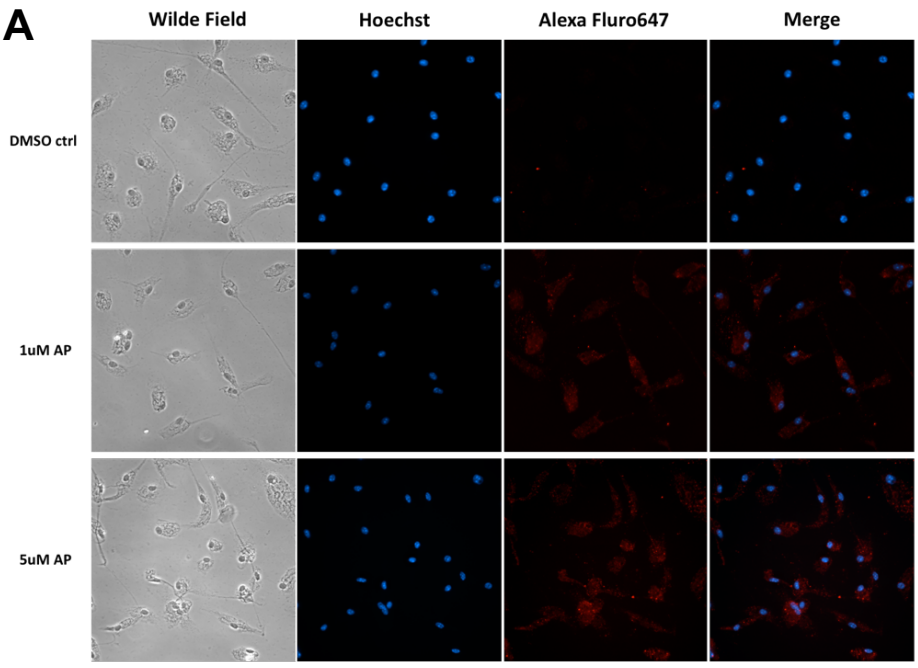
(second next page) Figure 7 AP-3-84 modulates p53 protein expression in myeloid cells from tumor-bearing mice and ascites from ovarian cancer patients. Panels a and B show mouse 3% Thioglycollate Induced macrophages were cultured with AP-3-84 treatment overnight and analyzed for p53 expression by microscopy and Western blot analysis. Panel C shows two ovarian patient ascites myeloid cells analyzed by flowcytometry for p53 expression over increasing concentrations of AP-3-84.

A



B

	Intracellular function	Symbol	fold change, pvalue and FDR for comparison of each treatment vs control									relative expression profile in a form of heatmap									
			4h, 10uM	18h, 5uM	18h, 10uM	4h, 10uM	18h, 5uM	18h, 10uM	4h, 10uM	18h, 5uM	18h, 10uM	ctr			4h, 10uM			18h, 5uM			18h, 10uM		
			fold	fold	fold	pv	pv	pv	fdr	fdr	fdr	1	2	3	1	2	3	1	2	3	1	2	3
RAD51 associated protein 2	p53 target	Rad51ap2	41.46	5.835	5.512	0.01	0.251	0.27	14%	100%	100%												
von Willebrand factor C and EGF domains	p53 target	Vwce	14.58	1.028	1.069	0.336	0.992	0.981	100%	100%	100%												
dual specificity phosphatase 1	p53 target	Dusp1	3.287	1.084	1.022	5E-29	0.455	0.837	0%	100%	100%												
dual specificity phosphatase 10	p53 target	Dusp10	2.395	1.294	-1.112	2E-04	0.292	0.673	1%	100%	100%												
dual specificity phosphatase 13	p53 target	Dusp13	2.178	1.109	1.267	0.002	0.694	0.366	4%	100%	100%												
sestrin 2	p53 target	Sesn2	2.143	-1.455	-1.271	9E-05	0.066	0.237	0%	100%	83%												
TNF receptor associated factor 4	p53 target, apoptosis regulator	Traf4	2.141	2.829	2.35	0.431	0.275	0.375	100%	100%	100%												
dual specificity phosphatase 14	p53 target	Dusp14	2.114	1.888	1.005	0.103	0.168	0.992	63%	100%	100%												
growth differentiation factor 15	p53 target	Gdf15	2.104	-1.162	1.015	1E-14	0.122	0.882	0%	100%	100%												
polo like kinase 2	p53 target	Plk2	1.731	1	1.071	5E-14	0.995	0.353	0%	100%	100%												
transformed mouse 3T3 cell double minute 2	p53 target	Mdm2	1.705	1.007	1.033	7E-17	0.908	0.612	0%	100%	100%												
immediate early response 5	p53 target	Ier5	1.705	1.045	1.03	2E-27	0.376	0.553	0%	100%	100%												
polo like kinase 1	p53 target	Plk1	1.698	1.33	1.241	0.041	0.278	0.417	36%	100%	100%												
colony stimulating factor 1 (macrophage)	p53 target	Csf1	1.69	1.558	1.276	0.005	0.017	0.196	8%	100%	48%												
phorbol-12-myristate-13-acetate-induced protein 1	p53 target, apoptosis regulator	Pmaip1	1.682	-1.107	1.066	0.001	0.538	0.696	2%	100%	100%												
malic enzyme 1, NADP(+)-dependent, cytosolic	p53 target	Me1	1.596	1.033	1.204	4E-04	0.809	0.163	1%	100%	100%												
growth arrest and DNA-damage-inducible 45 alpha	p53 target	Gadd45a	1.586	1.002	1.127	6E-08	0.986	0.163	0%	100%	100%												
B cell translocation gene 2, anti-proliferative	p53 target	Btg2	1.58	1.152	-1.043	1E-10	0.05	0.568	0%	100%	75%												
ring finger protein 19B	p53 target	Rnf19b	1.526	1.123	1.051	3E-10	0.088	0.468	0%	100%	92%												
ribonucleotide reductase M2 B (TP53 inducible)	p53 target, DNA repair	Rrm2b	1.447	1.188	1.213	8E-06	0.039	0.021	0%	55%	68%												
cyclin-dependent kinase inhibitor 1A (P21)	p53 target	Cdkn1a	1.434	1.082	-1.031	2E-06	0.297	0.686	0%	100%	100%												
microtubule associated monooxygenase, calponin an	p53 target	Micall1	1.354	-1.142	-1.068	2E-05	0.07	0.366	0%	100%	85%												
protein tyrosine phosphatase 4a1	p53 target	Ptp4a1	1.315	1.185	1.08	1E-06	0.002	0.17	0%	100%	17%												
BCL2/adenovirus E1B interacting protein 3	p53 target	Bnip3	1.28	1.126	-1.038	0.011	0.228	0.704	15%	100%	100%												
polo like kinase 3	p53 target	Plk3	1.267	-1.203	1.003	0.045	0.122	0.98	38%	100%	100%												
polo like kinase 3	p53 target	Plk3	1.267	-1.203	1.003	0.045	0.122	0.98	38%	100%	100%												
SERTA domain containing 1	p53 target	Sertad1	1.258	1.066	1.099	1E-04	0.291	0.117	0%	100%	100%												
G patch domain and ankyrin repeats 1	p53 target	Gpank1	1.24	1.096	1.03	0.019	0.322	0.751	22%	100%	100%												
sulfatase 2	p53 target	Sulf2	-1.25	1.143	-1.012	1E-04	0.021	0.844	0%	100%	53%												
family with sequence similarity 210, member B	p53 target	Fam210b	-1.377	-1	-1.119	0.012	0.998	0.374	16%	100%	100%												
MAD1 mitotic arrest deficient 1-like 1	p53 target	Mad1l1	-1.436	-1.005	1.033	0.005	0.968	0.8	8%	100%	100%												
ATP-binding cassette, sub-family B (MDR/TAP), mem	p53 target	Abcb1a	-1.443	-1.126	-1.335	6E-04	0.258	0.007	2%	30%	100%												
cell division cycle 25B	p53 target	Cdc25b	-1.474	-1.062	-1.137	8E-05	0.532	0.185	0%	100%	100%												
proline-serine-threonine phosphatase-interacting prot	p53 target	Pstpip2	-1.514	-1.393	-1.315	0.036	0.092	0.163	33%	100%	93%												
sestrin 1	p53 target	Sesn1	-1.605	1.234	1.05	6E-10	0.004	0.516	0%	100%	24%												
peptidyl arginine deiminase, type IV	p53 target	Padi4	-1.69	1.014	-1.027	0.004	0.934	0.879	7%	100%	100%												
PERP, TP53 apoptosis effector	p53 target, apoptosis regulator	Perp	-1.717	-3.068	-11.23	0.783	0.578	0.241	100%	100%	100%												
RAD51 associated protein 1	p53 target	Rad51ap1	-2.303	-1.118	-1.18	0.049	0.781	0.68	41%	100%	100%												
cell division cycle 25C	p53 target	Cdc25c	-12.56	-1.974	-23.57	0.108	0.627	0.045	65%	73%	100%												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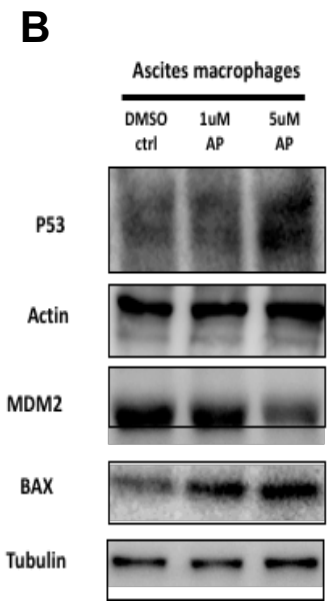
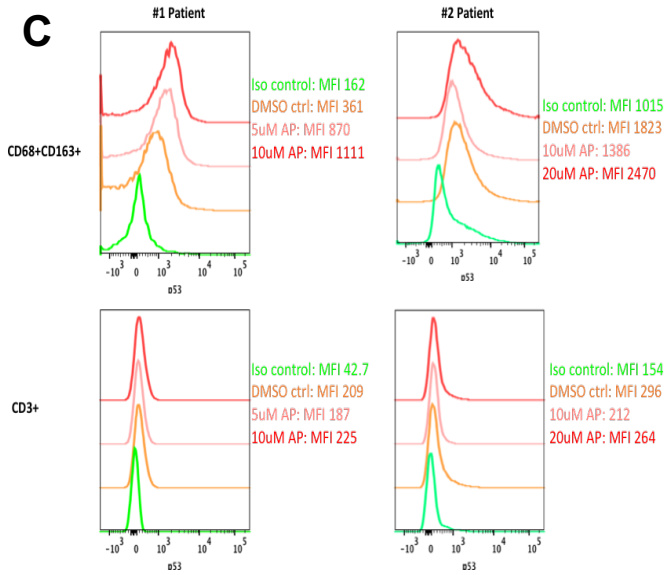
Wilde Field
Hoechst
Alexa Fluro647
Merge
DMSO ctrl
1uM AP
5uM AP


B
Ascites macrophages
DMSO ctrl
1uM AP
5uM AP
P53
Actin
MDM2
BAX
Tubulin


C
#1 Patient
#2 Patient
CD68+CD163+
Iso control: MFI 162
DMSO ctrl: MFI 361
5uM AP: MFI 870
10uM AP: MFI 1111
Iso control: MFI 1015
DMSO ctrl: MFI 1823
10uM AP: 1386
20uM AP: MFI 2470
CD3+
Iso control: MFI 42.7
DMSO ctrl: MFI 209
5uM AP: MFI 187
10uM AP: MFI 225
Iso control: MFI 154
DMSO ctrl: MFI 296
10uM AP: 212
20uM AP: MFI 264
p53

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What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

For Aim 1, during no cost extension we plan to receive more clinical samples to complete Major Task 1 (Subtasks 2 and 3) and Major Task 2 (subtask 2 and 3) in order to assess the effect on apoptosis, function and gene change of human myeloid and lymphoid cells. We will repeat experiments measuring gene induction of ascites myeloid cells exposed to AP-3-84 but also add sorted T-cells as comparator (as we expect not equal regulation in T-cells).

For Aim 2, during no cost extension we are planning to test combination between AP-3-84 with anti-PD1 (Major Task 4, Subtask 2).

We plan to complete final data analysis and manuscript preparation for submission (Major Task 5).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Data collected to date supports our central initial hypothesis that treatment with Rb modulators AP-3-84 can result in ovarian tumor regression and/or improved survival by increasing myeloid cell death and increasing anti-tumor immune-mediated responses. The ultimate impact of this study will be the introduction of targeting the Rb protein as a **new first-in-class molecular target**

in immunosuppressive myeloid cells in achieving control of ovarian cancer by promoting myeloid cell death within the tumor microenvironment, leading to a decrease in local immunosuppression, and enhanced immune-mediated cellular control.

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

As we had indicated in our last progress report, due to the COVID-19 pandemic which resulted in a state-mandated shutdown of our clinical sites for months, we had a delay in receiving clinical samples from those sites to complete our analyses. Dr. Muthumani also left his position before end of second year to be replaced by Dr. Evgenii Tcyganov. Once the clinical sites were permitted to re-open, we have been doing our best to make up for the delays but due to the recent resurgence in the area we are requesting an extension to ensure that we complete the project.

As we previously indicated, we are projecting an estimated carryforward of $65,156. While we have requested a one-time 12-month extension, in the event there are no further shutdowns, we should be able to complete the project with an additional 3 to 6 months no-cost extension.

Changes that had a significant impact on expenditures

As we had indicated in our last progress report, due to the COVID-19 pandemic which resulted in a state-mandated shutdown of our clinical sites for months, we had a delay in receiving clinical samples from those sites to complete our analyses. Therefore, an extension was requested due to a change in expenditure during year 2.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

As indicated above, we are projecting an estimated carryforward of $65,156. While we have requested a one-time 12-month extension, in the event there are no further shutdowns, we should be able to complete the project with an additional 3 to 6 months no-cost extension.

Significant changes in use or care of vertebrate animals
As indicated above, we are projecting an estimated carryforward of $65,156. While we have requested a one-time 12-month extension, in the event there are no further shutdowns, we should be able to complete the project with an additional 3 to 6 months no-cost extension.

Significant changes in use of biohazards and/or select agents
None

6. PRODUCTS:

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:		Luis J. Montaner
Project Role:		Principal Investigator
Researcher Identifier		
Nearest whole person month worked:		1
Contribution to Project:		Overall administration and guidance of research conducted. Training and management of personnel engaged in achieving specific aims outlined in proposal
Funding Support		This award

Name:		Evgenii Tcyganov
Project Role:		Postdoctoral Fellow
Researcher Identifier		
Nearest whole person month worked:		1
Contribution to Project:		Perform experiments and analysis outlined in grant application
Funding Support		This award

Name:		Taekyoung Kwak
Researcher Identifier		
Project Role:		Postdoctoral Fellow
Nearest whole person month worked:		3

Contribution to Project:		Perform experiments and analysis outlined in grant application
Funding Support		This award

Name:		Devivasha Bordoloi
Researcher Identifier		
Project Role:		Postdoctoral Fellow
Nearest whole person month worked:		6
Contribution to Project:		Perform experiments and analysis outlined in grant application
Funding Support		This award

Name:		Xue Yang
Researcher Identifier		
Project Role:		Postdoctoral Fellow
Nearest whole person month worked:		1
Contribution to Project:		Perform experiments and analysis outlined in grant application
Funding Support		This award

Name:		Laxminarasimha Donthireddy
Research Identifier		
Project Role:		Research Assistant
Nearest whole person month worked:		3
Contribution to Project:		Assist with experiments and analysis outlined in grant application
Funding Support		This Award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Muthumani left his position before second year to be replaced by Dr. Evgenii Tcyganov.

What other organizations were involved as partners?

Christiana Care Health System (CCHS)

Location of Organization: Delaware

Partner's contribution to the project (identify one or more)

- In-kind support: computers, equipment available to project staff

- Facilities (e.g., project staff use the partner's facilities for project activities)
- Collaboration (e.g., partner's staff work with project staff on the project)

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS:
See attached.

9. APPENDICES:

Not applicable.

Development of Novel Small-Molecule Rb Protein Modulator for Ovarian Cancer Immunotherapy
OC180193
W81XWH-19-1-0092

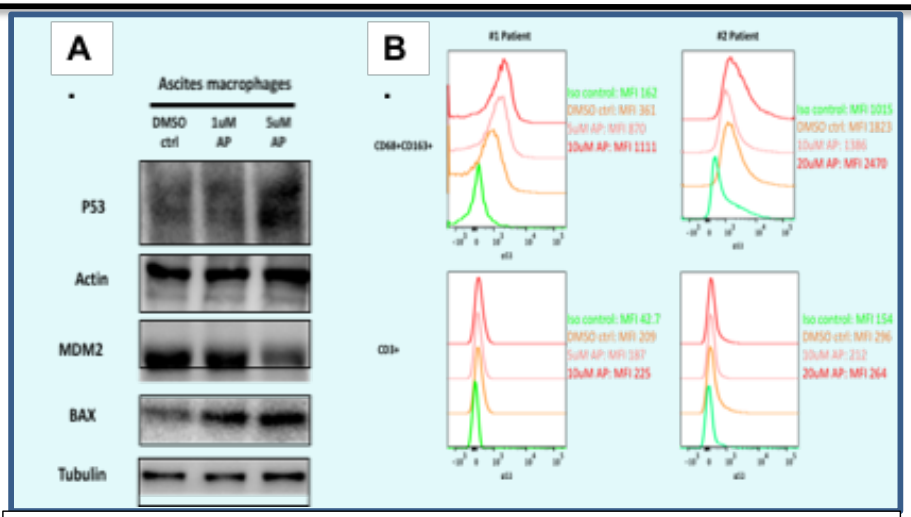
PI: Luis J. Montaner, Ph.D. **Org:** The Wistar Institute of Anatomy and Biology **Award Amount:** $444,334

Study/Product Aim(s)

- Specific Aim 1 – Establish the impact of exposure to AP3-84 on gene expression, viability, and function
- Specific Aim 2 - Defining the efficacy of treatment with AP-3-84 in achieve tissue and tumor myeloid cell depletion

Approach

We hypothesize that treatment with Rb modulators of AP-3-84 will result in ovarian tumor regression and improved survival by increasing myeloid cell death. We will establish the impact of AP-3-84 treatment on viability and function of purified myeloid and lymphoid cells from human or murine ovarian cancer tissue; define efficacy of treatment with AP-3-84 in achieving tissue and tumor myeloid cell depletion and the impact of myeloid cell depletion on T-cell cell activation, priming, infiltration, and altering tumor burden; identify molecular mechanism of action of AP-3-84.



Accomplishment: Ascites myeloid cells from ovarian tumor bearing mice (Panel A, Western) as well as human patients (Panel B, flowcytometry) show increased p53 activation after in vivo administration of AP-3-84 (Panel A) or ex vivo exposure of increasing doses of AP-3-84.

Timeline and Cost

Activities CY	19	20
Task 1 Direct apoptosis effects		
Task 2 Gene expression change		
Task 3 In vivo Anti-tumor AP-3-84		
Task 4 In vivo Cell Apoptosis & Immune Control		
Estimated Budget ($K)	**$152,451**	**$444,334**

Updated: March 31, 2021

Goals/Milestones CY19

***Specific Aim 1* Major Task 1 – Direct apoptosis effects**

Local IRB and DoD HRPO approval

Establish direct apoptosis effects on myeloid cells

***Specific Aim 1* Major Task 2 – Gene expression change**

Establishing gene expression: myeloid and lymphoid cells after Rb modulation

***Specific Aim 2* Major Task 1-3 – in vivo AP-3-84 therapy**

Local IACUC and DoD ACURO approval

First experiment – determine efficacy of AP-3-84 treatment in achieving tissue and tumor myeloid cell depletion

Goals/Milestones CY20

Completion of Aim 1 Task 1, Aim 2 Task , and Manuscript Publication

Comments/Challenges/Issues/Concerns

- Major Task 3 delay, COVID-19 pandemic impact on timeline to completion so no cost extension requested/granted to June '21.

Budget Expenditure to Date

Projected Expenditure: $444,334 (Direct and Indirect)

Actual Expenditure: $369,715 (Direct and Indirect)