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Award Number: W81XWH-11-1-0509

TITLE: Nuclear Factor-Kappa B Activity in the Host-Tumor Microenvironment of
Ovarian Cancer

PRINCIPAL INVESTIGATOR: Dr. Andrew Wilson

CONTRACTING ORGANIZATION: Vanderbilt University
Nashville, TN 37203-6869

REPORT DATE: August 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.				
1. REPORT DATE August 2013		2. REPORT TYPE Annual		3. DATES COVERED 25 July 2012 – 24 July 2013
4. TITLE AND SUBTITLE Nuclear Factor-Kappa B Activity in the Host-Tumor Microenvironment of Ovarian Cancer			5a. CONTRACT NUMBER W81XWH-11-1-0509	
			5b. GRANT NUMBER W81XWH-11-1-0509	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Andrew Wilson E-Mail: Andrew.j.wilson@vanderbilt.edu			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Nashville, TN 37203-6869			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)	
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT Overcoming tumor resistance to platinum chemotherapy is critical for prolonging life in women with advanced ovarian cancer. The nuclear factor-kappaB (NF-κB) signaling pathway is a key mediator of tumorigenesis by linking inflammatory pathways to cancer. Inhibitors of NF-κB such as thymoquinone (TQ) potentiate the effects of cytotoxic agents, including cisplatin, in ovarian cancer cells. Equally relevant are the potential effects of NF-κB inhibition in host cells such as peritoneal macrophages, thought to play pro-tumor (M2-like) or anti-tumor (M1-like) roles during ovarian cancer progression. We will define patterns of NF-κB activity in host cells using NF-κB reporter (NGL) transgenic mice injected with mouse ID8 ovarian cancer cells. NF-κB activity in tumor cells will be monitored through stable transfection of the NGL reporter. We have detected increased NF-κB reporter activity in tumor cells and in host macrophages, and increased markers of M2 macrophages in ascites fluid, in late stages of progression. Reducing NF-κB activity in tumor cells with TQ treatment was associated with a shift towards M1 macrophages, while longer-term TQ treatment lead to increased ascites, elevated NF-κB signaling and an M2 shift. Combined TQ and cisplatin treatment lead to synergistic anti-tumor effects in vitro, reduced tumor burden and apoptotic marks in tumors to a greater extent than treatment with cisplatin alone, and reduced M2, and induced M1, macrophage markers.				
15. SUBJECT TERMS NF-kappaB, ovarian cancer, Thymoquinone, macrophages				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U		
				19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Ovarian cancer is the most common cause of death from gynecologic malignancies in the United States [1]. Most women with epithelial ovarian cancers are diagnosed with advanced, metastatic disease characterized by widespread peritoneal carcinomatosis and abdominal ascites [2]. Overcoming tumor resistance to platinum chemotherapy is a key objective for prolonging life in women with advanced disease. The nuclear factor- κ B (NF- κ B) signaling pathway is known to play an important role in several malignancies, including ovarian cancer [3-8]. Constitutive activation of NF- κ B is observed in a large subset of ovarian tumors, is associated with tumor growth, progression and resistance to chemotherapy, and is an important molecular link between inflammation and cancer [3-8]. Inhibitors of NF- κ B are known to suppress angiogenesis and progressive tumor growth [9], and potentiate the anti-tumor activity of cytotoxic agents [10], in ovarian cancer cells. One example is thymoquinone, a product of the medicinal plant *Nigella sativa* [11]. Thus, a promising strategy in ovarian cancer treatment is the combination of NF- κ B inhibitors with current platinum-based regimens. Equally relevant, but far less understood are the potential effects of NF- κ B inhibition in host cells. Host macrophages are thought to play a pro-tumor role during ovarian cancer progression, at least in part via aberrant NF- κ B signaling activity [12] in these macrophages, designated M2. These M2-like, tumor-associated macrophages may be a target for therapy through “re-education” towards a cytotoxic (M1), anti-tumor function by NF- κ B inhibition [12]. **However, major gaps in knowledge still remain regarding the specific influence of NF- κ B, and the consequences of inhibiting its activity, in cancer cells and host cells during ovarian cancer progression.** We will use an innovative strategy to define patterns of NF- κ B activity in ovarian cancer cells and in host cells during peritoneal carcinomatosis *in vivo* in a unique preclinical model. Mouse ID8 ovarian cancer cells will be injected intra-peritoneally into NF- κ B reporter transgenic mice expressing a green fluorescent protein (GFP)/luciferase fusion product under the control of a synthetic NF- κ B-dependent promoter [13, 14], allowing intra-vital mapping of NF- κ B activity in the host during tumor development. In a complementary approach, ID8 cells stably transfected with the NF- κ B reporter will be monitored in wild-type mice to study measure NF- κ B activity in the developing tumor. Completion of the proposed work will not only provide a unique insight into the role of NF- κ B in ovarian cancer progression, but also provide a powerful tool for the preclinical testing of NF- κ B inhibitors as single agents, or as combination therapy to help overcome resistance to established treatment.

The ***hypothesis*** to be tested is that *ovarian tumor progression depends on NF- κ B activation in both malignant ovarian epithelial cells and host macrophages in the peritoneal microenvironment.* Our ***objectives*** are (1) defining the pattern of NF- κ B activity in the host-tumor microenvironment during ovarian cancer progression and (2) evaluating consequences of inhibiting NF- κ B activity in tumor and host cells during ovarian cancer progression via treatment with NF- κ B inhibitors alone and in combination with cisplatin chemotherapy.

BODY

Task 1. Define the pattern of NF- κ B activity in the host-tumor microenvironment during ovarian cancer progression:

1a. Validate response of ovarian cancer cells stably transfected with NF- κ B reporter (ID8-NGL).

We have further confirmed the specificity of our ID8-NGL stable cell line by now showing that two known activators of NF- κ B signaling, TNF- α and IL-1 β , robustly increase NF- κ B reporter activity in these cells (Fig 1A).

1b. Validate response of bone marrow-derived macrophages from NGL reporter transgenic C57BL/6 mice (BMDM-NGL).

We have further confirmed that bone marrow-derived macrophages harvested from NGL reporter mice, designated BMDM-NGL, also response to TNF- α stimulation with approximately 2-fold increase in NF- κ B reporter activity in these cells (Fig 1B).

1c. Evaluate NF- κ B activity in tumor cells during tumor progression in vivo.

The parental cell line for our ID8-NGL cells, ID8, has previously been shown to model the peritoneal carcinomatosis characteristic of advanced serous ovarian cancer [15]. To confirm that the ID8-NGL cells formed a similar pattern of abdominal tumorigenesis, we conducted an initial trial experiment. Wild-type BL6 mice were injected IP with ID8-NGL cells or IP with PBS only as a non-injected control. Mice were sacrificed at 30d, 60d or 90d. Bioluminescence imaging (BLI) of the 90d groups were performed at the 30d, 60d and 90d time points to non-invasively measure activity of the NGL reporter in the tumor cells (see Fig 2 for a schematic representation of our experimental design).

As shown in Fig 3A, at approximately 90d after tumor cell injection, mice developed prominent abdominal distension compared to control mice receiving mock (PBS) injection (designated “non-injected” throughout Task 1), indicative of ascites. This was consistent with significantly increased body weight and abdominal girth observed in mice at this time point compared to PBS-injected controls (Fig. 3B&C). Careful dissection of the abdominal cavities revealed that injected mice displayed a reproducible pattern of tumor development. As shown in Fig 3D, tumor nodules were detected embedded in the peritoneal wall (yellow arrows), and in the connective tissue (mesentery) of the intestines (white arrows). A representative tumor implant in the smooth muscle of the peritoneal wall is shown in Fig 3E. Therefore, measuring number of peritoneal implants and mesenteric tumor mass will be primary end-point indices for quantifying direct tumor burden.

Bioluminescence analysis was performed in the Vanderbilt University Small Animal Imaging Core using the Xenogen IVIS 200 bioluminescent image system with Living Image acquisition software (Caliper Life Science). Quantification of bioluminescence in the peritoneal cavity was performed through region of interest analyses of a standardized area based on anatomical landmarks. First, we confirmed that mice injected with ID8-NGL cells displayed high levels of luminescence compared to PBS-injected mice by bioluminescence imaging. Representative images are shown in Fig. 4A. ID8-NGL-injected mice displayed approximately 100-fold higher levels than non-injected controls (average of $3.7 \pm 2.3 \times 10^7$ Relative Luciferase Units [RLU] over the 30, 60 and 90 day time points examined compared to $0.013 \pm 0.009 \times 10^7$ RLU, respectively, $p < 0.001$, Mann-Whitney test), indicating the specificity of the signal. NF- κ B reporter activity increased in a time-dependent manner (Fig. 4B), which showed significant correlation with indices of tumor burden such as mesenteric tumor mass and ascites volume at 90 days (Figs. 4C&D). To differentiate between specific increases

in NF- κ B activity in tumor cells and an overall increase in NF- κ B signal due to increased tumor burden, we performed luciferase assays of snap-frozen tumor tissue. As shown in Fig. 4E, there was a specific increase in NF- κ B activity within tumor tissue (when corrected for cellular protein) in later phases of peritoneal tumor spread. We confirmed this observation independently of the NF- κ B reporter by measuring levels of p65 in nuclear extracts from harvested mesenteric tumors. As shown in Fig. 4F&G, the highest levels of nuclear p65 expression were observed in 90 day tumors.

We confirmed that peritoneal implants formed by ID8-NGL cells have characteristics representative of high-grade serous epithelial ovarian cancer. First, we performed H&E staining and demonstrated that tumors displayed the characteristic morphology of high-grade serous cancer (Fig. 5A). Furthermore, we stained tumors for expression of PAX8, an established marker of high-grade serous ovarian tumors [16]. We found strong nuclear immunoreactivity for PAX8 in epithelial tumor cells, but not in the stroma, as confirmed by staining for the epithelial marker, cytoplasmic pan-cytokeratin (Figs. 5B&C). Macrophage infiltration in stromal areas of the tumor was also demonstrated by staining with F4/80, a marker of mature macrophages (Fig. 5D). We then performed immunofluorescent analyses of molecular markers of proliferation, NGL reporter expression, and NF- κ B signaling in the epithelial component of tumors. Staining for Ki67/mib-1 demonstrated that the tumors were highly proliferative (Fig. 5E). Activity of the NGL reporter in tumors was shown by detection of the GFP component of the fusion reporter protein (Fig. 5F), and NF- κ B activity in tumors was independently confirmed by analysis of nuclear expression of phosphorylated-p65 (serine 276) (Fig. 5G). We then demonstrated that phospho-p65 and Ki67 were co-expressed in the nuclei of a subset of tumor cells, but that there was minimal overlap between the two within the nuclei (Fig. 5H). This is consistent with the localization of Ki67 to ribosomal RNA-encoding nucleoli in proliferating cells [17].

We confirmed that the abdominal distension observed in living mice was due to accumulation of ascites fluid at sacrifice. Characteristic of tumors from ID8 cells, the ascites contained significant amounts of blood. We were able to reliably collect the ascites fluid with a hypodermic needle. Therefore, volume of ascites collected at sacrifice will be another primary end-point of measuring extent of disease. Besides blood cells, we anticipated that ascites would contain inflammatory cells from the host and tumor cells. To assess effects during early stages of tumor progression, we performed peritoneal lavages with sterile PBS to collect peritoneal cell populations in injected mice prior to the development of ascites or in control non-injected mice.

Following collection of ascites or peritoneal lavages, we performed the procedures described below after lysis of red blood cells and cell counts:

- 1) Cells were centrifuged directly onto slides for cytopsin analyses of specific cell populations.
- 2) Cell pellets were snap-frozen for RNA extraction and for luciferase activity assays.

In our cytopsin analyses of H&E-stained slides, large cell clusters without definable borders consistent with tumor cells in the peritoneal fluid were excluded from our inflammatory cell counts (see arrows in Fig. 6A). Mononuclear cells were the predominant inflammatory cell population present in the peritoneal cavities of both non-injected and injected mice (Fig 6B). Furthermore, in injected mice mononuclear cells made up consistently 90% or greater of the inflammatory cells harvested in ascites fluid or in peritoneal lavages irrespective of duration of exposure to tumor cells (Fig 6B). The overall number of mononuclear cells harvested was elevated approximately 8-fold relative to non-injected controls at 90 days in the presence of tumor cells and with increasing duration of tumor spread (approximately 15-fold increase from the 30 day to 90 day time point in injected mice) (Fig. 6C).

The tumor cell component of ascites fluid or peritoneal lavages was confirmed in luciferase activity assays of the tumor cell-specific NF- κ B reporter in cell pellets. Quantification of raw NF- κ B reporter activity indicated that there was a time-dependent increase (Fig. 6D). These data suggest that the overall number of tumor cells in peritoneal fluid increases with ovarian cancer progression, which we would anticipate. The presence of tumor cells in peritoneal fluid was also demonstrated by immunofluorescent staining of cytopsins for the epithelial-

specific marker, pan-cytokeratin (Fig. 7A). Since macrophages are a major subset of mononuclear cells, and given the established role of peritoneal macrophages in ovarian cancer progression [12], we also stained cytopins for the mature macrophage marker, F4/80 (Fig. 7A). These analyses confirmed that the tumor cells were a major component of ascites fluid at later time points (Fig. 7B), and that mature macrophages account for at least 60% of the non-epithelial cells present. Collectively, these results suggest an elevated inflammatory response is associated with the presence of tumor cells in the peritoneal cavity. Currently, the precise contributions to this inflammatory response by host-initiated defenses against the tumor cells or by tumor-directed mechanisms promoting tumorigenesis are unknown. We have begun to address this issue in this proposal, through examining expression of markers of cytotoxic, anti-tumor M1 and pro-tumor M2 macrophages.

During the second year of this proposal, we have optimized a method for the isolation of peritoneal macrophages from ascites/peritoneal lavage fluid. Our method is based on the differential adhesion method originally described by Kumagai *et al* [18], which takes advantage of the rapid adhesive properties of macrophages compared to other cell types. As shown in Fig. 7C, F4/80-positive macrophages can be efficiently isolated from ascites fluid as the “adherent” cell population following plating on fetal calf serum-coated tissue culture dishes. In contrast, pan-cytokeratin-positive tumor cells form a major component of the “non-adherent” cell population. We also validated our isolation method by quantitative RT-PCR (QPCR) analysis of enrichment of steady-state mRNA expression of the epithelial-specific gene CK18 and the macrophage markers CC chemokine ligand 3 (CCL3) and mannose-receptor (mann-R) in “non-adherent” and “adherent” cell populations, respectively (Fig. 7D).

CCL3 and mann-R are well-established markers of M1 and M2 macrophages, respectively [19]. Expression of mann-R was significantly increased in injected mice compared to non-injected controls at both the 30 day and 90 day time points, with an approximately 20-fold increase at 90 days (Fig. 8A). There was a markedly smaller increase in CCL3 expression at 90 days in tumor-bearing mice (approximately 5-fold), with no significant difference observed at 30 days (Fig. 8B). Consistent with these observations, the ratio of mann-R to CCL3 expression was significantly higher in injected mice at both time points (Fig. 8C). We have also developed an immunofluorescent assay for detecting expression of arginase-1, another marker of M2 macrophages, in cytospin slides. As shown in Fig. 8D, co-expression of arginase-1 and F4/80 was observed in macrophages isolated from tumor-bearing mice, but not mock-injected mice.

1d. Assess NF- κ B activity in host cells during tumor progression.

We have continued to experience delayed breeding of our NGL reporter transgenics. Consequently, we have not been able to conduct an experiment with as many mice as originally planned (3 time points x 10 injected mice + 5 non-injected mice = 45).

During the initial reporting period (FY12), we performed an initial trial with 4 NGL reporter mice injected with ID8 cells and measured BLI at baseline (prior to tumor cell injection) and 30, 60 and 69d after injection (Fig 9). In NGL mice, BLI measures NF- κ B reporter in host tissue. Our underlying hypothesis is that increases in NF- κ B activity in both tumor and host cells contribute to ovarian cancer progression. As described above, we have identified markedly increased NF- κ B activity in tumor cells at later stages of progression and increased infiltration of inflammatory cells into the peritoneal cavity. Therefore, we anticipated that we would observe increased reporter activity in the peritoneal cavity in NGL reporter mice during progression. In contrast, there was a trend towards decreased detection of NGL reporter activity at later time points following ID8 cell injection, which reached statistical significance at day 60 (Fig 10A-C). Similar results were obtained for raw abdominal luciferase activity (Fig 10B) and when abdominal activity was normalized to corresponding brain signal (Fig 10C).

We acknowledge that this is a small group of mice, and are cautious in our interpretation. In fact, results presented in Task 2c shows no statistically significant difference between any time points in mice receiving

only vehicle treatment. It is possible that the sensitivity of detection of reporter activity in individual cells in the peritoneal cavity is below the limits of the in vivo imaging device, particularly when the signal may also be “damped” by the presence of ascites fluid. This contrasts to signal from the ID8-NGL tumor cells, which form a solid mass. It is clear, therefore, that more sophisticated analyses will be required to produce a definite conclusion about NF- κ B reporter activity in host cell populations such as macrophages.

We repeated the experiment comparing uninjected to ID8-injected mice in order to dissect more carefully the host inflammatory cell component of ascites fluid or peritoneal lavages. Mice were sacrificed at 30 days and 60 days after ID8 cell or mock (PBS) injection and macrophages isolated using our differential adhesion method (Fig. 11B). We performed luciferase assays on the isolated adherent cell population, and showed that there was (i) increased NF- κ B reporter activity in macrophages harvested from tumor-bearing mice compared to mock-injected mice and (ii) NF- κ B activity increased in macrophages over the duration of tumorigenesis, since reporter activity was markedly elevated at 60 days compared to 30 days in tumor-bearing mice (Fig. 11B).

1e. Determine the contribution of peritoneal macrophages to early and late tumor progression and NF- κ B responses.

We have performed a pilot experiment to determine the effect of the macrophage inhibitor, liposomal clodronate, on tumorigenesis. This study was conducted following technical advice from the laboratory of Dr Timothy Blackwell to help guide the technically challenging task of preparing the clodronate formulation. Dr Blackwell is a consultant on this grant who has published previously on the use of clodronate [20].

Clodronate was administered IP from 30-60 days following injection of ID8-NGL cells into BL/6 mice. Control groups used were vehicle only (PBS) and mice receiving empty liposomes (EL). As shown in Figs. 12A-C, clodronate treatment significantly inhibited ascites formation and reduced the number of peritoneal implants and mesenteric tumor mass. These results suggest the importance of peritoneal macrophages to ovarian cancer progression. We confirmed a reduction in macrophages by F4/80 staining of cytopsin slides (Figs. 12D&E).

Milestone #1: We have demonstrated that there is specific upregulation of NF- κ B reporter activity in ID8-NGL cells in response to multiple activators of NF- κ B. We confirmed that ID8-NGL cells form tumors in the abdominal cavity of mice that are representative of high-grade serous ovarian cancer, and that there is a marked increase in NF- κ B activity during late stages of tumorigenesis. We have validated various methods of analyzing tumor cells and peritoneal M2 and M1 macrophage populations from harvested tumors and the collection of ascites fluid or peritoneal lavages. Collectively, the result of subtasks 1a and 1c indicate the robustness of our ID8-NGL cells as a model for monitoring NF- κ B activity in tumor cells during ovarian cancer progression and regression following drug treatment (Tasks 2 and 3). These aims include sufficient controls to enable preparation of a manuscript for peer-reviewed publication in an ovarian cancer journal. We have also established the complementary model of growing ID8 cells in NGL reporter mice in order to track NF- κ B activity in host cells during ovarian cancer progression. We have preliminary evidence that there is increased NF- κ B reporter activity in host macrophages during ovarian cancer progression, and studies with the macrophage-depleting drug clodronate showed peritoneal macrophages play a role in ovarian cancer progression. No further mouse experiments are anticipated from Task 1, but we will continue to analyze existing material (eg tumor blocks, cytopsin slides, frozen tumor samples) to optimize end-points for molecular and cellular analysis of ovarian cancer progression as part of FY13 no-cost extension.

Task 2. To determine the consequences of reducing NF- κ B response in tumor and host cells by treatment with NF- κ B inhibitors.

2a. Treatment of ID8, ID8-NGL or BMDM-NGL cells with NF- κ B inhibitors (thymoquinone (TQ) and curcumin).

Effects of the NF- κ B inhibitor thymoquinone (TQ) in cells cultured *in vitro* are summarized in Fig 13. We have validated that short-term treatment with TQ induces decreased the stimulatory effects of TNF- α on NF- κ B reporter activity in ID8-NGL (Fig. 13A&B) and BMDM-NGL cells (Fig. 13C). When cells were treated for longer periods with TQ, we observed a concentration-dependent reduction in NF- κ B reporter activity after 24h (Fig. 13D) and cell growth after 72h (Fig. 13E), and induction of cell apoptosis (Fig. 13E).

2b. Measure NF- κ B activity in ovarian tumors during treatment with NF- κ B inhibitors and evaluate efficacy of treatment *in vivo*.

We reported the results of an initial experiment using TQ treatment for 4 weeks in WT mice injected with ID8-NGL cells in the Progress Report for Year 1. Mice were treated with Vehicle or TQ from day 30-60 following tumor cell injection, and sacrificed at day 70. 8 mice per group were used. BLI was performed at 30d, 60d and 70d in a subset of the mice (Fig 14A).

Treatment of tumors with TQ produced several unanticipated results. We have subsequently repeated experiments with TQ and have confirmed these results are reproducible (described in later sections of Task 2 and in Task 3).

First, TQ treatment resulted in significantly greater ascites volume at sacrifice (Fig. 15A&B). In contrast, in counts directly measuring tumor burden, there were no significant differences in the number of peritoneal implants and mesenteric tumor mass between vehicle and TQ-treated mice (Fig. 15C&D).

Second, levels of NF- κ B reporter activity in tumor cells from TQ-treated mice measured *in vivo* were significantly higher compared to Vehicle-treated mice (Fig. 14B). We confirmed increased NF- κ B reporter activity in TQ-treated tumors by luciferase activity assays in snap-frozen tumors harvested at sacrifice (Fig 14C). Representative BLI images are shown in Fig 14D. Up-regulated NF- κ B activity in tumors was also demonstrated by western blot detection of nuclear p-65 expression in tumors harvested from TQ-treated mice (Fig. 16A&B).

We performed immunofluorescence staining of tumors to determine effects of TQ on cell proliferation and apoptosis. Consistent with its *in vitro* effects, TQ treatment resulted in a significant but modest reduction in the percentage of tumor cells positive for the proliferation mark, Ki67/mib-1 (Fig. 17A&B), and a significant but small increase in apoptosis (Fig. 18A&B). We also demonstrated that nuclear phosphorylated p65 was co-expressed with Ki67/mib-1 in a subset of cells from both vehicle and TQ-treated tumors (Fig 17A). However, the proportion of Ki67-positive cells also expressing phosphorylated p65 was significantly increased in TQ-treated tumors compared to vehicle (Fig. 17C), consistent with our luciferase assay and western blot data.

Cytospin analyses revealed that macrophages were the predominant inflammatory cell population present in the peritoneal cavity of either vehicle or TQ-treated mice (Fig 19A). In contrast, the overall number of macrophages harvested increased significantly with TQ treatment (Fig 19B), suggestive of an elevated inflammatory response. We then quantified the presence of tumor cells in ascites fluid by luciferase assay of the tumor cell-specific NF- κ B reporter in cell pellets from ascites fluid. As shown in Fig 19C, there was increased NF- κ B reporter activity in tumor cells in the ascites from TQ-treated mice.

To directly compare the effects of short-term versus longer-term exposure to TQ in our model system, we conducted a pilot experiment where ID8-NGL-injected mice were treated with TQ for either 10 days or 30d days, starting at 1 month after tumor cell injection. Because of the limited amount of macroscopic tumor observed at the time of sacrifice for the 10 day treatment group, we were unable to accurately quantify drug effects on tumor burden. Furthermore, as expected, no ascites was observed at this relatively early stage of tumor progression. We prioritized snap-freezing tumors for luciferase assays, and determined that TQ treatment reduced NGL reporter activity by approximately 25% in tumors in luciferase assays compared to vehicle-treated

tumors at 10 days of treatment (Fig. 20A). In contrast, 30 day treatment with TQ resulted in upregulation of NF- κ B reporter activity (Fig. 20A).

Reduced NF- κ B activity with short-term TQ treatment was associated with significantly reduced levels of the M2 macrophage markers mann-R and IL-10 in peritoneal lavage fluid (Fig. 20B). Therefore, macrophage populations may be shifted towards an “anti-tumor” M1 phenotype by reducing NF- κ B activity in tumor cells. In contrast, elevated NF- κ B activity in tumor cells resulting from long-term TQ treatment induced robust increases in both M2 macrophage marks with a smaller increase in CCL3 (Fig. 20B). These disparate effects were reflected in a significant reduction in the mannR:CCL3 expression ratio at 10 days and a marked increase at 30 days (Fig. 20C).

2c. Evaluate NF- κ B response in the host microenvironment during treatment with NF- κ B inhibitors.

We also performed an experiment evaluating the effects of TQ on NF- κ B reporter activity and tumor burden in NGL reporter mice injected with ID8 cells. Mice were treated with Vehicle or TQ from day 30-60 following tumor cell injection, and sacrificed following the end of treatment. 5 mice per group were used. BLI was performed at baseline, 30d, and 60d in a subset of the mice (Fig 21A). Minimal overall changes were observed between vehicle and TQ-treated groups when NF- κ B reporter activity was quantified (Fig 21B). In contrast to the experiment described in Task 1d, there was no significant reduction in NF- κ B reporter activity during ovarian cancer progression under basal conditions (that is, in vehicle-treated mice).

We repeated the experiment comparing ID8-injected mice treated with vehicle or TQ to dissect more carefully the host inflammatory cell component of ascites fluid or peritoneal lavages. Mice were sacrificed at 60 days and macrophages isolated using our differential adhesion method. Luciferase assays on the isolated macrophage population showed that TQ treatment induced an approximately 2-fold overall increase in NF- κ B reporter activity (Fig. 22A), accompanied by significant increases in mRNA expression of both M2 mann-R and IL-10 macrophage marks and the M1 CCL3 mark (Fig. 22B). Consistent with the results from our ID8-NGL cells, ascites volume at sacrifice was significantly higher with TQ treatment (Fig 22C), but the number of peritoneal implants and mesenteric tumor mass were not significantly different between vehicle and TQ-treated mice (Fig 22D&E).

It is unclear at present if NF- κ B activity is elevated in M2 or M1 populations or both. A possible immunological mechanism for promotion of tumor progression through systemic NF- κ B inhibition is that anti-tumor M1 cytotoxic macrophages may require NF- κ B signaling for normal function, and NF- κ B inhibitors impair their ability to efficiently target tumor cells [19]. However, this will remain speculative until NF- κ B activity can be tracked in specific M2 and M1 macrophage populations. Like other NF- κ B inhibitors utilized clinically, such as curcumin and bortezomib, TQ is not a specific inhibitor of NF- κ B. This may also potentially underlie, at least in part, the augmented inflammatory response observed in our Task 2 studies. This would also be consistent with a recent study in a lung cancer model showing that prolonged treatment with the NF- κ B inhibitor bortezomib has pro-inflammatory effects and promotes tumor progression [21]. In contrast, short exposure to bortezomib produces the anticipated effects of inhibiting tumor cell growth in that study.

Milestone #2: Our results suggest that TQ has more complex effects *in vivo* than *in vitro*. We have identified contrasting effects of TQ on NF- κ B activity in tumor cells depending on the duration of exposure, which likely influences resident macrophage cell populations. Reduced NF- κ B activity induced by short-term TQ treatment induced a shift towards M1 cytotoxic macrophages, whereas a dramatic shift towards M2-like pro-tumor macrophages was observed with long-term TQ treatment, which led to increased overall NF- κ B activity in tumors and peritoneal macrophages. This is consistent with increased ascites formation with long-term TQ treatment in spite of direct anti-proliferative and pro-apoptotic effects on tumors. These aims include sufficient controls to enable preparation of a manuscript for peer-reviewed publication in an ovarian cancer journal. No further mouse experiments are anticipated from Task 2, but we will continue to analyze existing material such

as paraffin-fixed tumor sections, frozen tumor samples, macrophage populations on cytospin slides, cytokine levels in the supernatants from ascites fluid and mRNA expression levels of various macrophage markers via cDNA as part of FY13 no-cost extension.

Task 3. To evaluate NF- κ B activity in host and tumor cells and efficacy of treatment with NF- κ B inhibitors in combination with cisplatin (Months 6-24).

3a. Treat ID8-NGL or BMDM-NGL cells with NF- κ B inhibitor in the presence or absence of cisplatin.

Effects of TQ in combination with cisplatin in ID8-NGL cells cultured *in vitro* are summarized in Fig 23. In SRB assays, we showed that TQ and cisplatin act synergistically to reduce cell growth through isobologram analysis [22] (Fig. 23A&B). Moreover, the pro-apoptotic effects of cisplatin are markedly enhanced by combination TQ treatment (Fig. 23C), indicating apoptosis induction is an important mechanism of action of the anti-tumor effects of the combined drugs.

3b. Measure NF- κ B activity in ovarian tumors during treatment with NF- κ B inhibitors and cisplatin and evaluate efficacy of treatment in vivo.

In these studies, mice injected with ID8-NGL cells were treated with vehicle, TQ, cisplatin or the TQ/cisplatin combination from day 30-60 following tumor cell injection, and then sacrificed. 5 mice per group were used because of limited wild-type BL/6 mouse numbers. We will repeat this experiment during the FY13 no-cost extension to ensure reproducibility of the results.

As shown in Fig. 24A-C, TQ treatment produced the expected increase in ascites volume, but had no significant effect of direct tumor burden indices, number of peritoneal implants and mesenteric tumor mass. Cisplatin treatment significantly reduced all three indices compared to vehicle-treated mice, and the combination of TQ and cisplatin showed co-operative anti-tumor effects significantly above that of cisplatin alone. The TQ/cisplatin combination also significantly reduced the effects of TQ alone (Fig. 24A-C).

Having established the anti-tumor efficacy of the TQ/cisplatin combination, we then probed underlying molecular and cellular effects that mediated this response. Measuring NF- κ B reporter activity in tumors in luciferase assays revealed that cisplatin alone had minimal effects but that the TQ/cisplatin combination led to a significant reduction in the stimulatory effect of TQ alone (Fig. 24D). In tumors, cisplatin treatment also significantly reduced cell proliferation and induced apoptosis (Fig. 25&26), while the TQ/cisplatin combination significantly enhanced the effects of TQ alone on both indices. Compared to cisplatin alone, the combination induced significantly greater levels of apoptosis (Fig. 26), consistent with our *in vitro* results.

Finally, in RNA extracted from ascites fluid/peritoneal lavages, cisplatin significantly reduced both M2 macrophage marks and increased the M1 mark CCL3 (Fig. 24E). The TQ/cisplatin combination also led to significant reduction of the stimulatory effects of TQ alone on the M2 marks (Fig. 24A-C) and tended to increase CCL3 expression compared to cisplatin alone, an effect that just failed to reach statistical significance ($p=0.056$, Mann-Whitney test).

3c. Evaluate NF- κ B response in the host microenvironment during treatment with NF- κ B inhibitors and cisplatin.

The studies listed under Tasks 3c were scheduled for year 2 of this funding, but were not attempted because of limited numbers of NGL reporter mice. In order to determine effects of combining TQ and cisplatin on NF- κ B activity in peritoneal macrophage populations, we will perform an experiment in ID8-injected NGL reporter mice during the no-cost extension for FY13.

Milestone #3: Combined TQ and cisplatin treatment lead to synergistic anti-tumor effects *in vitro*, reduced tumor burden and apoptotic marks in tumors to a greater extent than treatment with cisplatin alone, and reduced

M2, and induced M1, macrophage markers. Since improving the response to cisplatin is a critical question in the clinical management of ovarian cancer, our results provide preclinical evidence for the considerable potential of NF- κ B inhibition in sensitizing tumor cells to cisplatin-induced apoptosis and growth arrest. Our studies also suggest that strong caution needs to be employed in using systemic NF- κ B inhibitors and emphasizes the need for therapy directly targeting NF- κ B signaling in tumor cells and directly modulating its activity in specific macrophage populations. Our collaborative studies have provided a framework for grant applications exploring these exciting new directions in ovarian cancer therapy. The remainder of Task 3 will be undertaken during the FY13 no-cost extension.

KEY RESEARCH ACCOMPLISHMENTS

During this reporting period:

- 1) We have further strengthened the collaborative efforts of the Khabele/Wilson and Yull laboratories. We continue to have regular joint lab meetings for data sharing and interpretation, and are actively seeking grant funding for ovarian cancer research as a collaborative team.
- 2) We have obtained data indicating that NF- κ B activity is elevated in ovarian cancer cells and in host peritoneal macrophages, particularly in the late stages of progression.
- 3) We have demonstrated that the macrophage-depleting drug clodronate reduced tumor burden, suggesting an important role for resident peritoneal macrophages in ovarian cancer progression.
- 4) We have validated a method of isolating macrophages from ascites and peritoneal fluid by exploiting the rapid adhesive properties of macrophages to fetal calf serum-coated tissue culture plates, based on a method described by Kumagai et al (ref 18).
- 5) We have validated multiple new end-points for molecular and cellular analysis of tumor progression and drug effects, such as: luciferase assays in isolated macrophages from NGL reporter mice; immunofluorescent detection of nuclear phosphorylated p65; western blot detection of nuclear p65; immunofluorescent detection of the macrophage markers F4/80 and arginase-1 (M2), and the epithelial marker pan-cytokeratin, in cytospin slides.
- 6) We have shown that reducing NF- κ B activity in tumor cells with TQ treatment was associated with a shift towards M1 macrophages, while longer-term TQ treatment lead to increased ascites, elevated NF- κ B signaling in tumors and in host peritoneal macrophages, and shift towards an M2 phenotype.
- 7) We have shown that combined TQ and cisplatin treatment lead to synergistic anti-tumor effects *in vitro*, reduced tumor burden and apoptotic marks in tumors to a greater extent than treatment with cisplatin alone, and reduced M2, and induced M1, macrophage markers.

REPORTABLE OUTCOMES

Manuscripts, Abstracts, Presentations

1. **AJ Wilson**, W Barham, J Saskowski, O Tikhimorov, L Chen, H-J Lee, F Yull, D Khabele. TrackingNF- κ B activity during ovarian cancer progression in a syngeneic mouse model. **Manuscript under second review at *Journal of Ovarian Research*. [manuscript file pages 52-86]**
2. **AJ Wilson**, JL Saskowski, WJ Barham, L Chen, D Khabele, F Yull. Opposing effects of the NF-kappaB inhibitor thymoquinone in a syngeneic mouse model of ovarian cancer. (2013 Washington DC: AACR Annual Meeting, peer-reviewed). **[abstract page 87]**

Funding applied for based on work supported by this award

Yull F (PI), Wilson (Co-Investigator at 1.2 calendar months): Inducing cytotoxic functions of macrophages to limit ovarian tumor progression: Mannose receptor-targeting nanoparticles as siRNA delivery tools, \$125,000/yr. The goal of this proposal is to determine if targeted modulation of nuclear factor-kappa B (NF- κ B) in macrophages by a novel nanoparticle-based strategy can limit ovarian cancer progression and synergize with chemotherapy. OCRP Pilot Award 2013. **[no appendix entry]**

Yull F (PI), Wilson (Co-Investigator at 1.2 calendar months): Targeted activation of macrophages to limit ovarian cancer progression, \$125,000/yr. Dr Yull will test the hypothesis that targeted activation of nuclear factor-kappa B (NF- κ B) in macrophages can limit ovarian cancer progression and synergize with chemotherapy using the ID8-NGL cell model. R21 2013. **[no appendix entry]**

CONCLUSIONS

The ***hypothesis*** to be tested is that *ovarian tumor progression depends on NF- κ B activation in both malignant ovarian epithelial cells and host macrophages in the peritoneal microenvironment*. Our ***objectives*** are (1) defining the pattern of NF- κ B activity in the host-tumor microenvironment during ovarian cancer progression and (2) evaluating consequences of inhibiting NF- κ B activity in tumor and host cells during ovarian cancer progression via treatment with NF- κ B inhibitors alone and in combination with cisplatin chemotherapy.

In order to reach our ultimate goal of testing the efficacy of combination NF- κ B inhibitor and cisplatin therapy in preclinical ovarian cancer models in the immunocompetent host, four major milestones will need to be reached; 1) development and characterization of reproducible syngeneic *in vivo* models of ovarian cancer progression (manuscript in second review); 2) understanding the role of NF- κ B signaling in both host and tumor cells during ovarian cancer progression and regression following drug treatment (underway); 3) defining measurable end-points to determine overall effects on tumor burden and molecular and cellular markers of response of tumors and host macrophage properties to drug intervention (underway), 4) precisely defining and fully understanding the effects of NF- κ B inhibitors alone and in combination with cisplatin on both tumor and host cell populations during ovarian cancer progression (underway).

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APPENDICES

Appendix materials start with Supporting Data [pages 19-44]

Biographical Sketches for PI Andrew J. Wilson and Co-PI Fiona E. Yull during the award period (July 2011 – July 2012). Please note that Research Assistant II Jeanette Saskowski and Research Assistant II Whitney Barham are also supported in part by this grant.

1. Wilson Andrew J., Biographical Sketch [pages 45-47]
2. Yull, Fiona E., Biographical Sketch [48-51]

Following Biographical Sketches, each item identified as a ‘reportable outcome’ for this project is included in appendices.

1. **AJ Wilson**, W Barham, J Saskowski, O Tikhimorov, L Chen, H-J Lee, F Yull, D Khabele. TrackingNF- κ B activity during ovarian cancer progression in a syngeneic mouse model. **Manuscript under second review at *Journal of Ovarian Research*. [manuscript file pages 52-86]**
2. **AJ Wilson**, JL Saskowski, WJ Barham, L Chen, D Khabele, F Yull. Opposing effects of the NF-kappaB inhibitor thymoquinone in a syngeneic mouse model of ovarian cancer. (2013 Washington DC: AACR Annual Meeting, peer-reviewed). **[abstract page 87]**

APPENDICES

Supporting Data

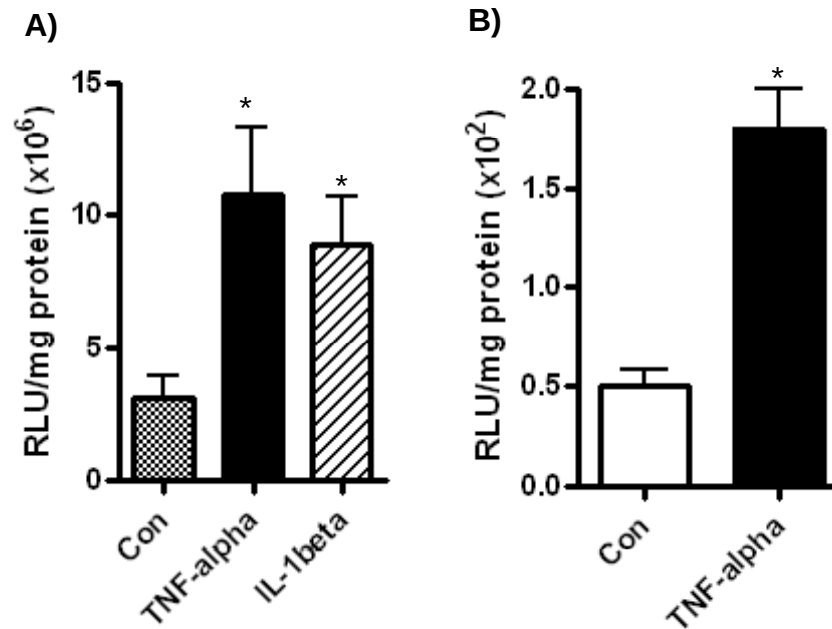


Fig 1. A) Similar to TNF- α (10ng/ml), the established activator of NF- κ B signaling IL-1 β (10ng/ml) also significantly increased NF- κ B reporter activity in ID8-NGL cells grown *in vitro* (4h exposure). B) TNF- α (10ng/ml) also stimulates NF- κ B reporter activity in BMDM-NGL cells (6h treatment). Values are mean + SD of 3 independent experiments. * $p < 0.01$ relative to control, Student's T test.

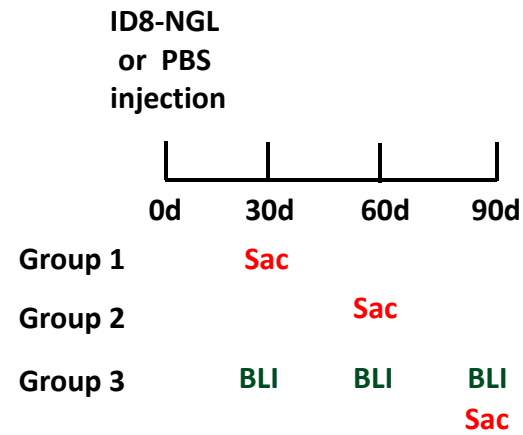


Fig 2. Schematic representation of the timeline for our initial trial experiment in WT BL/6 mice injected with ID8-NGL cells or mock-injected (PBS) at Day 0. BLI: bioluminescence imaging; Sac: sacrifice.

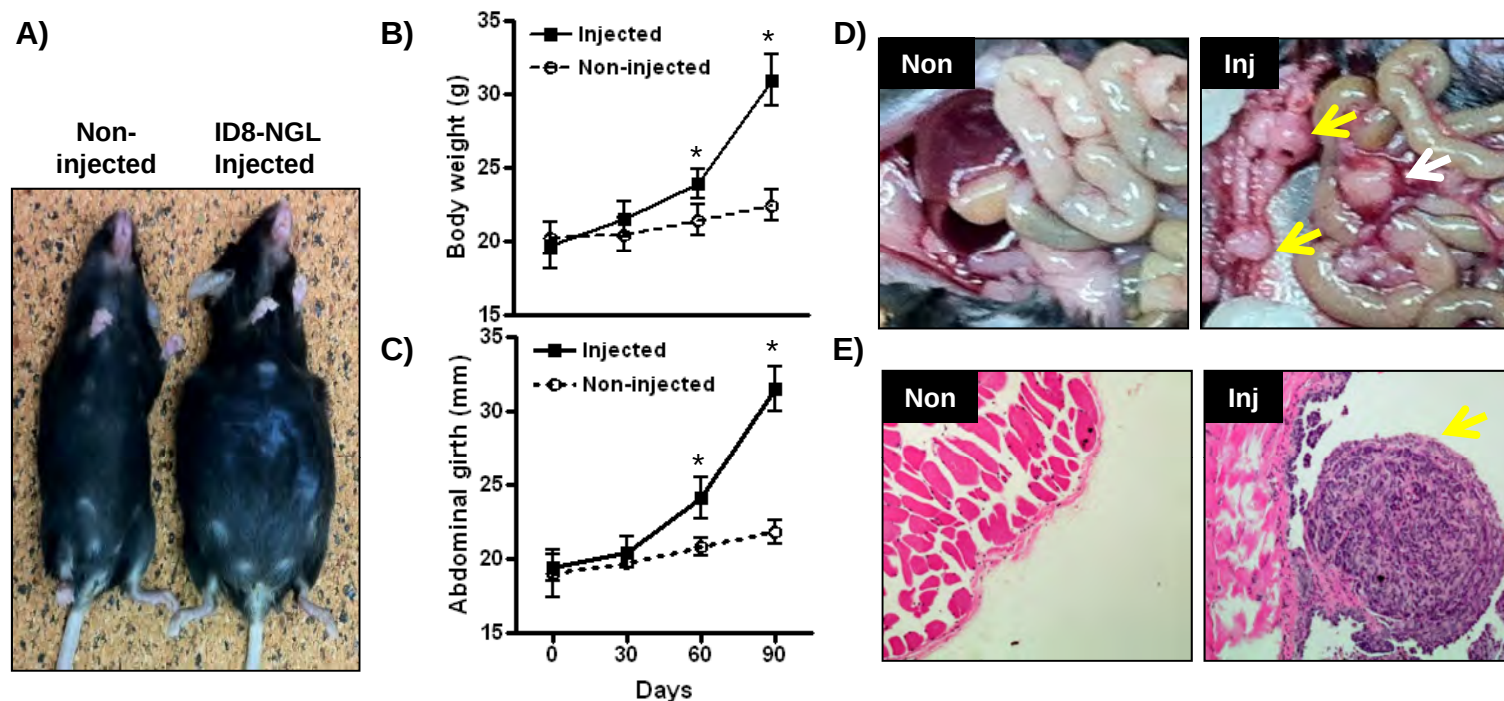


Fig. 3. (A) Mice injected with ID8-NGL cells show distended abdomen indicative of ascites formation at 90d after injection compared to PBS-injected mice (non-inject). Changes in (B) body weight and (C) abdominal girth were measured. Values are mean+SD for 5 mice per group. * $p < 0.01$ relative to non-injected mice, Mann-Whitney test. (D) Mice injected with ID8-NGL cells display abdominal dissemination of tumor cells. The main sites of tumor implantation are the peritoneal wall (yellow arrows) and in the mesentery (white arrow). (E) H&E staining example of tumor implantation in the peritoneal wall.

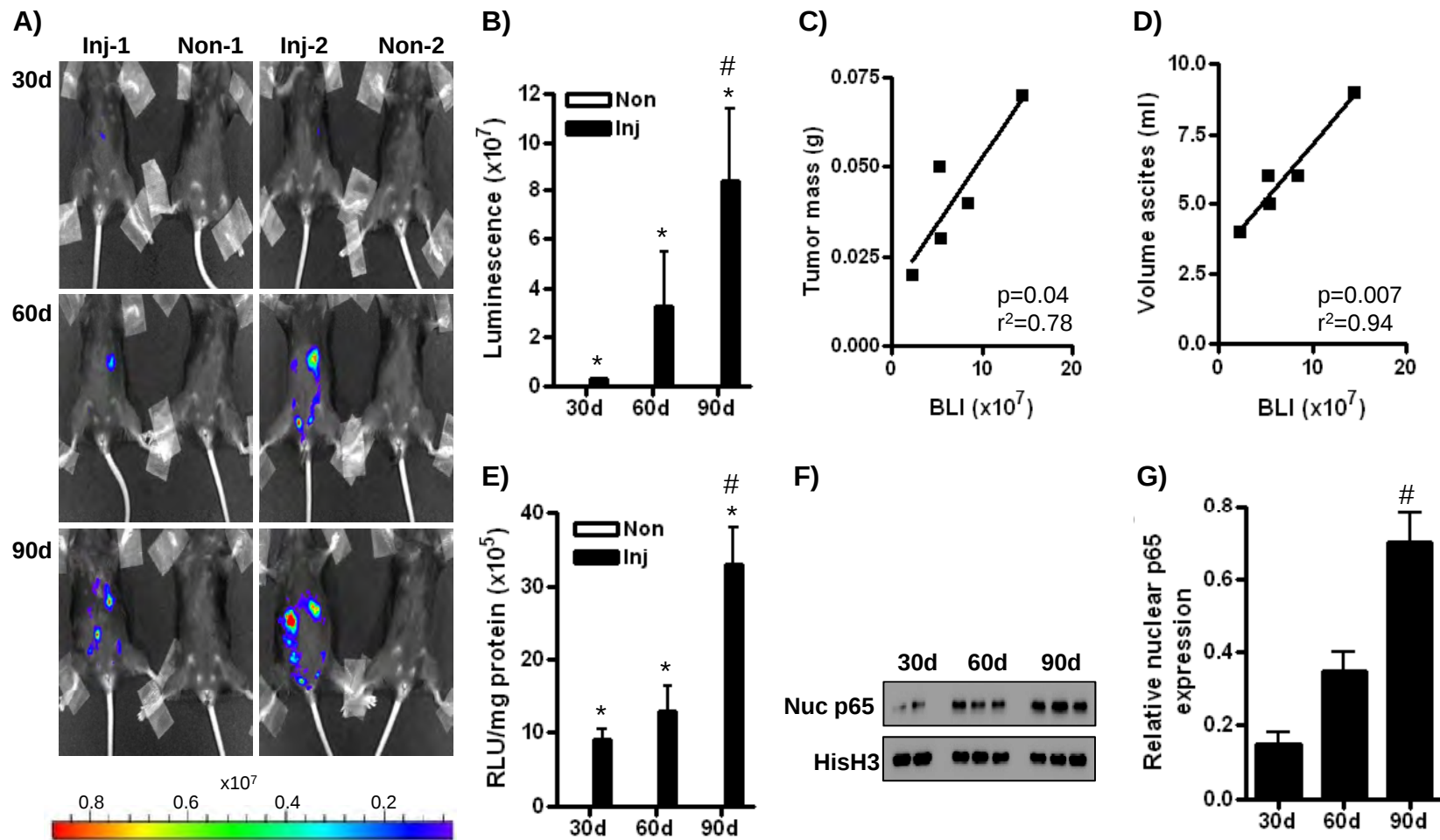


Fig 4. (A) Representative BLI of WT mice injected with ID8-NGL cells or PBS (non-inject) over 90d following injection showing NF-κB reporter signal in tumor cells. Quantification of BLI in the abdominal cavity is shown in (B). BLI was significantly correlated with (C) mesenteric tumor mass and (D) volume of ascites at 90d (Spearman correlation). (E) Luciferase activity of the NF-κB reporter was measured in harvested tumors and expressed relative to cellular protein. (F) Western blot analysis of p53 in nuclear extracts from harvested tumors. Equal loading was shown by probing for the nuclear-specific protein, histone H3. (G) Nuclear p53 expression relative to corresponding histone H3 levels was measured by densitometry. Values shown are mean + SD from 5 mice per group. * $p < 0.01$ relative to non-injected mice; # $p < 0.01$ relative to 30d or 60d, Mann-Whitney test.

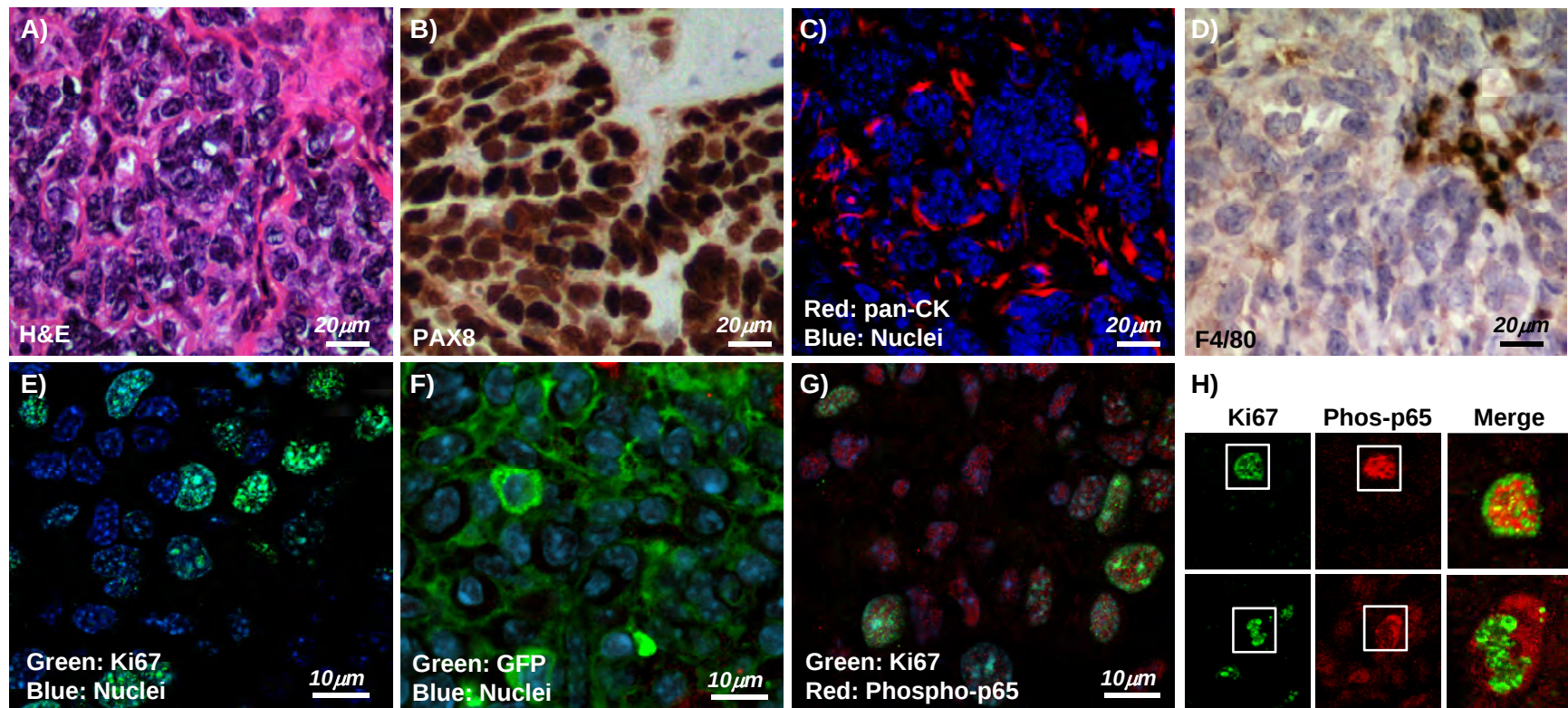


Fig. 5. (A) H&E stain of a representative area of ID8-NGL tumors. (B) Immunohistochemical detection of the marker of high-grade serous ovarian tumors, PAX8. (C) Fluorescent microscopy showing expression of the epithelial marker pan-cytokeratin (CK) expression relative to TO-PRO-3-stained nuclei. (D) Immunohistochemical detection of the mature macrophage marker, F4/80, in tumor sections. High power (x100) confocal images of the representative tumor section showing immunofluorescent detection of (E) the proliferation marker Ki67/mib-1, (F) the GFP reporter and (G) overlap of Ki67/mib-1 and phospho-p65 (serine 276) (red) in the epithelial component of the tumor. TO-PRO-3-stained nuclei are in blue. (H) Co-expression of phospho-p65 (red) and Ki67/mib-1 (green) is observed in the nuclei of a subset of cells. The panels on the right show merged images of the corresponding boxed areas for two representative nuclei.

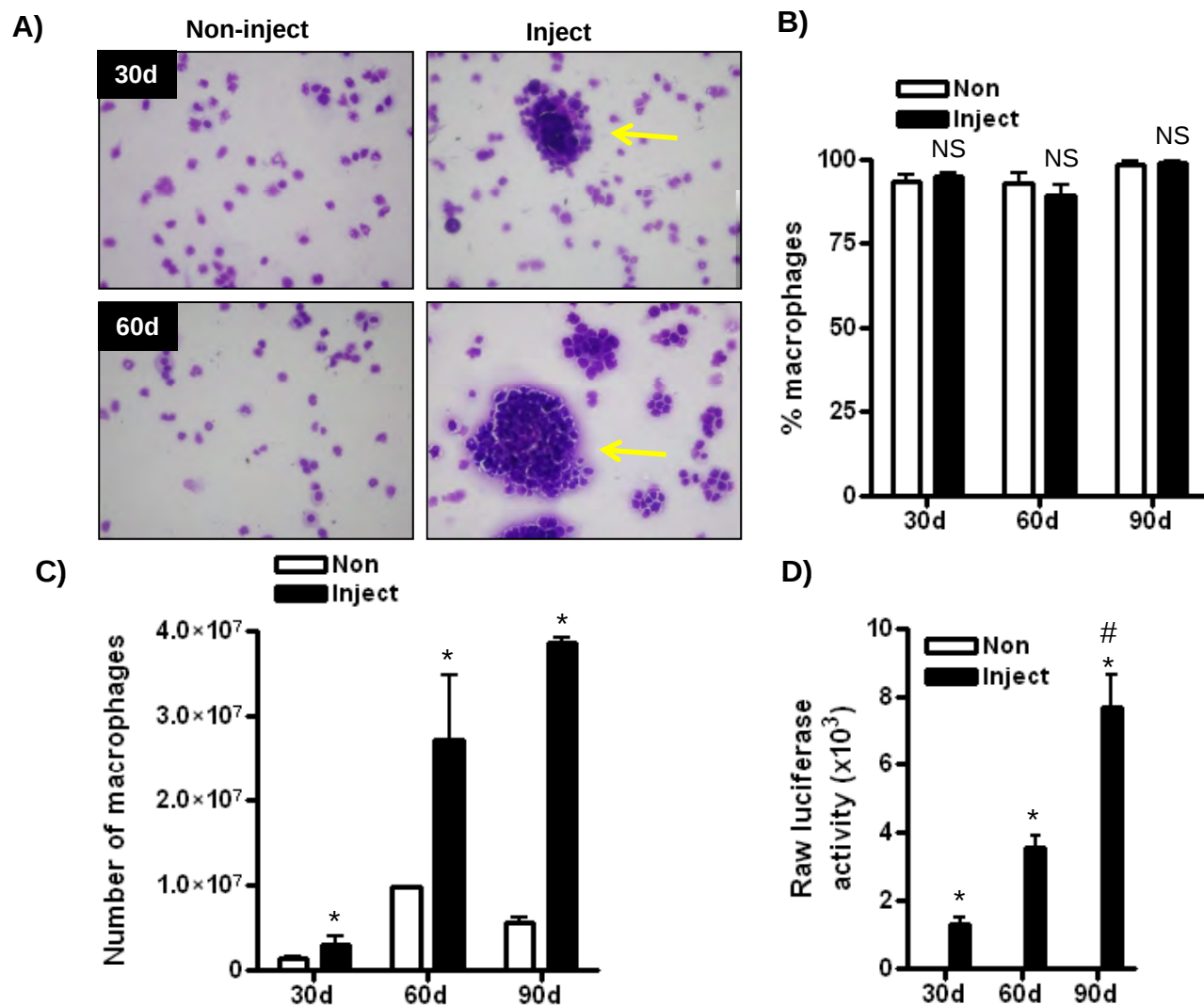


Fig 6. (A) Representative images of cytopspins showing the presence of tumor cells (yellow arrows). (B) No significant differences in the proportion of mononuclear cells counted in cytopspins from ascites fluid or peritoneal lavages collected from injected or non-injected mice at sacrifice were seen. (C) Overall number of mononuclear cells collected increased in mice with tumors and with duration of progression. (D) Raw luciferase activity in cell pellets from ascites fluid or peritoneal lavages show an increase with duration of progression. * $p < 0.01$ relative to non-injected mice; NS: not significant relative to vehicle; # $p < 0.01$ relative to 30d or 60d; Mann-Whitney test. Values are mean + SD for at least 3 mice per group.

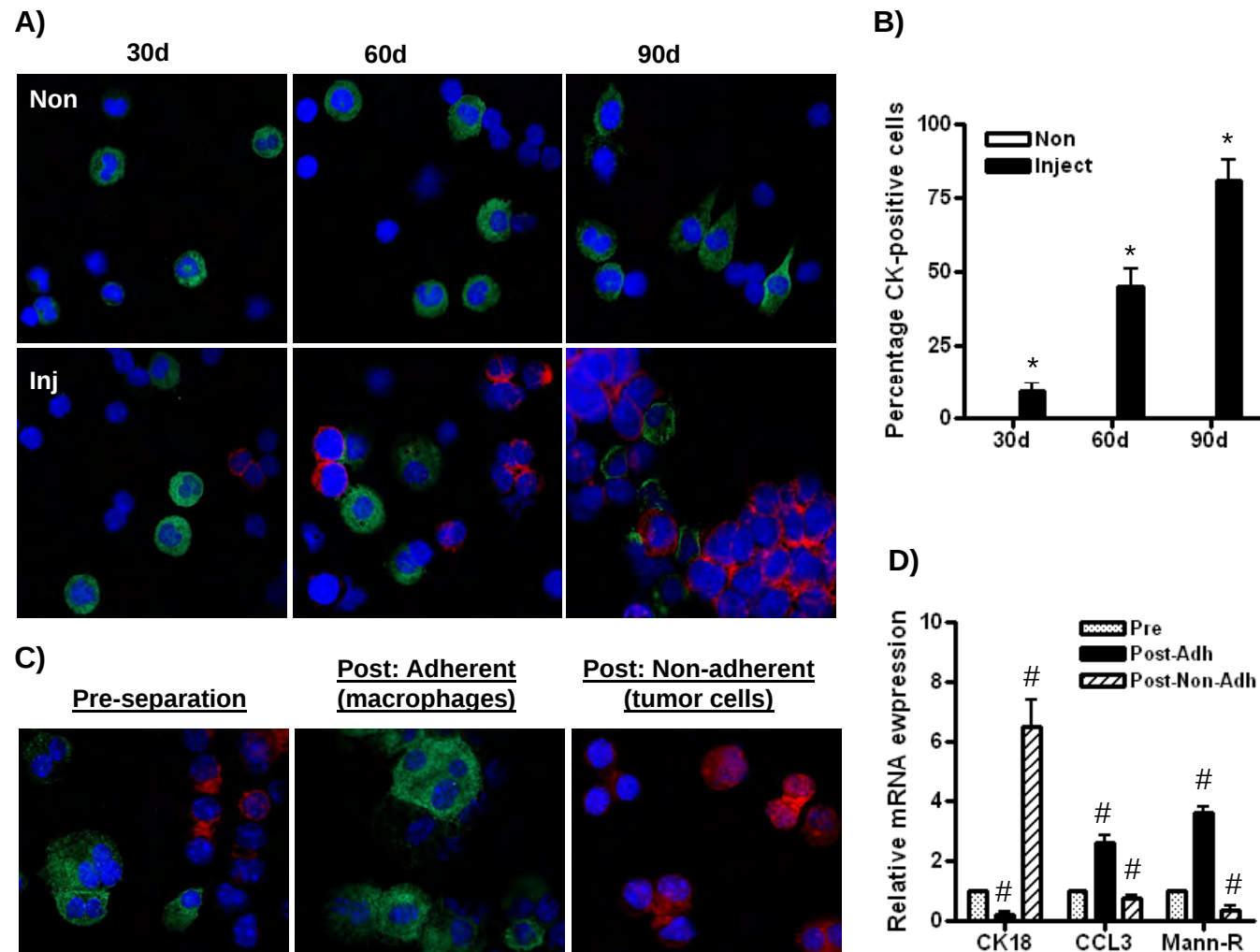


Fig 7. (A) Expression of the epithelial marker, pan-cytokeratin (CK; red), and the mature macrophage marker, F4/80 (green), in ascites fluid on cytopsin slides. DAPI-stained nuclei are in blue. Percentage of CK-positive cells is shown in (B). Values are mean+SD of counts from 200 cells from each of 3 mice per group. * $p < 0.01$ relative to non-injected mice Mann-Whitney test. (C) Staining for cell nuclei, pan-CK and F4/80 in ascites fluid prior to macrophage isolation and post-isolation. (D) QPCR analysis of the mRNA expression of epithelial specific marker (cytokeratin 18) and the macrophage markers, CCL3 and mannose-receptor, of cells from ascites pre- and post-macrophage isolation. # $p < 0.05$ relative to levels in pre-separated fluid, Mann-Whitney test.

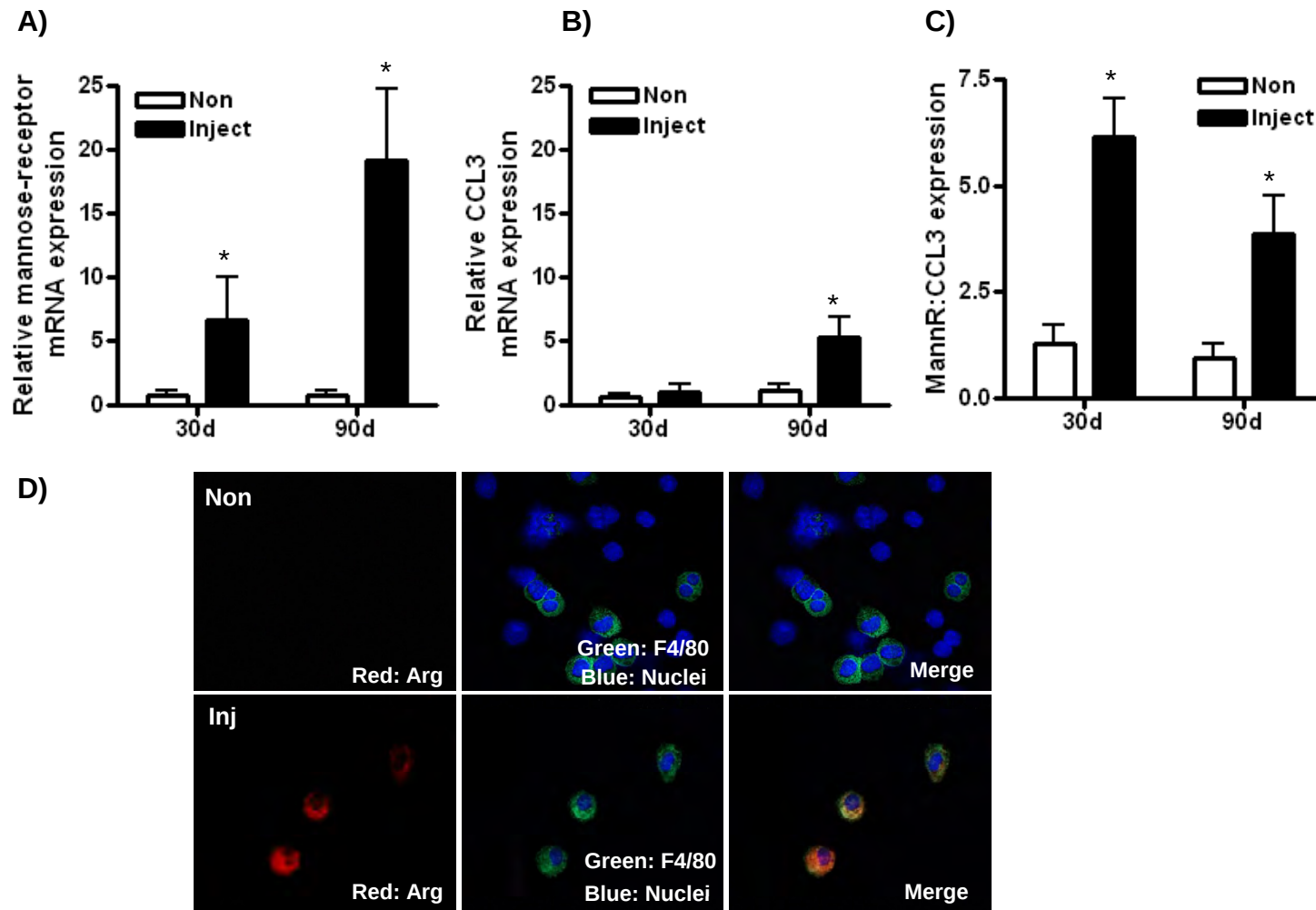


Fig. 8. QPCR analysis of the mRNA expression of the markers of (A) M2 macrophages, mannose-receptor (mann-R) and (B) M1 macrophages, CCL3, in RNA extracted from peritoneal lavages or ascites fluid. Values were normalized to corresponding levels of GAPDH mRNA expression and also CK18 mRNA levels, to account for epithelial content. The expression ratio of mann-R to CCL3 is shown in (C). (D) Immunofluorescent analysis of expression of arginase-1, an established M2 macrophage marker. Values are mean+SD for 3 mice per group. * $p < 0.01$ relative to non-injected mice, Mann-Whitney test.

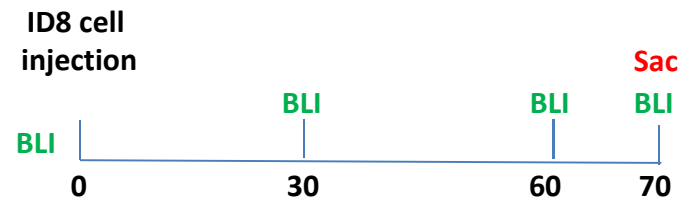


Fig 9. Schematic representation of the timeline for our initial trial experiment in NGL reporter mice injected with ID8 cells at Day 0. Baseline BLI were taken prior to injection.

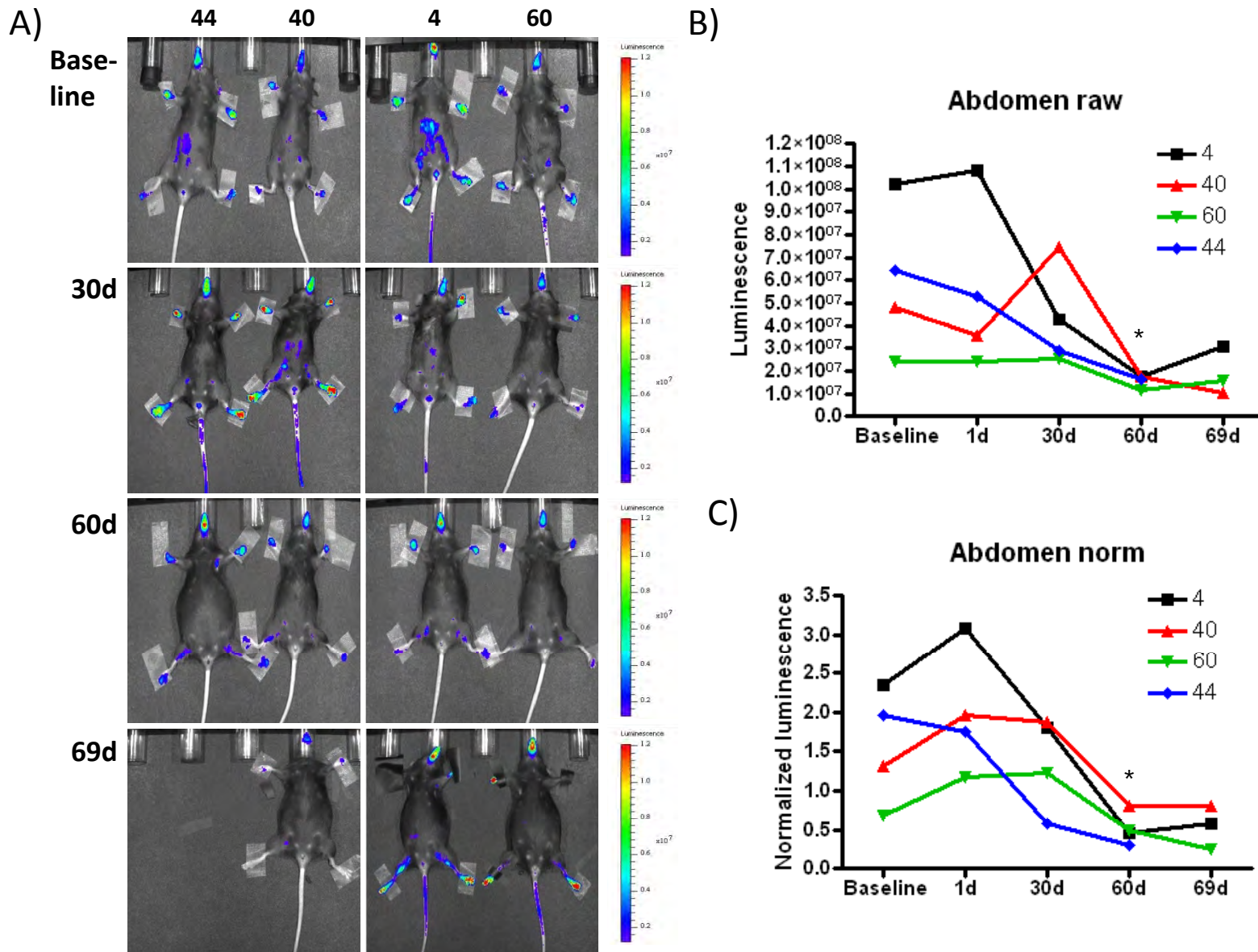


Fig 10. (A) Representative BLI of NGL reporter mice injected with ID8 cells showing specific NF- κ B reporter signal in **host** cells. (B) Quantification of raw BLI in the peritoneal cavity shows decreased NF- κ B reporter activity during ovarian cancer progression. (C) When abdominal BLI was normalized to corresponding brain BLI, similar results were observed. * $p < 0.05$ relative to baseline, Mann-Whitney test.

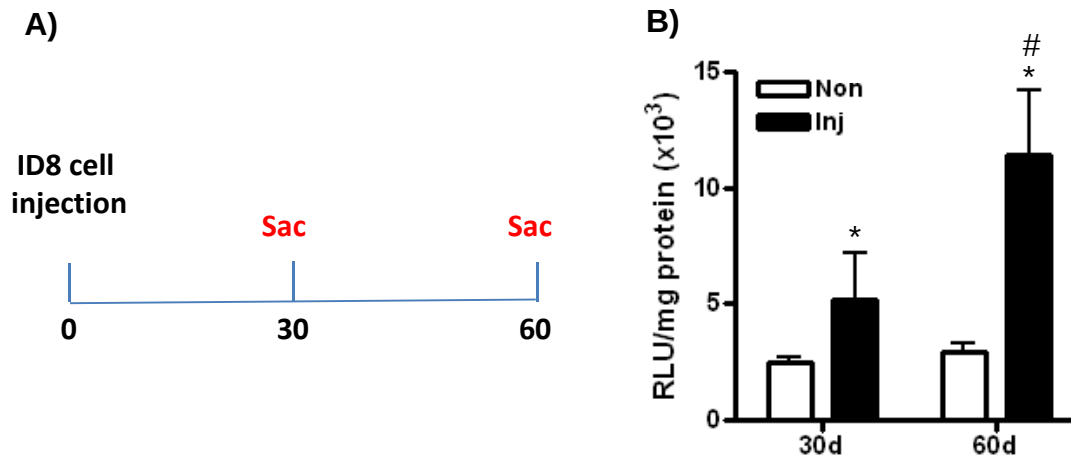


Fig. 11. (A) Schematic representation of the timeline for our experiment comparing NF- κ B activity in non-injected versus ID8-injected NGL reporter mice. Mice were injected with ID8 cells at Day 0. (B) Luciferase activity of the NF- κ B reporter was measured in isolated macrophages from ascites or peritoneal lavage fluid and expressed relative to cellular protein. Values shown are mean + SD from 5 mice per group. * $p < 0.01$ relative to non-injected; # $p < 0.01$ relative to 30d injected, Mann-Whitney test.

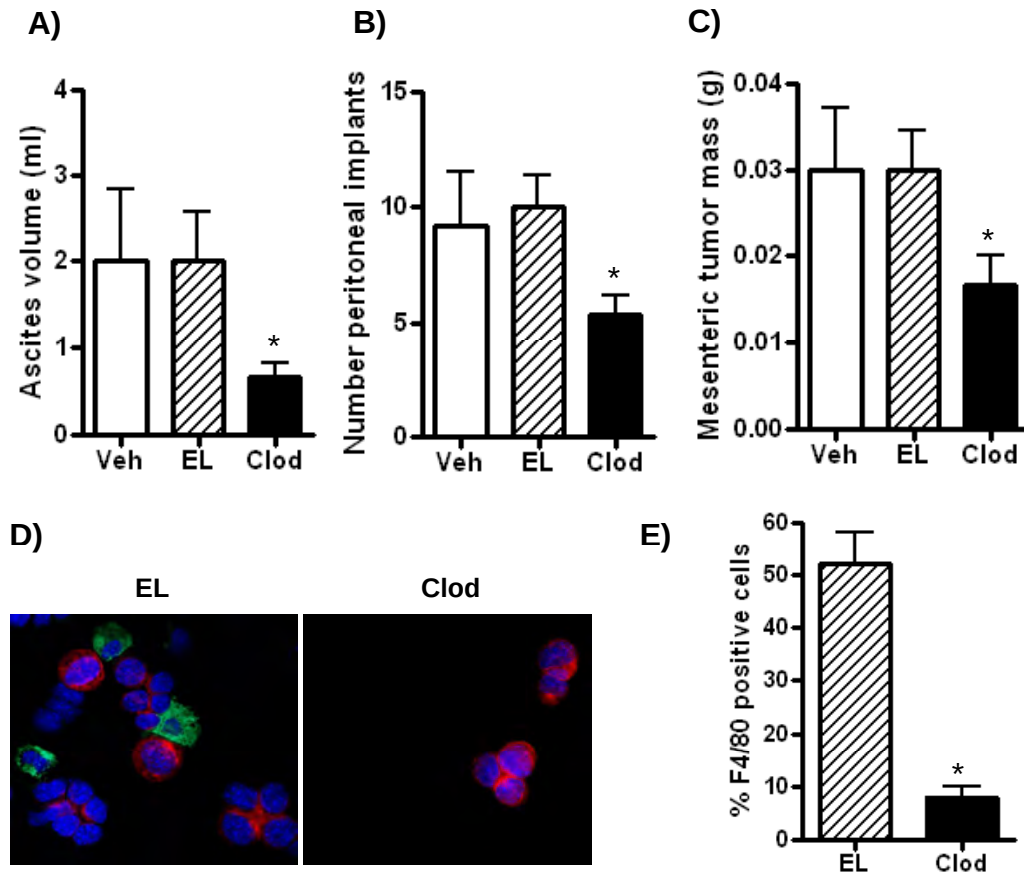


Fig. 12. Quantification of (A) ascites fluid volume, (B) number of peritoneal implants and (C) mesenteric tumor mass (g) at sacrifice in mice treated with Vehicle (PBS), empty liposomes (EL) or liposomal clodronate (Clod). (D) Representative fluorescent images of cytopins from EL or Clod-treated mice showing expression of the macrophage marker F4/80 (green) and the epithelial marker, pan-cytokeratin (red). DAPI-stained nuclei are in blue. (E) Quantification of the number of F4/80-positive cells. Values are mean+SD for 5 mice per group. * $p < 0.02$ relative to EL, Mann-Whitney test.

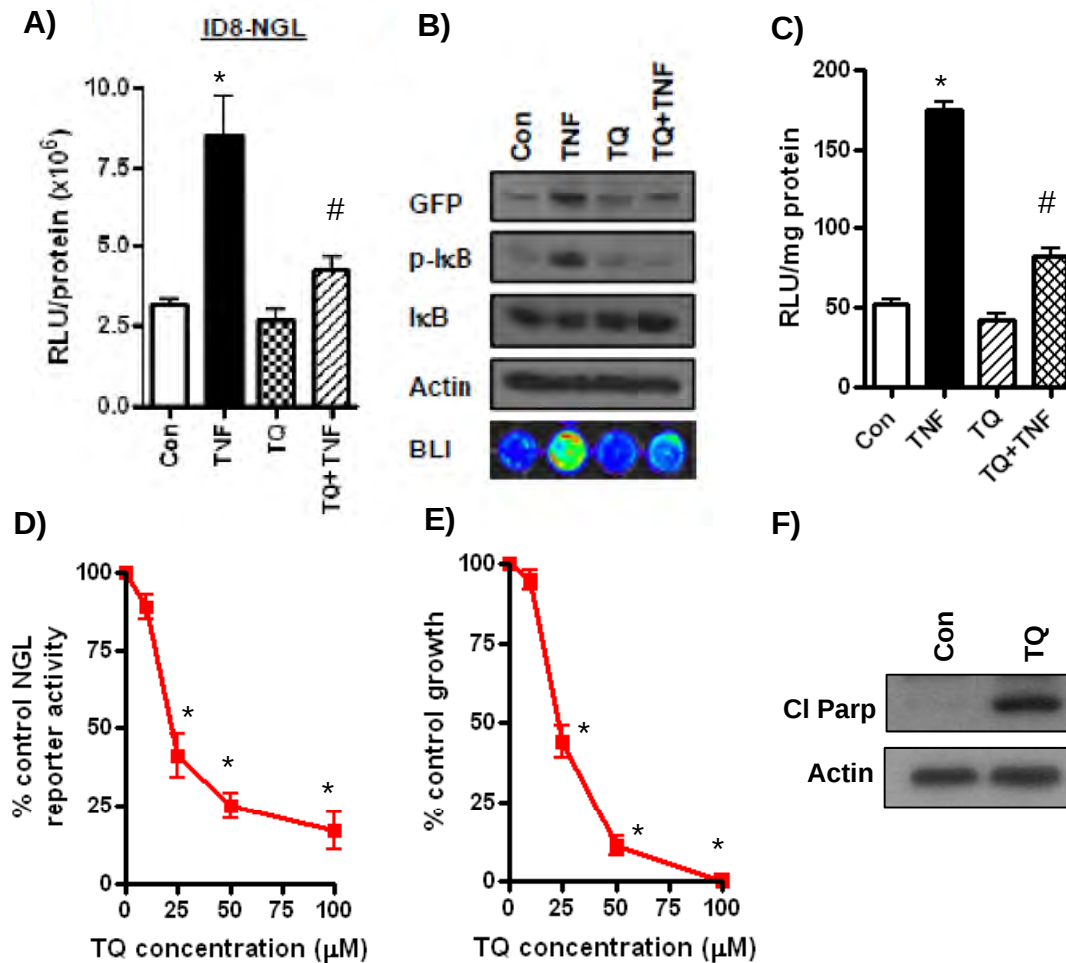


Fig 13. Effects of NF- κ B inhibitor TQ in ID8-NGL cells grown *in vitro*. (A) 2h pre-treatment with 50 μ M TQ inhibited the stimulatory effect of 10 ng/ml TNF- α on NF- κ B reporter activity in protein extracts. (B) Western Blot showing the stimulatory effect of TNF- α on GFP and phospho-I κ B expression after 4h, again inhibited by TQ. Representative BLI of ID8-NGL cells cultured *in vitro* showing similar effects is also shown. (C) 2h pre-treatment with 50 μ M TQ also inhibited the stimulatory effect of 10 ng/ml TNF- α on NF- κ B reporter activity in BMDM-NGL cells. (D) Reduction in NF- κ B reporter activity following 24h treatment with increasing concentrations of TQ. (E) SRB assay showing that TQ inhibits growth in a dose-dependent manner (72h treatment). (F) Western Blot detection of the apoptotic marker, cleaved PARP (Cl parp) in cells treated with 50 μ M TQ for 24 hours. * $p < 0.01$ relative to control, # $p < 0.01$ relative to TNF alone, both Student's T test. Results are from a representative experiment; experiments were performed at least 3 times.

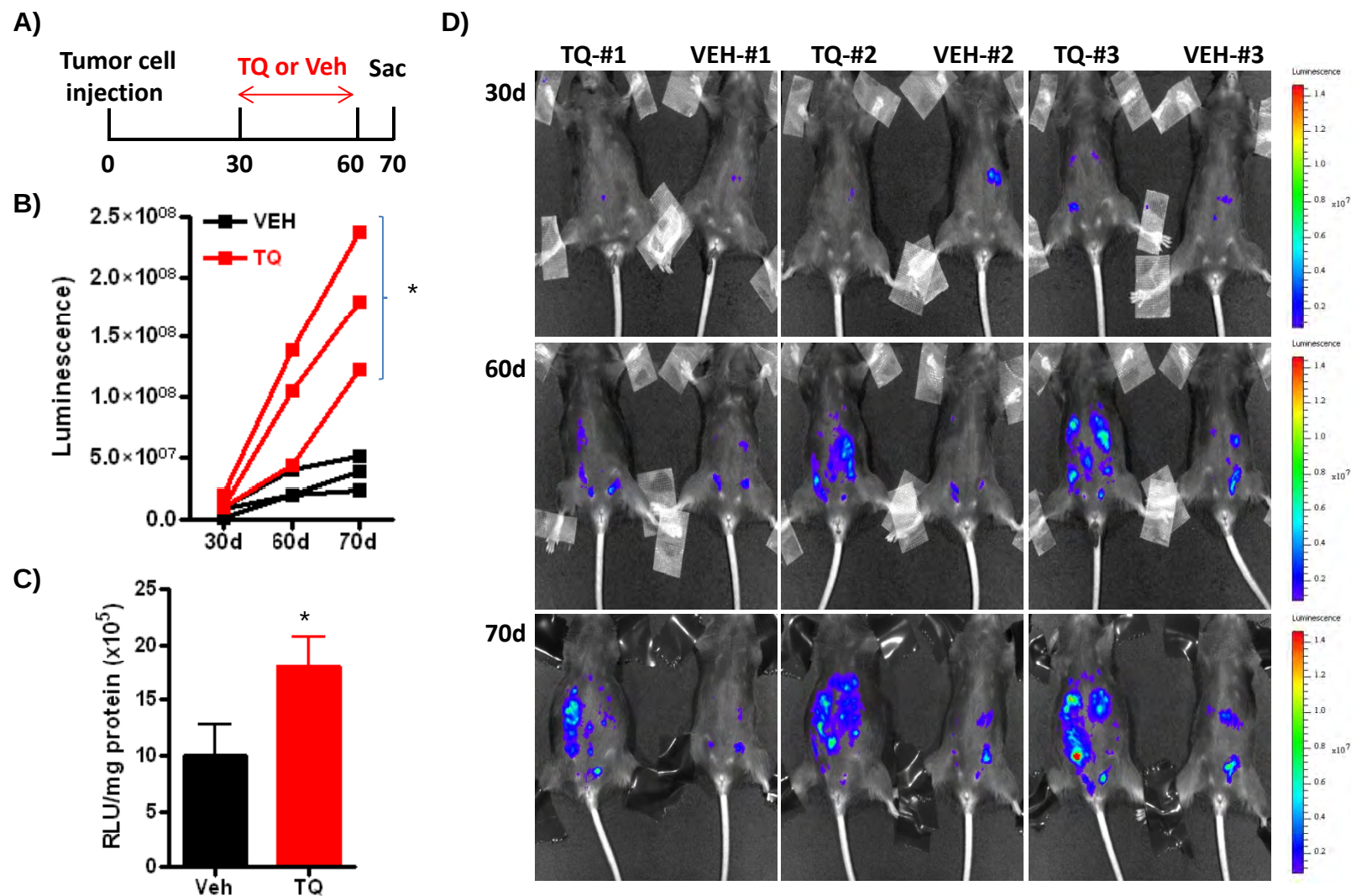


Fig 14. A) Schematic diagram showing treatment schedule for Vehicle or TQ in WT mice injected with ID8-NGL cells. B) Quantification of BLI in the peritoneal cavity shows increased NF- κ B reporter activity in TQ-treated mice. C) Luciferase activity was measured in harvested tumors and expressed relative to cellular protein. Data show a specific increase in NF- κ B reporter in tumors from TQ-treated mice. D) Representative BLI images comparing NF- κ B reporter signal in vehicle and TQ-treated cells. Values are mean+SD for 8 mice per group * $p < 0.01$ relative to vehicle, Mann-Whitney test.

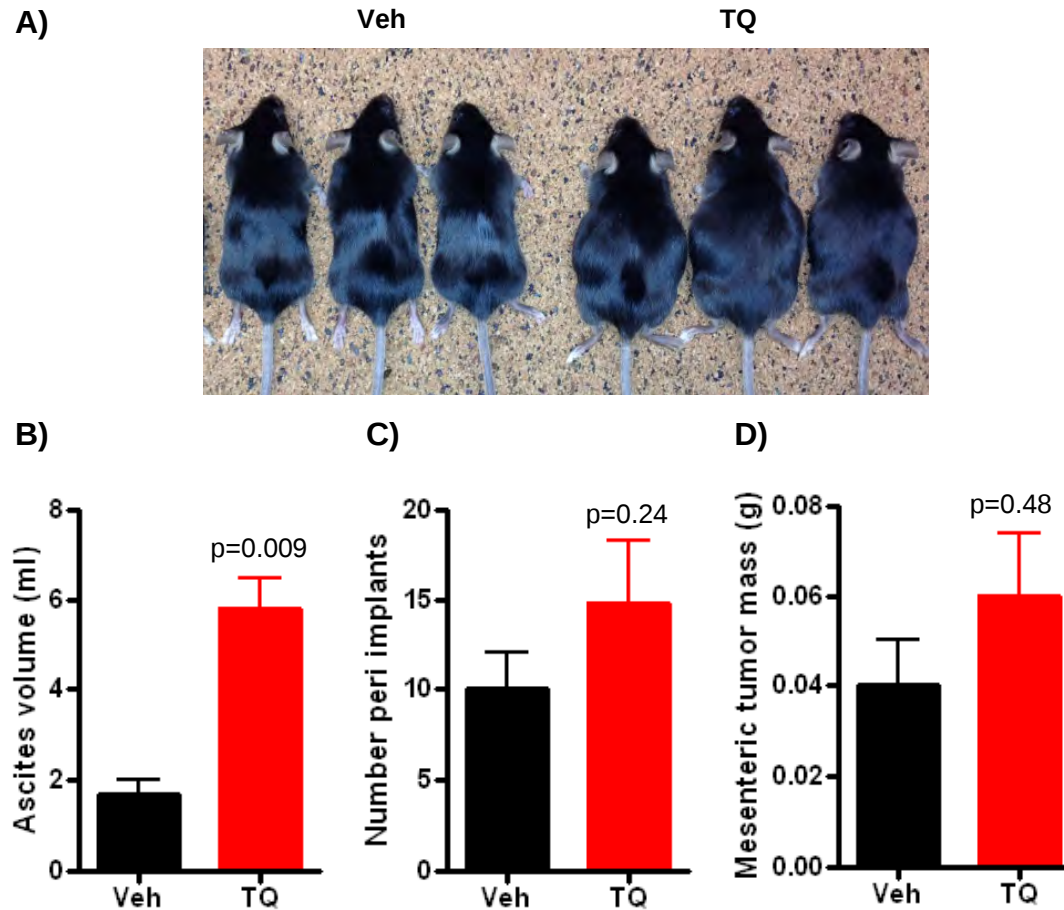


Fig 15. A) Representative images of vehicle and TQ-treated mice shows greater abdominal distension in TQ-treated mice, indicative of ascites. B) Quantification of ascites fluid volume at sacrifice confirmed increased ascites in TQ-treated mice. In contrast, there were no significant differences in tumor burden between Vehicle and TQ-treated mice when C) peritoneal implants or D) mesenteric tumor mass were quantified. P values show the effect of TQ relative to vehicle and were generated by Mann-Whitney test.

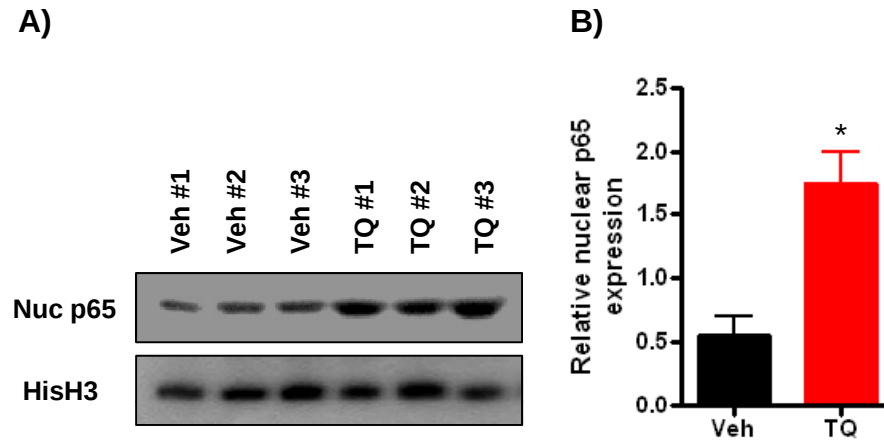


Fig. 16 (A) Western blot analysis of p65 in nuclear extracts from harvested Vehicle or TQ-treated tumors. Equal loading was shown by probing for the nuclear-specific protein, histone H3. (B) Nuclear p65 expression relative to corresponding histone H3 levels was measured by densitometry. Values are mean+SD for 3 mice per group * $p < 0.01$ relative to vehicle-treated mice, Mann-Whitney test.

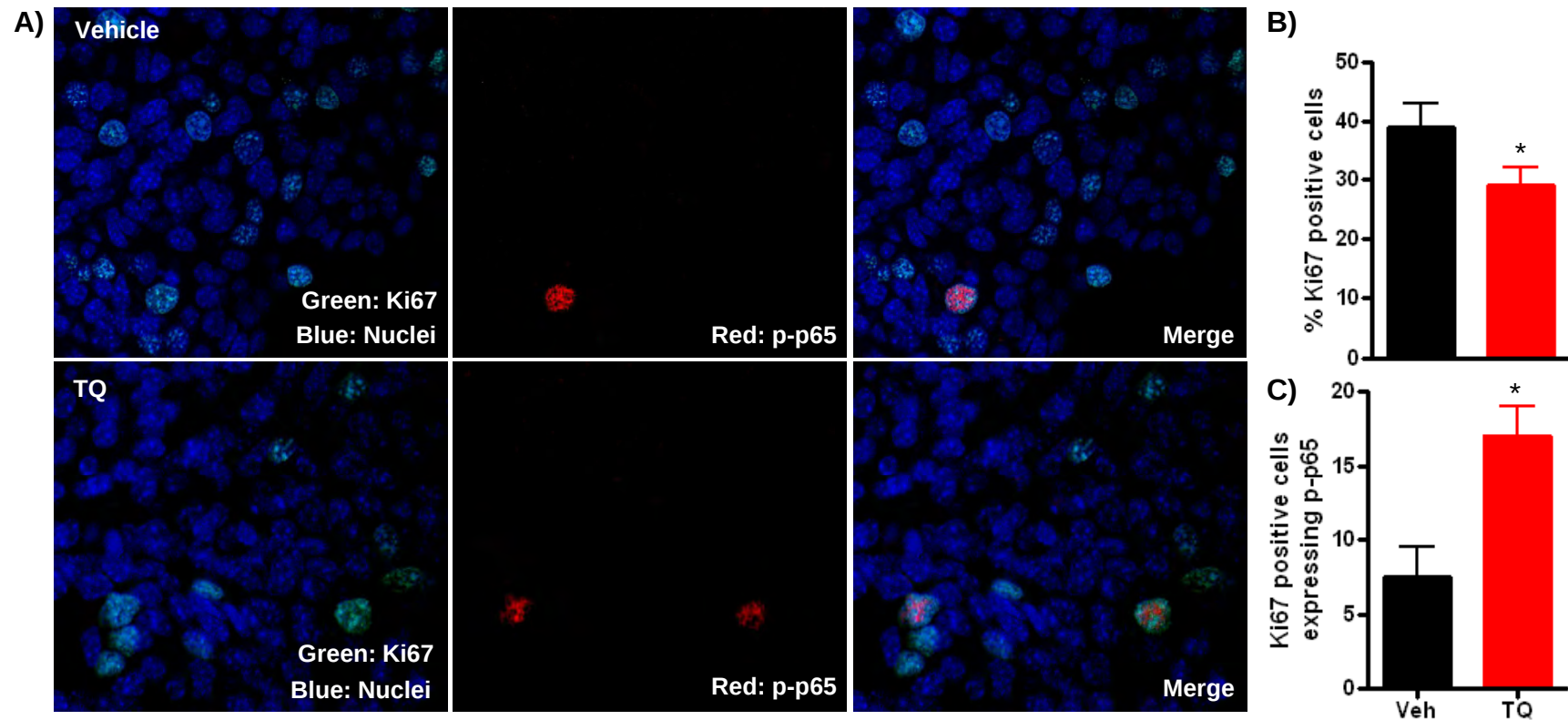
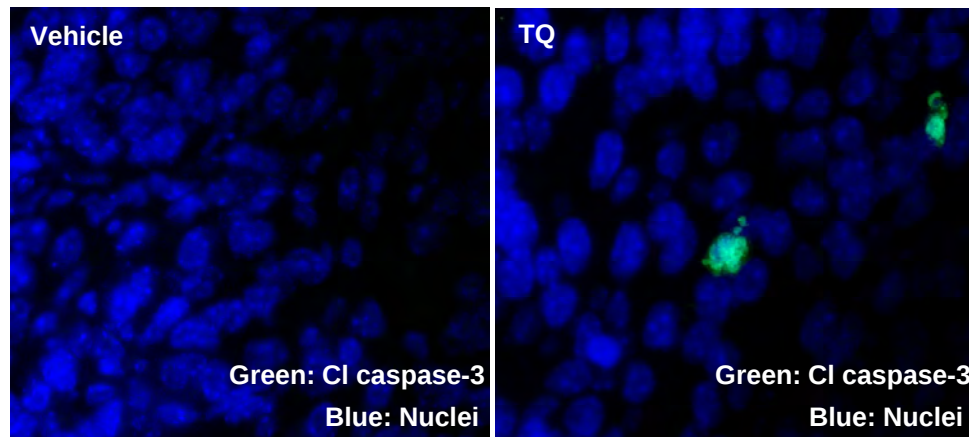


Fig. 17 (A) Immunofluorescent detection of the proliferation marker Ki67/mib-1 and the phosphorylated p65 (p-p65) in ID8-NGL tumors harvested from vehicle or TQ-treated mice. TO-PRO-3-stained nuclei are in blue. Co-expression of phospho-p65 (red) and Ki67/mib-1 (green) is observed in the nuclei of a subset of cells. (B) The percentage of tumor cells positive for Ki67 staining. (C) The percentage of cells staining positively for Ki67 that also express nuclear p-p65. Values are mean+SD for 3 mice per group. * $p < 0.01$ relative to vehicle-treated mice, Mann-Whitney test.

A)



B)

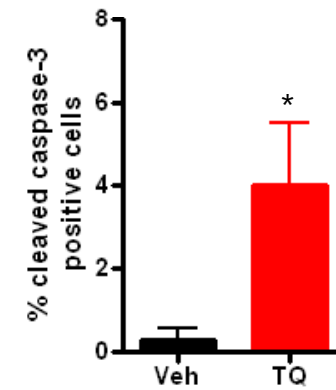


Fig. 18 (A) Immunofluorescent detection of the apoptosis marker cleaved caspase-3 in ID8-NGL tumors harvested from vehicle or TQ-treated mice. TO-PRO-3-stained nuclei are in blue. (B) The percentage of tumor cells positive for cleaved caspase-3 staining. Values are mean+SD for 3 mice per group. * $p < 0.01$ relative to vehicle, Mann-Whitney test.

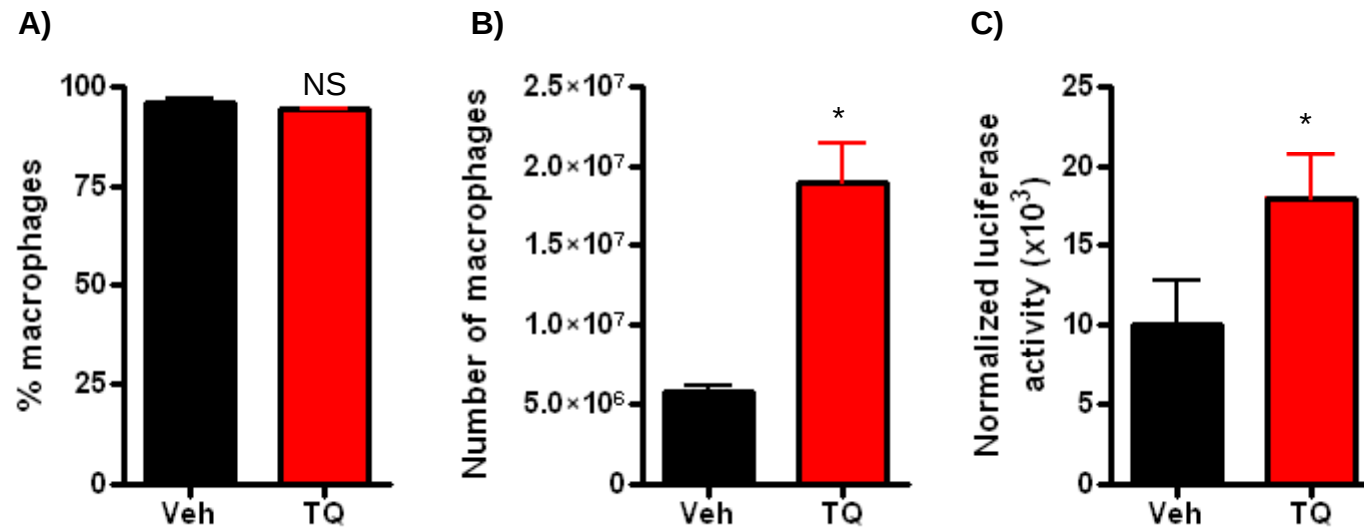


Fig 19. (A) No significant differences in the proportion of mononuclear cells counted in cytopins from ascites fluid or peritoneal lavages collected from vehicle or TQ-treated mice were seen. (B) Overall number of mononuclear cells increased in ascites collected from TQ-treated mice. (C) TQ treatment significantly increased NF- κ B reporter activity in tumor cells harvested from ascites fluid. Luciferase activity was normalized to corresponding cellular protein. Values are mean+SD for 3 mice per group. * $p < 0.01$ relative to vehicle; NS: not significant relative to vehicle, Mann-Whitney test.

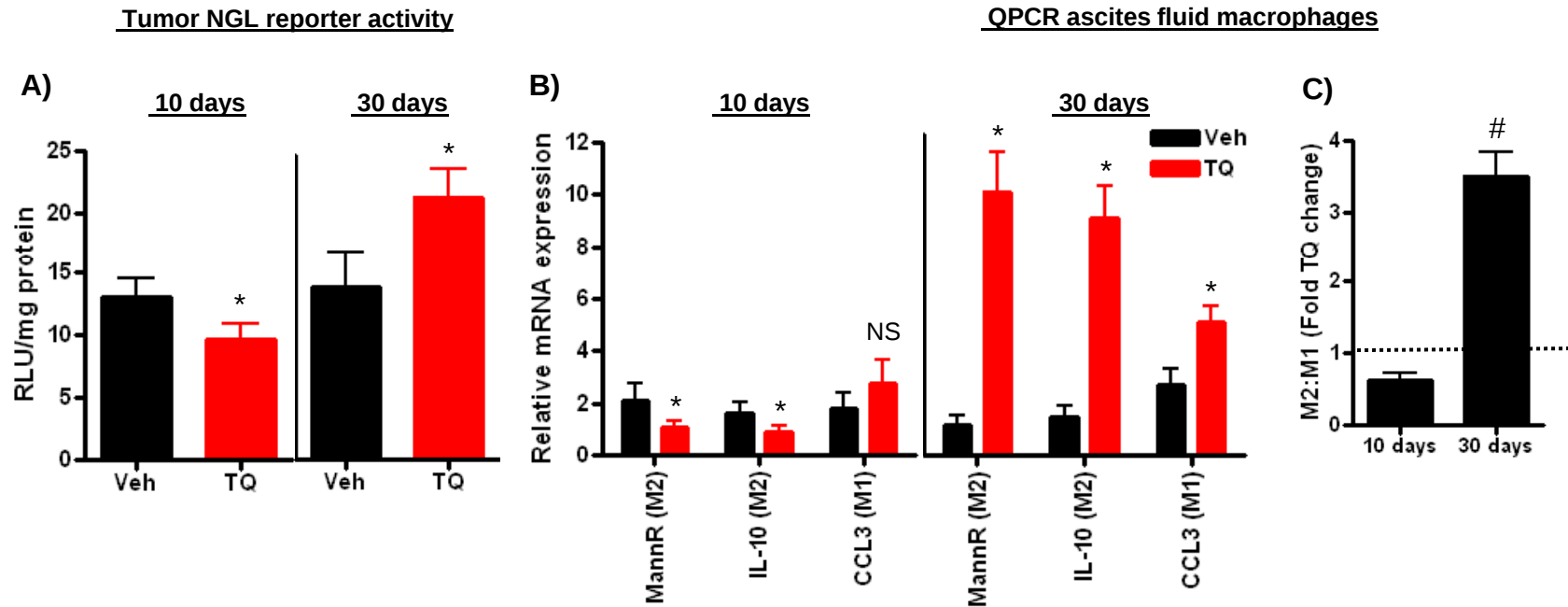


Fig. 20. (A) Relative luciferase activity in harvested ID8-NGL tumors following 10 or 30 days' TQ treatment. (B) QPCR analysis of the mRNA expression of the markers of M2 macrophages, mannose-receptor (mann-R) and interleukin-10 (IL-10) and M1 macrophages, CCL3, in RNA extracted from peritoneal lavages or ascites fluid. Values were normalized to corresponding levels of GAPDH mRNA expression levels. The expression ratio of the fold-change induced by TQ at 10 and 30 days' treatment on expression of mann-R/IL-12 to CCL3 is shown in (C). Values are mean+SD for 5 mice per group. * $p < 0.01$ relative to vehicle-treated mice; # $p < 0.001$ relative to 10d timepoint, Mann-Whitney test.

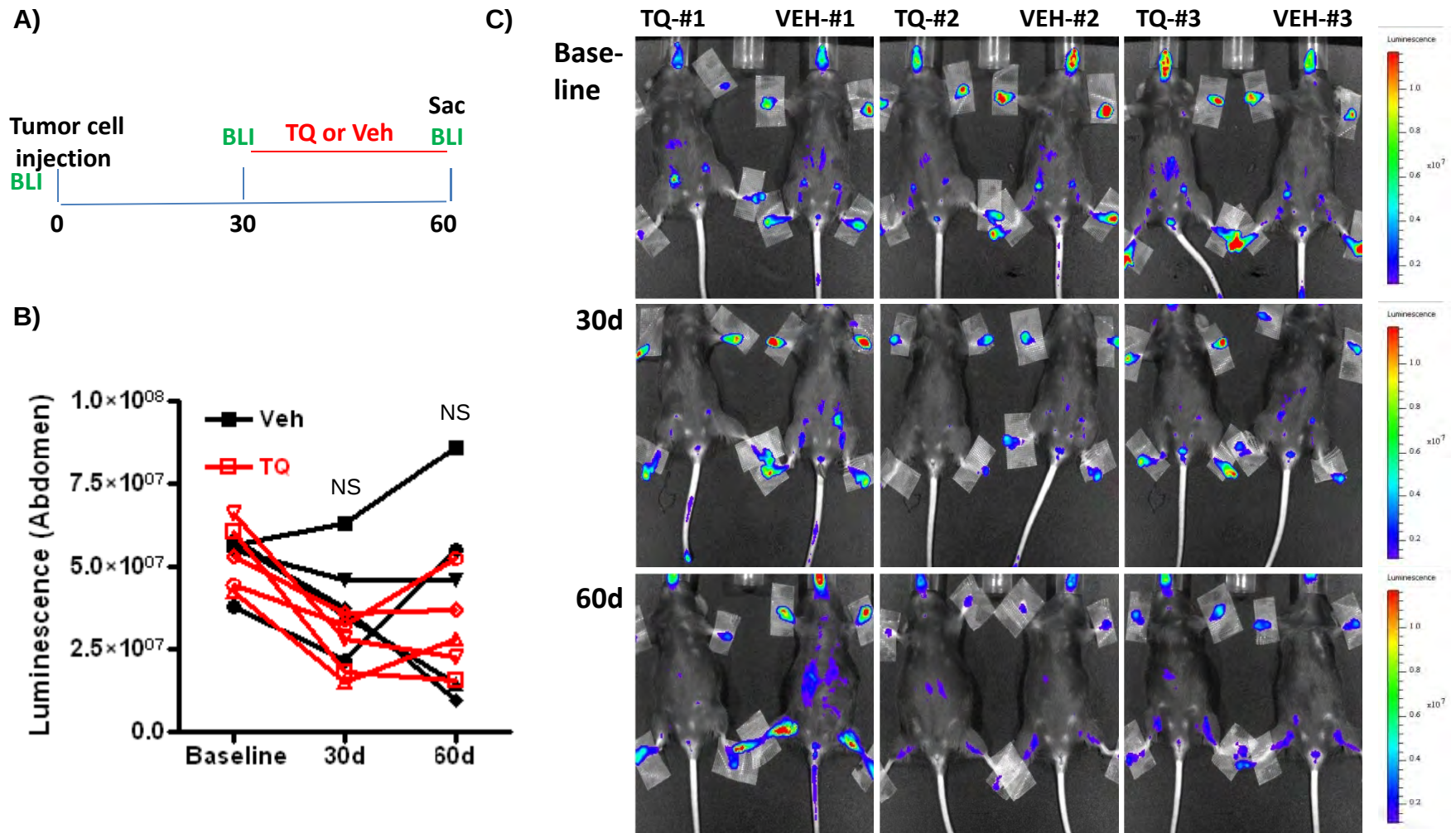


Fig 21. (A) Schematic diagram showing treatment schedule for vehicle or TQ in NGL reporter mice injected with ID8 cells. (B) Quantification of BLI in the peritoneal cavity shows no differences between NF- κ B reporter activity in Vehicle or TQ-treated mice. Values are mean+SD from 5 mice per group. P value determined by Mann-Whitney test revealed no difference (NS) between baseline BLI and 30d or 60d values in vehicle and TQ-treated mice. (C) Representative BLI images comparing NF- κ B reporter signal in Vehicle and TQ-treated cells.

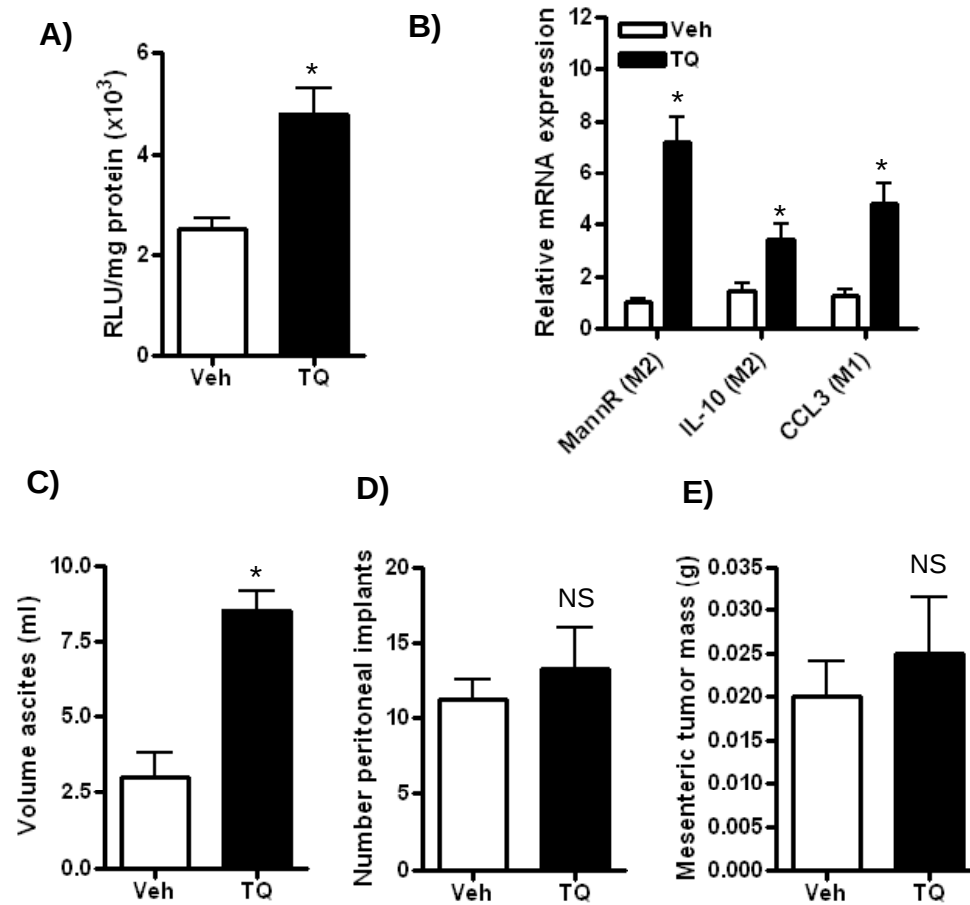


Fig 22. (A) Luciferase activity of the NF- κ B reporter was measured in isolated macrophages from ascites or peritoneal lavage fluid collected from vehicle or TQ-treated NGL reporter mice injected with ID8 cells, and expressed relative to cellular protein. (B) QPCR analysis of the mRNA expression of the markers of M2 macrophages, mannose-receptor (mann-R) and interleukin-10 (IL-10) and M1 macrophages, CCL3, in RNA extracted from peritoneal lavages or ascites fluid. Values were normalized to corresponding levels of GAPDH mRNA expression. (C) Quantification of ascites fluid volume at sacrifice showed increased ascites with TQ treatment, but no significant differences in (D) the number of peritoneal implants or (E) mesenteric tumor mass. Values are mean+SD for 5 mice per group. * $p < 0.01$ relative to vehicle-treated mice; NS: not significant relative to vehicle, Mann-Whitney test.

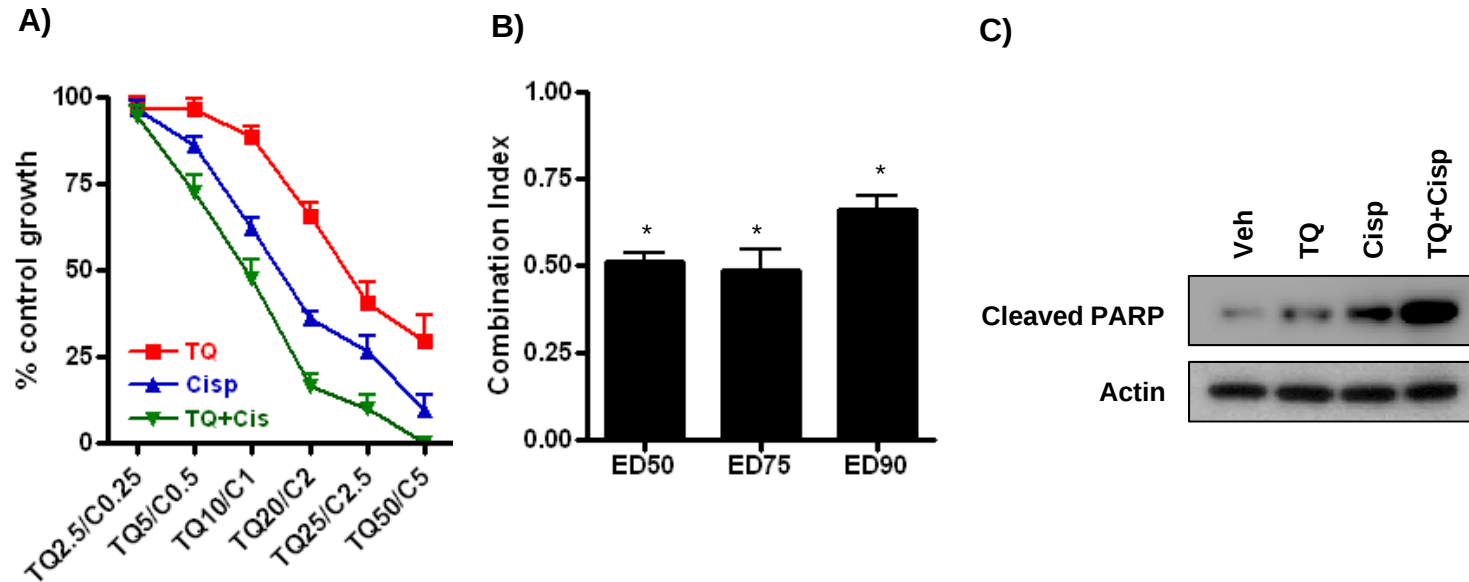


Fig 23. (A) SRB assays showed that treatment of ID8-NGL cells with the combination of TQ and cisplatin (concentrations in μM , 72 hours) induced greater inhibitory effects on cell growth than either drug alone. (B) Isobologram analysis using Calcsyn software that the Combination Index was significantly less than 1 for Effective Doses (ED) ED50, ED75 and ED90, indicating synergistic drug interaction. Values are mean $\pm$ SD for 3 experiments; * $p < 0.01$ relative to the individual effect of each drug alone. (C) Western Blot analysis of cleaved PARP following 24 hour treatment of ID8-NGL cells with TQ (25 μM), cisplatin (1 μM) or the combination. Actin was used as a loading control.

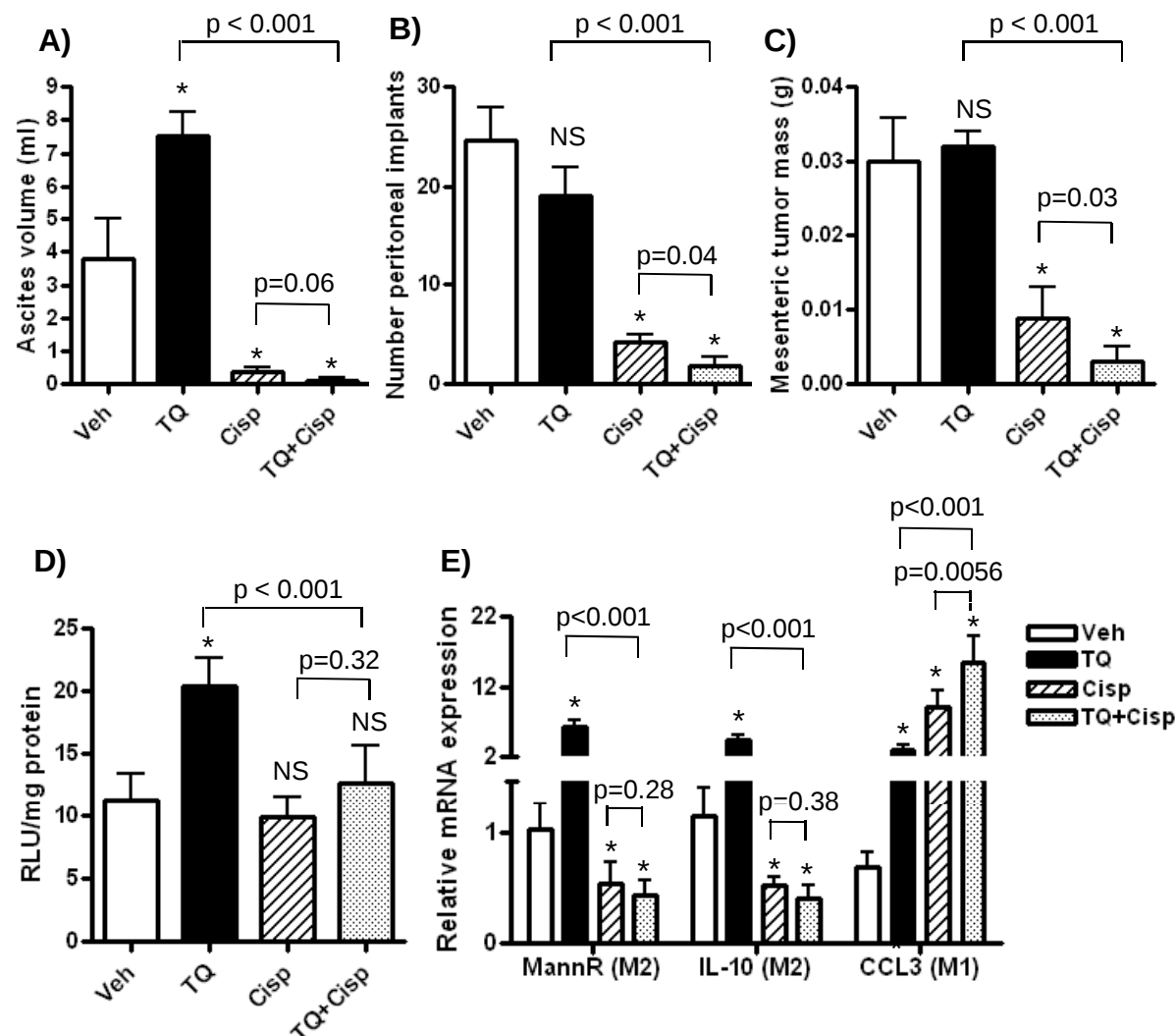
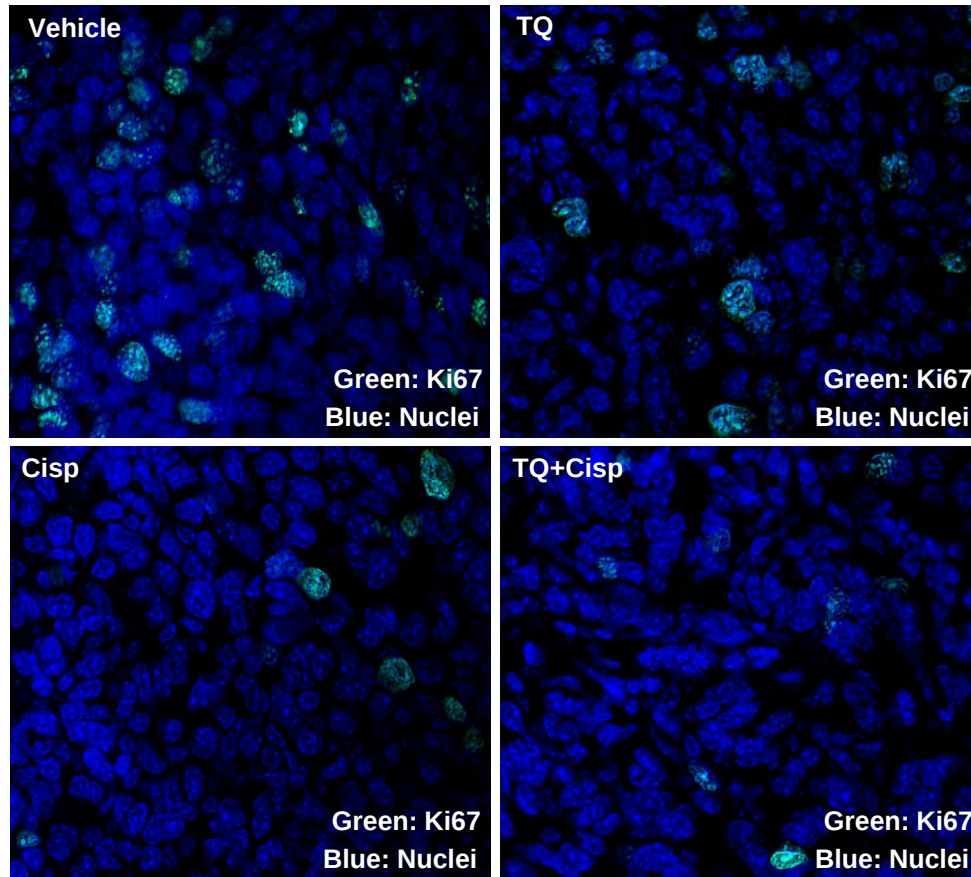


Fig 24. In BL/6 mice injected with ID8-NGL cells and treated with vehicle, TQ, cisplatin or the combination. (A) ascites fluid volume, (B) number of peritoneal implants and (C) mesenteric tumor mass was measured at sacrifice. (D) Luciferase assay in tumors harvested at sacrifice. (E) QPCR analysis of the mRNA expression of the markers of M2 macrophages, mannose-receptor (mann-R) and interleukin-10 (IL-10) and M1 macrophages, CCL3, in RNA extracted from peritoneal lavages or ascites fluid. Values were normalized to corresponding levels of GAPDH mRNA expression. Values are mean+SD for 5 mice per group. * $p < 0.01$ relative to vehicle; NS: not significant relative to vehicle; all p values determined by Mann-Whitney test.

A)



B)

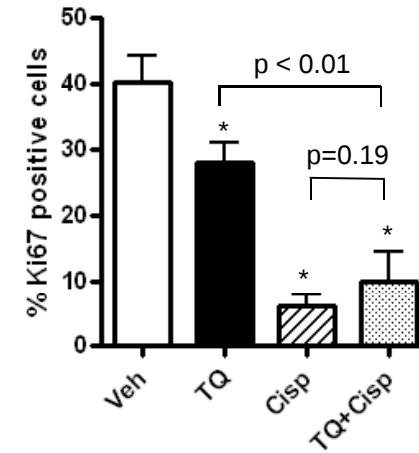
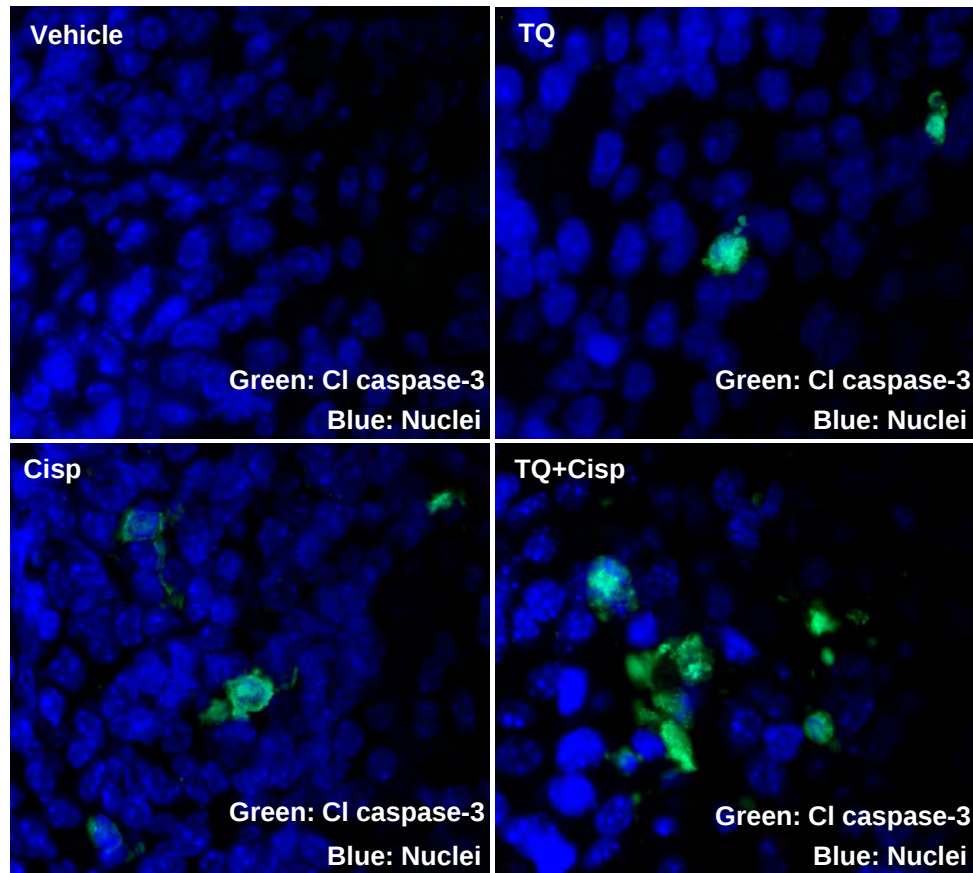


Fig. 25 (A) Immunofluorescent detection of the proliferation marker Ki67/mib-1 in ID8-NGL tumors harvested from mice treated with vehicle, TQ, cisplatin or the combination. TO-PRO-3-stained nuclei are in blue. (B) The percentage of tumor cells positive for Ki67 staining. * $p < 0.01$ relative to vehicle; all p values determined by Mann-Whitney test.

A)



B)

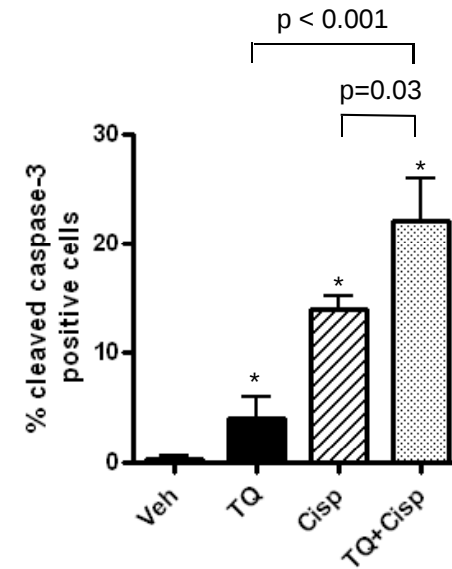


Fig. 26 (A) Immunofluorescent detection of the apoptosis marker cleaved caspase-3 in ID8-NGL tumors harvested from mice treated with vehicle, TQ, cisplatin or the combination. TO-PRO-3-stained nuclei are in blue. (B) The percentage of tumor cells positive for cleaved caspase-3 staining. * $p < 0.01$ relative to vehicle; all p values determined by Mann-Whitney test.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Andrew James Wilson, Ph.D	POSITION TITLE Research Assistant Professor		
eRA COMMONS USER NAME (credential, e.g., agency login) WILSONAJ			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Melbourne University, Australia	B.Sc (Hons)	1992-1994	Immunology/Pathology
Department of Medicine, Melbourne University, Australia	Ph.D.	1995-1998	Medicine
Department of Medicine, Melbourne University, Australia	Postdoctoral	1998-1999	Colon biology
Albert Einstein Cancer Center, Bronx, NY	Postdoctoral	2001-2003	Colon cancer

A. Personal Statement

I have a long-standing 11 year experience in cancer research including dissecting signaling pathways driving the tumorigenic process in cancer cells, generation and characterization of stable cell lines in tissue culture, and investigating efficacy of anti-cancer drugs in preclinical studies in vitro and in mouse models in vivo. At Vanderbilt, I have been able to apply this knowledge to discovering new treatment strategies for ovarian cancer in the laboratory of Assistant Professor Dineo Khabele, a physician-scientist and practicing gynecologic oncologist. I am a Principal Investigator on a currently funded Department of Defense grant examining the patterns of nuclear factor-kappaB (NF-κB) activity in ovarian cancer (Dr Yull, Co-PI). We have established an excellent working relationship that has formed the basis of several grant applications. Our overarching goal is to determine effects of modulating NF-κB activity in tumor cells and in host peritoneal macrophages on the progression of ovarian cancer in unique preclinical syngeneic models. This innovative approach will lead to increased understanding of molecular pathways involved in ovarian cancer progression, and guide novel strategies aimed at limiting this progression. With the assistance of Dr Khabele, we also have a pipeline to the clinic of drugs that show promising preclinical efficacy.

B. Positions and Honors**Positions and Appointments**

1999-2000	Post-Doctoral Researcher, Department of Medicine, Melbourne University, Australia
2001-2003	Post-Doctoral Fellow, Albert Einstein Cancer Center, Montefiore Medical Center, Bronx, NY
2003-2008	Instructor of Medicine, Albert Einstein Cancer Center, Montefiore Medical Center, Bronx, NY
2008-2011	Research Instructor, Department of Obstetrics and Gynecology, Vanderbilt University, Nashville, TN
2011-Present	Research Assistant Professor, Department of Obstetrics and Gynecology, Vanderbilt University, Nashville, TN

C. Selected peer-reviewed publications (out of 37 total publications)

1. A Basu, NE Bodycombe, JH Cheah, EV Price, K Liu, GI Schaefer, RY Ebright, ML Stewart, D Ito, S Wang, AL Bracha, T Liefeld, M Wawer, JC Gilbert, **AJ Wilson**, Nicolas Stransky, GV Kryukov, V Dancik, J Barretina, LA Garraway, C Hon, B Munoz, JA Bittker, BR Stockwell, D Khabele, AM Stern, PA Clemons, AF Shamji, SL Schreiber. An interactive resource to identify cancer genetic. *Cell* In Press.
2. **AJ Wilson**, AY Liu, JT Roland, OB Adebayo, SA Fletcher, JC Slaughter, J, Saskowski MA Crispens, HW Jones 3rd, S James, O Fadare, D Khabele. TR3 modulates platinum resistance in ovarian cancer. *Cancer Res.* 73: 4758-69, 2013. **PMID: 23720056**
3. **AJ Wilson**, AS Lalani; E Wass, JL Saskowski, D Khabele. Romidepsin (FK228) combined with cisplatin stimulates DNA damage-induced cell death in ovarian cancer. *Gyn. Onc.* **127**: 579-86, 2012. **PMID: 23010348**
4. **AJ Wilson**, TQ Cheng, D Khabele. Thailandepsins are new small molecule class I HDAC inhibitors with potent cytotoxic activity in ovarian cancer cells: a preclinical study of epigenetic ovarian cancer therapy. *Cancer Biol. Ther.* J Ovarian Res. 2012 Apr 24;5(1):12. **PMID: 22531354**
5. **AJ Wilson**, E Holson, F Wagner, Y-L Zhang, DM Fass, SJ Haggarty, S Bhaskara, SW Hiebert, SL Schreiber, D Khabele. The DNA damage mark pH2AX differentiates the cytotoxic effects of small molecule HDAC inhibitors in ovarian cancer cells. *Cancer Biol. Ther.* **12**: 484-493, 2011. **PMID: 22295145**
6. D Khabele, O Fadare, AY Liu, **AJ Wilson**, E Wass, K Osteen. MA Crispens. An orthotopic model of platinum-sensitive high grade serous fallopian tube carcinoma. *Int. J. Clin. Exp. Pathol.* **5**: 37-45, 2012. **PMID: 21738006**
7. Bhaskara S, Knutson SK, Jiang G, Chandrasekharan MB, **Wilson AJ**, Zheng S, Yenamandra A, Locke K, Yuan JL, Bonine-Summers AR, Wells CE, Kaiser JF, Washington MK, Zhao Z, Wagner FF, Sun ZW, Xia F, Holson EB, Khabele D, Hiebert SW. Hdac3 is essential for the maintenance of chromatin structure and genome stability. *Cancer Cell.* **18**: 436-47, 2010. **PMID: 21075309**
8. Son DS, **Wilson AJ**, Parl AK, Khabele D. The effects of the histone deacetylase inhibitor romidepsin (FK228) are enhanced by aspirin (ASA) in COX-1 positive ovarian cancer cells through augmentation of p21. *Cancer Biol. Ther.* **9**: 928-35, 2010. **PMID: 20404564**
9. **AJ Wilson**, L Togel, A Chueh, GA Corner, N Ahmed, S Goel, DS Byun, S Nasser, D Arango, MA Houston, M Jhawer, HJM Smartt, LB Murray, C Nicholas, BG Heerdt, LH Augenlicht, JM. Mariadason. Apoptotic sensitivity of colon cancer cells to histone deacetylase inhibitors is mediated by an Sp1/Sp3-activated transcriptional program involving immediate-early gene induction. *Cancer Res.* 70: 609-620, 2010. **PMID: 20068171**
10. Z Yuan Z, J Shin, **AJ Wilson**, S Goel, YH Ling, N Ahmed, H Dopeso, M Jhawer, S Nasser, C Montagna, K Fordyce, LH Augenlicht, LA Aaltonen, D Arango, TK Weber, JM Mariadason. An A13 repeat within the 3'-untranslated region of epidermal growth factor receptor (EGFR) is frequently mutated in microsatellite instability colon cancers and is associated with increased EGFR expression. *Cancer Res.* **69**: 7811-7818, 2009. **PMID: 19789347**
11. **AJ Wilson**, DS Byun, S Nasser, LB Murray, D Arango, A Melnick, M Figueroa, G Kao, LH. Augenlicht, JM Mariadason. HDAC4 promotes growth of colon cancer cells by repression of p21. *Mol. Biol. Cell* **19**:4062-475, 2008. **PMID: 18632985**
12. M Jhawer, S Goel, **AJ Wilson**, C Montagna, Yi-He Ling, DS Byun, S Nasser, D Arango, J Shin, L Klampfer, LH Augenlicht, R Perez-Soler, JM Mariadason. PIK3CA/PTEN expression status predicts response of colon cancer cells to the EGFR inhibitor cetuximab. *Cancer Res.* **68**: 1953-1961, 2008. **PMID: 18339877**
13. **AJ Wilson**, DS Byun, N Popova, LB Murray, K L'Italien, Y Sowa, D Arango, A Velcich, LH. Augenlicht, JM. Mariadason. HDAC3 and other Class I HDACs regulate colon cell maturation and p21 expression, and are deregulated in human colon cancer. *J. Biol. Chem.* **281**: 13548-13558, 2006. **PMID: 16533812**
14. **AJ Wilson**, D Arango, JM Mariadason, BG Heerdt, LH Augenlicht. TR3/Nur77 in colon cancer cell apoptosis. *Cancer Res.* **63**: 5401-5407, 2003. **PMID: 14500374**

D. Research Support

Ongoing research support

W81XWH-11-1-0509 (Wilson) 07/25/11-07/24/13 (No cost extension – 07/24/14)

Department of Defense

Nuclear factor-kappaB Activity in the Host-tumor Microenvironment of Ovarian Cancer

Study the patterns of nuclear factor-kappa B activity in the host versus the tumor epithelium during progression of ovarian cancer in a murine model. Bioluminescent reporters in ovarian cancer cell lines or transgenic mice will determine patterns of NF- κ B activity and responses to pharmacologic interventions during tumor progression.

K08CA148887 Khabele (PI) 5/1/10-4/30/15

NCI

Targeting Histone Deacetylases with Small Molecule Inhibitors in Ovarian Cancer

Study the efficacy of Class I histone deacetylases, particularly HDAC3, as targets for therapy in ovarian cancer cells, as single agents and in combination with DNA damaging agents such as cisplatin.

Pending support

13082724 (Yull) 09/01/13-08/31/15 1.20 cm

NIH/NCI \$125,000

Targeted activation of macrophages to limit ovarian cancer progression

Macrophages are the major cell population in the microenvironment of ovarian cancer, therefore targeted modulation of their functions represents a novel therapeutic approach. We will use doxycycline-inducible transgenics, adoptive transfer of activated macrophages or treatment with Mifamurtide (a drug that activates macrophages) to provide evidence that activation of NF- κ B signaling specifically targeted to macrophages can limit tumor progression and can synergize with chemotherapy.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Yull, Fiona Elizabeth, D.Phil.	POSITION TITLE Associate Professor of Cancer Biology		
eRA COMMONS USER NAME (credential, e.g., agency login) fiona_yull			
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	MM/YY	FIELD OF STUDY
University of St. Andrews, St. Andrews, UK	B.Sc. (Hons)	1985	Biochemistry w/Microbiology
University College Oxford, Oxford, U.K.	D.Phil.	1989	Biochemistry

A. Personal Statement

I have 21-years of experience with the design, generation and characterization of multi-mutation transgenic and knockout mice. My group develops murine models to investigate the role of the NF- κ B family of transcription factors in disease, particularly cancer. Development of these murine models represents a major investment but we have shared the resulting models with more than 60 groups worldwide, resulting in successful collaborative studies relating to the contribution of NF- κ B signaling in several types of cancer including lung, breast, prostate and skin. To address the roles of NF- κ B in cancer initiation, promotion and progression we have developed a modular inducible "tool kit" of transgenics. These enable up and down-regulation of canonical NF- κ B signaling directed to specific cell types (mammary epithelium, macrophages and lung epithelium). These models provide the opportunity to address the roles of NF- κ B in specific cells in the tumor and microenvironment during defined stages of progression. We recently made the unexpected discovery that targeted activation of NF- κ B signaling within macrophages can produce cells with tumor cytotoxic functions. Given the potential for macrophages to play critical roles during ovarian cancer progression, we wish to investigate modulation of NF- κ B specifically within macrophages using nanoparticle targeting, a methodology with relatively rapid translational potential, to attempt to define new therapeutic approaches. My overarching goal is to determine the contributions of NF- κ B signaling within specific cell types to cancer progression in order to discover and develop novel therapeutic approaches.

B. Positions and Honors

Positions and Employment

1985-1989	Predocotoral Fellow, mentored by Drs. A. and S. Kingsman, Oxford University, UK
1989-1995	Postdoctoral Fellow, mentored by Dr. J. Clark, The Roslin Institute, Roslin, Edinburgh, UK
1995-1998	Postdoctoral Fellow, Dept. of Microbiology and Immunology, mentored by Dr. L. Kerr, Vanderbilt University Medical Center, Nashville, Tennessee
1998-1998	Research Instructor, Dept. of Cell Biology, working with Dr. L. Kerr, Vanderbilt University. Awarded US Army Breast Cancer Research Program Fellowship
1998-1999	Research Asst. Professor, Dept. of Cell Biology. Acting Principal Investigator during absence of Dr. L. Kerr on Robert Woods Johnson Fellowship in Washington. Awarded American Cancer Society Institutional Research Grant
2000-2004	Research Assistant Professor, Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee
2004-Pres.	Assistant Professor of Cancer Biology, Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee
2010-Pres.	Associate Professor, Department of Cancer Biology, VUMC, Nashville, Tennessee

Other Experience and Professional Memberships

2000-Pres. Deputy Director, Department of Medicine PPG Core B

2003 Deputy Director of Cancer Biology Graduate Course
 2004-2006 Reviewer for Susan G Komen Foundation Postdoctoral Fellowships
 2004-Pres. Director of Cancer Biology Graduate Course
 2007-Pres. Member of Nashville VA Research Safety Subcommittee
 2011-Pres. Vice Chairman of Nashville VA Research Safety Subcommittee
 2009-Pres. Member of Vanderbilt University IACUC Committee
 2010,11,13 Ad hoc member, DOD BCRP FY10 Programmatic Review Panel
 2011 Ad hoc reviewer NIH MESH study section
 2012-Pres. Member of Editorial Board of ISRN Inflammation
 2013 Ad hoc reviewer NIH SEP Cancer Health Disparities and Diversity in Basic Cancer Research
 2013 Ad hoc reviewer NIH MESH study section

1986-Pres. Member of Society of General Microbiology
 1997-Pres. Member of Society of Developmental Biology
 1998-Pres. Member of American Association of Cancer Research

Honors

2000-2004 Aventis Leadership Development Program Fellow

C. Selected Peer-reviewed Publications (from 66 peer-reviewed publications)

1. Connelly L, Robinson-Benion C, Chont M, Saint-Jean L, Li H, Polosukhin VV, Blackwell TS, Yull FE, A transgenic model reveals important roles for the NF- κ B alternative pathway (p100/p52) in mammary development and links to tumorigenesis. *J Biol Chem.* 282: 10028-35, 2007. PMID: 17261585.
2. Stathopoulos GT, Sherrill TP, Cheng DS, Scoggins RM, Han W, Polosukhin VV, Connelly L, Vasiliou S, Karatza M, Yull FE, Fingleton B, Blackwell TS. Epithelial nuclear factor- κ B activation promotes urethane-induced lung carcinogenesis. *Proc Natl Acad Sci.* 104(47):18514-18519, 2007. PMCID: PMC2141808
3. Han W, Joo W, Everhart MB, Christman JW, Yull FE, Blackwell TS. Myeloid cells control termination of lung inflammation through the NF- κ B pathway. *Am J Physiol.: Lung Cell Mol Physiol.* 296(3): L320-7, 2009. PMCID: PMC2660215.
4. Connelly, L., Barham, W., Pigg, R., Saint-Jean, L., Sherrill, T., Cheng, D-S., Chodosh, L.A., Blackwell, T.S. Yull FE. Activation of NF-kappaB in mammary epithelium promotes milk loss during mammary development and infection. *Journal of Cellular Physiology* 222:73-81, 2010. PMCID: PMC2783968.
5. Connelly L, Barham W, Onishko HM, Sherrill T, Chodosh LA, Blackwell TS, Yull FE. Inhibition of NF-kappaB activity in mammary epithelium increases tumor latency and decreases tumor burden. *Oncogene* 30:1402-12, 2010. PMCID: PMC3063854
6. Zaynagetdinov R, Stathopoulos GT, Sherrill TP, Cheng DS, McLoed AG, Ausborn JA, Polosukhin VV, Connelly L, Zhou W, Fingleton B, Peebles RS, Prince LS, Yull FE, Blackwell TS. Epithelial nuclear factor- κ B signaling promotes lung carcinogenesis via recruitment of regulatory T lymphocytes. *Oncogene.* 2011. PMID: 22002309.
7. Zaynagetdinov R, Sherrill TP, Polosukhin VV, Han W, Ausborn JA, McLoed AG, McMahon FB, Gleaves LA, Degryse AL, Stathopoulos GT, Yull FE, Blackwell TS. A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. *J Immunol.* 187:5703-11, 2011. PMID: 22048774.
8. Blackwell, TS, Hipps, AN, Yamamoto, Y, Han, W, Barham, WJ, Ostrowski, MC, Yull, FE, Prince, LS. NF-kappaB Signaling in Fetal Lung Macrophages Disrupts Airway Morphogenesis. *J Immunol.* 187:2740-7, 2011. PMID: 21775686.
9. Connelly L, Barham W, Onishko HM, Chen L, Sherrill T, Zabuwalla T, Ostrowski MC, Blackwell TS, Yull FE. NF-kappaB activation within macrophages leads to an anti-tumor phenotype in a mammary tumor lung metastasis model. *Breast Cancer Res.* 13:R83. 2011. PMID: 21884585.
10. Karabela SP, Psallidas I, Sherrill TP, Kairi CA, Zaynagetdinov R, Cheng DS, Vassiliou S, McMahon F, Gleaves LA, Han W, Stathopoulos I, Zakynthinos SG, Yull FE, Roussos C, Kalomenidis I, Blackwell TS, Stathopoulos GT. Opposing effects of bortezomib-induced nuclear factor- κ B inhibition on chemical lung carcinogenesis. *Carcinogenesis.* 2012. PMID:22287559.

11. Swartz MA, Iida N, Roberts EW, Sangaletti S, Wong MH, Yull FE, Coussens LM, Declerck YA. Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy. *Cancer Res.* 72:2473-80, 2012 PMID: 22414581.
12. Barham W, Sherrill T, Connelly L, Blackwell TS, Yull FE. Intraductal injection of LPS as a mouse model of mastitis: signaling visualized via an NF- κ B reporter transgenic. *J Vis Exp.* 67:e4030, 2012. PMID:22971993
13. Yu SS, Lau CM, Barham WJ, Onishko HM, Nelson CE, Li H, Smith CA, Yull FE, Duvall CL, Giorgio TD. Macrophage-Specific RNAi Targeting via 'Click', Mannosylated Polymeric Micelles. *Mol Pharm.* 2013 PMID:23331322.
14. Han W, Li H, Cai J, Gleaves LA, Polosukhin VV, Segal BH, Yull FE, Blackwell TS. 2013. NADPH Oxidase Limits Lipopolysaccharide-Induced Lung Inflammation and Injury in Mice through Reduction-Oxidation Regulation of NF- κ B Activity. *J Immunol* 190:4786-94. PMID:23530143 PMCID: PMC3633681.
15. Barham W, Frump AL, Sherrill TP, Garcia CB, Saito-Diaz K, Vansaun MN, Fingleton B, Gleaves L, Orton D, Capecchi MR, Blackwell TS, Lee E, Yull F, Eid JE. Targeting the Wnt Pathway in Synovial Sarcoma Models. *Cancer Discov.* 2013 Aug 6. [Epub ahead of print] PMID: 23921231.

D. Research Support

Ongoing Research Support

W81XWH-11-1-0242 (Yull) 07/01/11-06/30/13 (No cost extension – 06/30/14)

Department of Defense

“Assessment of nanobiotechnology-Targeted siRNA Designed to **Inhibit** NF-kappaB Classical and Alternative signaling in Breast Tumor Macrophages”

Goals are; 1) exploration of macrophage response to **inhibition** of NF- κ B activation by the canonical and alternative pathways using siRNA *in vitro*, 2) develop nanobiotechnology delivery vehicle for specific delivery of siRNA to tumor associated macrophages *in vivo* to modulate NF- κ B activity.

W81XWH-11-1-0509 (Wilson) 07/25/11-07/24/13 (No cost extension – 07/24/14)

Department of Defense

Nuclear factor-kappaB Activity in the Host-tumor Microenvironment of Ovarian Cancer

Study the patterns of nuclear factor-kappa B activity in the host versus the tumor epithelium during progression of ovarian cancer in a murine model. Bioluminescent reporters in ovarian cancer cell lines or transgenic mice will determine patterns of NF- κ B activity and responses to pharmacologic interventions during tumor progression.

1 R01 HL 116358-01 (Blackwell/Prince, Co-PI's) 09/25/12 - 06/30/15

NIH/NHLBI

Imaging Activated Macrophages in the Lungs

Lung macrophages are critical for initiating the innate immune response to microbial and environmental stimuli, resolving acute inflammation, and promoting repair following injury. In this proposal, we hypothesize that developing molecular imaging techniques to identify functional subsets of activated macrophages will advance understanding of inflammatory lung diseases and could lead to novel, macrophage-targeted therapies. These studies will optimize imaging probes based on FR β expression and explore new imaging targets present on the surface of activated macrophages. These new strategies can then be applied to the study of inflammatory lung diseases in humans.

5 R01 HL 097195-02 (Prince) 09/14/09 - 07/31/14

NIH/NHLBI

“Role of Fetal Lung Macrophages in Bronchopulmonary Dysplasia”

Goal: This proposal tests the role of fetal lung macrophages in bronchopulmonary dysplasia pathogenesis. Specifically, we will test if macrophages are required for inhibition of normal lung development by innate

immune stimuli, if NF- κ B activation in macrophages mediates global fetal lung inflammation, and how early exposure of fetal lung macrophages to inflammatory stimuli alters macrophage phenotype as lungs mature. Aims: Identify the role of NF- κ B in inhibition of lung development following innate immune activation. To determine if early activation of fetal lung macrophages alters the macrophage phenotype as lungs mature.

5 R01 HL 085317 (Blackwell)

04/01/12 - 02/28/16

NIH/NHLBI

"Epithelial-Fibroblast Interactions in Lung Fibrosis"

Goal: This proposal uses novel mouse models to test the hypothesis that specific phenotypic alterations in alveolar epithelial cells affect the response to injurious stimuli, impact fibroblast activation, and determine the severity and progression of lung fibrosis.

Aims: To define the extent of epithelial-mesenchymal transition as a source of fibroblasts in experimental pulmonary fibrosis. In these studies, we will determine the proportion of lung fibroblasts derived from epithelium via EMT and examine the phenotypic characteristics of epithelial-derived fibroblasts.

5 R01 78188-02 (McGuinness)

06/01/09 - 05/31/14

NIH/NCI

Impact of Inflammation on the control of muscle

Goal: Identify the steps controlling MGU that are impacted by inflammation so that future therapies can have a more targeted approach in correcting MGU during an inflammatory stress such as sepsis

Aims: The impact of LPS on the relative control glucose transport and glucose phosphorylation have in determining MGU. If inflammatory stress amplifies the impact LCFA and glucose availability have in modulating MGU. If modulating oxidative stress (Nitric oxide availability and NF- κ B activation) will improve MGU by augmenting glucose phosphorylation and mitochondrial ATP flux.

Pending:

13041064 (Richmond)

07/01/13-06/30/18

1.20 cm

NIH/NCI

\$250,000

NIK Modulation of B-catenin: Targets for melanoma therapy

13072575 (Yull)

09/01/13-08/31/18

3.60 cm

NIH/NCI

\$250,000

Alternative NF-kappaB signaling impacts breast cancer development and progression (not discussed)

13082724 (Yull)

09/01/13-08/31/15

1.80 cm

NIH/NCI

\$125,000

Targeted activation of macrophages to limit ovarian cancer progression

Macrophages are the major cell population in the microenvironment of ovarian cancer, therefore targeted modulation of their functions represents a novel therapeutic approach. We will use doxycycline-inducible transgenics, adoptive transfer of activated macrophages or treatment with Mifamurtide (a drug that activates macrophages) to provide evidence that activation of NF- κ B signaling specifically targeted to macrophages can limit tumor progression and can synergize with chemotherapy.

1R21CA185672-01 (Yull)

04/01/14-03/31/16

1.80 cm

NCI

\$150,000

Induction of tumoricidal tumor-associated macrophages as lung cancer therapy

1R01CA177945-01A1 (Yull)

04/01/14-03/31/19

3.60 cm

NIH

\$250,000

Targeted activation of macrophages as breast cancer therapy

Overlap: There is no overlap between the currently funded grants listed above and this application.

Tracking NF- κ B activity in tumor cells during ovarian cancer progression in a syngeneic mouse model.

Andrew J. Wilson^{1*}, Whitney Barham², Jeanette Saskowski¹, Oleg Tikhomirov², Lianyi Chen², Hye-Jeong Lee¹, Fiona Yull^{2, 3#} and Dineo Khabele^{1, 3#}

¹Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Vanderbilt University School of Medicine, Nashville, TN; ²Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN; ³Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, TN.

*Correspondence to: Andrew J. Wilson, Ph.D., Research Assistant Professor, Department of Obstetrics and Gynecology, Vanderbilt University Medical Center, B1100 Medical Center North, Nashville, TN 37232; (615) 875-8968 (phone); (615) 343-8403 (fax); andrew.j.wilson@vanderbilt.edu

Contributed equally to the manuscript.

ABSTRACT

Background: Nuclear factor-kappa B (NF-kappaB) signaling is an important link between inflammation and peritoneal carcinomatosis in human ovarian cancer. Our objective was to track NF-kappaB signaling during ovarian cancer progression in a syngeneic mouse model using tumor cells stably expressing an NF-kappaB reporter.

Methods: ID8 mouse ovarian cancer cells stably expressing an NF-kappaB-dependent GFP/luciferase (NGL) fusion reporter transgene (ID8-NGL) were generated, and injected intra-peritoneally into C57BL/6 mice. NGL reporter activity in tumors was non-invasively monitored by bioluminescence imaging and measured in luciferase assays in harvested tumors. Ascites fluid or peritoneal lavages were analyzed for inflammatory cell and macrophage content, and for mRNA expression of M1 and M2 macrophage markers by quantitative real-time RT-PCR. 2-tailed Mann-Whitney tests were used for measuring differences between groups in *in vivo* experiments.

Results: In ID8-NGL cells, responsiveness of the reporter to NF-kappaB activators and inhibitors was confirmed *in vitro* and *in vivo*. ID8-NGL tumors in C57BL/6 mice bore histopathological resemblance to human high-grade serous ovarian cancer and exhibited similar peritoneal disease spread. Tumor NF-kappaB activity, measured by the NGL reporter and by western blot of nuclear p65 expression, was markedly elevated at late stages of ovarian cancer progression. In ascites fluid, macrophages were the predominant inflammatory cell population. There were elevated levels of the M2-like pro-tumor macrophage marker, mannose-receptor, during tumor progression, and reduced levels following NF-kappaB inhibition with thymoquinone.

Conclusions: Our ID8-NGL reporter syngeneic model is suitable for investigating changes in tumor NF-kappaB activity during ovarian cancer progression, how NF-kappaB activity influences immune cells in the tumor microenvironment, and effects of NF-kappaB-targeted treatments in future studies.

KEY WORDS:

NF- κ B activity; ovarian cancer; syngeneic mouse model; macrophages; bioluminescence.

BACKGROUND

Ovarian cancer is the most lethal gynecologic malignancy in the United States [1]. Most women diagnosed with epithelial ovarian cancers have advanced, metastatic disease characterized by abdominal ascites and peritoneal carcinomatosis [2]. Inflammation is a hallmark of cancer [3], and there is ample evidence linking chronic inflammation to pro-tumorigenic effects in ovarian cancer development and progression [4-9]. Pro-inflammatory cytokines and chemokines produced by peritoneal macrophages contribute to the distinct clinical features of ovarian cancer, specifically malignant ascites and widespread peritoneal tumor implants [4, 10, 11].

The nuclear factor-kappaB (NF- κ B) pathway is a critical molecular link between inflammation and cancer. NF- κ B signaling is known to play an important role in several malignancies, including ovarian cancer [9, 12-16]. There are five known NF- κ B subunits in humans: NF- κ B 1 (p105/p50), NF- κ B 2 (p100/p52), RelA (p65), RelB and c-Rel [17]. In a quiescent state, NF- κ B subunits are bound to inhibitors of κ B protein (I κ Bs) and are sequestered in the cytoplasm. Activation of the classical NF- κ B cascade is initiated by growth factors, microbes, cytokines and genotoxic stress, which in turn activate the I κ B kinase (IKK) complex. Activated IKK phosphorylates I κ B α , which is then ubiquitinated. As a result, the p50-RelA dimer translocates to the nucleus, where it binds to the promoter/enhancer regions of genes involved in the regulation of cell growth, apoptosis and inflammation [16].

Several lines of evidence link NF- κ B activity to ovarian cancer progression. Constitutive activation of NF- κ B is observed in a large subset of ovarian tumors and is associated with tumor growth and hallmarks of progression [4, 13-16]. Moreover, NF- κ B is known to link a specific subset of pro-inflammatory cytokines and chemokines, including a TNF cytokine network to human epithelial ovarian cancer [6, 10, 15, 18]. Such functions of NF- κ B have led to successful preclinical testing of NF- κ B inhibitors in ovarian cancer model systems, such as thymoquinone, a product of the medicinal plant

Nigella sativa [19]. However, most preclinical models are limited by the fact that drug effects are tested on cancer cells in the absence of the supporting tumor microenvironment, essential for cancer progression *in vivo*.

Ovarian tumors are known to polarize macrophages to display pro-tumorigenic characteristics in a NF- κ B-dependent manner [4, 20]. Classically activated or cytotoxic anti-tumorigenic macrophages (also called M1) and “alternatively” activated pro-tumorigenic macrophages (M2) represent two extremes in the spectrum of the macrophage phenotype [21]. This polarization is part of a complex interplay of signaling and responses between tumor cells and inflammatory cells such as macrophages, T cells and dendritic cells [22-24]. Despite current knowledge, substantial gaps remain regarding the specific influence of NF- κ B activation in the peritoneum during ovarian tumor dissemination.

Herein, we describe the generation of mouse ID8 ovarian cancer cells stably expressing a green fluorescent protein (GFP)/luciferase fusion product under the control of a synthetic NF- κ B-dependent promoter [25, 26]. These reporter cells allow the intra-vital mapping of NF- κ B activity during tumor progression in a syngeneic mouse model of ovarian cancer. Successful generation of this model provides unique insight into the role of NF- κ B activation in different phases of ovarian cancer progression, effects of modulating NF- κ B activity on host cell immune responses in the tumor microenvironment, and will serve as a powerful tool for pre-clinical testing of agents that target NF- κ B in ovarian cancer.

MATERIALS AND METHODS

Generation of ID8-NGL cells

Mouse epithelial ovarian cancer (ID8) cells are a well-established cell line derived from C57BL/6 mice that are routinely used in syngeneic mouse models of ovarian cancer [4, 11, 27, 28]. Here, we have generated ID8 cells stably expressing an NF- κ B-dependent reporter plasmid, termed ID8-NGL. To generate the NGL plasmid, four tandem copies of the 36-base enhancer from the 5' HIV-long terminal repeat, each containing two NF- κ B binding sites GGGACTTTCC, were cloned into the pEGFPluc vector backbone [25, 26] (Fig. 1A).

The NGL plasmid was transfected into ID8 cells according to manufacturer's instructions using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Clones with genomic incorporation of plasmid DNA were selected by growth in 10% fetal bovine serum (FBS)-supplemented DMEM High-Glucose medium (Invitrogen) with 400 μ g/ml G418 (Sigma Chemical Co., St Louis, MO). Clones were selected using an inverted light microscope and initially tested for NGL expression in luciferase assays. Further validation of ID8-NGL clones with basal luciferase activity greater than 1×10^6 RLU/mg protein was performed by bioluminescence imaging of cell cultures and Western Blot detection of GFP expression.

The ID8-NGL clone selected for *in vivo* experiments was cultured in 10% FBS-supplemented DMEM High-Glucose medium with 400 μ g/ml G418, and passaged by standard techniques. Parental ID8 cells and lung epithelial cells stably transfected with the NGL plasmid (A549-NGL), a kind gift of Dr. Timothy Blackwell (Vanderbilt University, Nashville, TN) [29], were used for comparison in these characterization studies. Finally, we confirmed that the ID8-NGL clone selected responded appropriately to classical activators of NF- κ B signaling, TNF- α and IL-1 β (R&D Systems, Minneapolis, MN), and the NF- κ B inhibitor, thymoquinone (Sigma).

Animal model and drug treatment

Wild-type C57BL/6 mice were injected intra-peritoneally (IP) with 1×10^7 ID8-NGL cells in 200 μ l PBS or mock-injected with an equal volume of sterile PBS. 21 gauge safety hypodermic needles (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for these injections. Tumor cells were allowed to grow in mice for up to 90 days before humane sacrifice. A subset of mice underwent bioluminescence imaging at defined time points. At 30 days after ID8-NGL injection, a subset of mice (5 per group) was randomly selected to receive thrice weekly 20 mg/kg thymoquinone or PBS vehicle by IP injection for 10 days [30]. No signs of drug toxicity were observed in the TQ-treated mice.

Tumor progression was also monitored by body weight and abdominal girth measurements. At time of sacrifice, abdominal ascites fluid was extracted with hypodermic syringe, and volume measured. If no measurable ascites was present, peritoneal lavages were performed by injecting 8 ml PBS intra-peritoneally and carefully extracting the fluid with a hypodermic syringe. Tumor implants in the peritoneal wall and the mesentery were harvested and snap frozen or formalin-fixed for further analysis. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

Luciferase assays

Luciferase activity was measured in harvested tumors following tissue homogenization in 1 ml passive lysis buffer, and in whole cell protein extracts from cells grown *in vitro*, using the Promega Luciferase Assay system (Madison, WI). Activity was analyzed using a GloMax Luminometer (Promega). Results were expressed as relative light units (RLU) normalized for protein content, as measured by the Bradford assay (Bio-Rad, Hercules, CA).

Bioluminescence imaging

Mice were anesthetized and luciferin (Biosynth AG, Rietlistr, Switzerland) administered by retro-orbital injection (1 mg/mouse in 100 µl isotonic saline). Mice were imaged using a Xenogen IVIS 2000 imaging device (Caliper Life Sciences., Hopkinton, MA). Light emission was detected by an ICCD camera and quantified using image processing Living Image v 2.5 software (Caliper Life Sciences). For bioluminescence of cell cultures, a final concentration of 150 µg/ml luciferin was added to cells just prior to imaging with the Xenogen IVIS 2000.

Analysis of ascites/peritoneal lavage fluid

Ascites or peritoneal lavage fluid was centrifuged at 1500 rpm for 5 minutes to separate cells from supernatant. Where applicable, red blood cells were lysed by ACK lysing buffer according to manufacturer's instructions (Invitrogen, Carlsbad, CA). An aliquot of cells were suspended in PBS with 1% BSA for total cell counts using a grid haemocytometer. Cells were then either snap-frozen for RNA extraction, or centrifuged onto microscope slides using a Thermo Cytospin II Cytocentrifuge (500 rpm for 10 minutes) for differential counts of inflammatory cells in hematoxylin and eosin-stained cells or immuofluorescence analysis.

RNA extraction and quantitative RT-PCR

RNA from ascites fluid or peritoneal lavages was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). DNase-treated samples were subjected to real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Steady-state mRNA levels of the M1 macrophage mark, CC chemokine ligand 3 (CCL3), and M2 macrophage mark, mannose receptor (mann-R) were expressed relative to corresponding GAPDH levels using the comparative $2^{\Delta\Delta Ct}$ method [31]. Relative expression

values were also normalized to levels of the epithelial marker cytokeratin-18 (CK18) to account for the epithelial (tumor) cell component of ascites or peritoneal lavage fluid. Primers sequences used were:

Cytokeratin-18 (CK18): forward: 5'-ACGGAGCTGAGACGCACCCT-3', reverse: 5'-GCCTCCACATCCCCGAGGCT-3';

CC chemokine ligand 3 (CCL3): forward: 5'-TGCCCTTGCTGTTCTTCTCT-3', reverse: 5'-GATGAATTGGCGTGGAATCT-3';

Mannose receptor (mann-R): forward: 5'-CAAGGAAGGTTGGCATTGT-3', reverse: 5'-CCTTTCAGTCCTTTGCAAGC-3';

GAPDH: forward: 5'-TGAGGACCAGGTTGTCTCCT-3', reverse: 5'-CCCTGTTGCTGTAGCCGTAT-3'.

Immunofluorescence/Immunohistochemistry

Processing, embedding and sectioning of formalin-fixed ID8-NGL tumor tissue, and hematoxylin and eosin staining for histology, were performed in The Allergy/Pulmonary & Critical Care Med Division Immunohistochemistry Core at Vanderbilt [32]. Immunofluorescent analysis of formalin-fixed paraffin-embedded tumor tissue was performed using standard deparaffinization and rehydration in 100%-90%-80%-70% ethanol. To reduce tissue autofluorescence, the samples were incubated in 0.5% Sudan Black in 70% ethanol for 10 min, 70% ethanol without Sudan Black for 10 min, and 50% ethanol for 2 min. The samples were permeabilized in 0.4% Triton X-100 for 10min and subjected to antigen retrieval by boiling in 10mM sodium citrate for 7 min. The following primary antibodies were used: rabbit polyclonal anti-Ki67/Mib-1 (Abcam, Cambridge, UK; 1:200 dilution), goat polyclonal anti-GFP (Abcam, 1:200), mouse monoclonal anti-pan-cytokeratin (Abcam, 1:100), and rabbit polyclonal anti-phospho-p65 (serine 276) (Santa Cruz Biotechnology, Dallas, TX, 1:100. Secondary antibodies

used were goat anti-rat or -rabbit or -mouse conjugated with Molecular Probes Alexa488 or Alexa594 (Invitrogen). After staining with secondary antibodies, the slides were washed 3 times with TBS-Tween20, then washed once in TBS and then stained with Molecular Probes TO-PRO-3 (Invitrogen) for 15 min (1 μ M in TBS). The slides were washed with TBS and the coverslips mounted using Molecular Probes ProlongGold antifade reagent (Invitrogen). Images were acquired using an LSM 510 Meta confocal microscope in the Vanderbilt University Medical Center Imaging Core.

For ascites fluid, macrophages and tumor cells were identified by immunofluorescent staining for F4/80 (AbD Serotec; Raleigh, North Carolina, 1:200) and pan-cytokeratin, respectively (Abcam, 1:1000) as described [32]. Secondary antibodies used were as described above. The percentage of F4/80-positive/CK-negative cells was counted in 3 separate fields at 40x using an immunofluorescent microscope. At least 200 cells were counted per sample.

Immunohistochemical analysis of PAX8 and F4/80 expression in tumor tissue was performed as described [32], using rabbit polyclonal anti-PAX8 (Proteintech, Chicago, IL, 1:50) and rat polyclonal anti-F4/80 (AbD Serotec; 1:50), respectively.

Western blotting

Whole cell protein isolation, subcellular fractionation, western blotting and signal detection and quantification were performed as described previously [33, 34]. Primary antibodies used were mouse monoclonal anti-GFP (Invitrogen), mouse monoclonal anti-phospho-I κ B (Cell Signaling Technology, Danvers, MA; 1:500), rabbit polyclonal anti-p65 (Cell Signaling Technology; 1:1000), mouse monoclonal anti-histone H3 (Millipore; 1:1000) and anti- β -actin (Sigma; 1:10000).

Statistical analysis

Unless otherwise indicated, values shown for *in vitro* experiments were the mean + SD of 3 independent experiments, with comparison of groups performed by 2-tailed Student's t test. Comparison of groups in *in vivo* experiments was performed by 2-tailed Mann-Whitney test. A p value < 0.05 is considered statistically significant. The relationship between BLI and indices of tumor burden was analyzed by Spearman correlation.

RESULTS

Effects of stimulation or inhibition of NF- κ B can be quantified in ID8 mouse ovarian cancer cells stably expressing the NF- κ B reporter (ID8-NGL). Mouse epithelial ovarian cancer (ID8) cells are a well-established cell line derived from C57BL/6 mice that are routinely used in syngeneic mouse models of ovarian cancer [4, 11, 27, 28]. Here, we generated ID8 cells stably expressing an NF- κ B-dependent reporter plasmid, termed ID8-NGL. The NGL plasmid expresses a green fluorescent protein (GFP)/luciferase fusion product under the control of a synthetic NF- κ B dependent promoter with a total of 8 NF- κ B binding sites [25, 26] (Fig. 1A). As expected in this tumor cell line, we confirmed detectable baseline levels of luciferase activity (Fig. 1B&C) and GFP expression (Fig. 1D) in ID8-NGL cells compared to parental ID8 cells. Lung tumor cells (A549-NGL) carrying the same reporter construct were used as a positive control.

To test the ability to measure NF- κ B responses, ID8-NGL cells were treated with known activators of NF- κ B, TNF- α and IL-1 β . Both activators stimulated a significant increase in luciferase activity in protein extracts compared to controls after 4 hours' treatment ($p < 0.01$, Student's t test) (Fig. 1E). Results were supported by bioluminescence imaging of cell monolayers (Fig. 1F). We then demonstrated the specificity of this effect by the abrogation of TNF- α -mediated stimulation of luciferase activity and GFP expression by 2 hours pre-treatment with the NF- κ B inhibitor, thymoquinone (TQ) (Figs. 1F&G). In addition, western blot analysis was consistent with the known ability of TQ to inhibit TNF- α induction of I κ B phosphorylation (Fig. 1F).

Intra-peritoneal injection of ID8-NGL cells produces ascites and peritoneal carcinomatosis in mice.

The parental ID8 cell line for the ID8-NGL cells has been shown to model the features of advanced serous ovarian cancer with the development of malignant, bloody ascites and peritoneal carcinomatosis

[27, 28, 35]. Here, we confirmed that ID8-NGL cells form a similar pattern of ascites and peritoneal spread in our syngeneic model. By 90 days after tumor injection, mice developed prominent abdominal distension indicative of ascites (Supplementary Fig. 1A). This was consistent with markedly increased body weight and abdominal girth observed in mice at this time point compared to PBS-injected controls (Supplementary Fig. 1B&C). Careful dissection of the peritoneal cavities revealed that tumor cell-injected mice displayed a reproducible pattern of tumor dissemination. As shown in Supplementary Fig. 1D tumor nodules were detected embedded in the peritoneal wall (yellow arrows), and in the mesentery of the intestines (white arrows). A representative tumor implant in the smooth muscle of the peritoneal wall is shown in Supplementary Fig. 1E.

Increased NF- κ B activity is detected during progressive ovarian cancer dissemination in the peritoneal cavity. First, we confirmed that mice injected with ID8-NGL cells displayed high levels of luminescence compared to PBS-injected mice by bioluminescence imaging. Representative images are shown in Fig. 2A. When region of interest analysis was performed to quantify abdominal luminescence, ID8-NGL-injected mice displayed approximately 100-fold higher levels than non-injected controls (average of $3.7 \pm 2.3 \times 10^7$ RLU over the 30, 60 and 90 day time points examined compared to $0.013 \pm 0.009 \times 10^7$ RLU, respectively, $p < 0.001$, Mann-Whitney test), indicating the specificity of the signal. NF- κ B reporter activity increased in a time-dependent manner (Fig. 2B), which showed correlation with indices of tumor burden such as mesenteric tumor mass and ascites volume at 90 days (Figs. 2C&D). To differentiate between specific increases in NF- κ B activity in tumor cells and an overall increase in NF- κ B signal due to increased tumor burden, we performed luciferase assays of snap-frozen tumor tissue. As shown in Fig. 2E, there was a specific increase in NF- κ B activity within tumor tissue (when corrected for cellular protein) in later phases of peritoneal tumor spread. We confirmed this observation

independently of the NF- κ B reporter by measuring levels of p65 in nuclear extracts from harvested mesenteric tumors. As shown in Fig. 2F&G, the highest levels of nuclear p65 expression were observed in 90 day tumors.

Peritoneal implants formed by ID8-NGL cells have characteristics representative of high-grade serous epithelial ovarian cancer. To determine if ID8-NGL tumors were representative of high-grade serous epithelial ovarian cancer, we first performed H&E staining and confirmed that tumors displayed the characteristic morphology of high-grade serous cancer (Fig. 3A). Furthermore, we stained tumors for expression of PAX8, an established marker of high-grade serous ovarian tumors [36]. We found strong nuclear immunoreactivity for PAX8 in epithelial tumor cells, but not in the stroma, as confirmed by staining for the epithelial marker, cytoplasmic pan-cytokeratin (Fig. 3B&C). Macrophage infiltration in stromal areas of the tumor was also demonstrated by staining with F4/80, a marker of mature macrophages (Fig. 3D).

We then performed immunofluorescent analyses of molecular markers of proliferation, NGL reporter expression, and NF- κ B signaling in the epithelial component of tumors. Staining for Ki67/mib-1 demonstrated that the tumors were highly proliferative (Fig. 3E). Activity of the NGL reporter in tumors was shown by detection of the GFP component of the fusion reporter protein (Fig. 3F), and NF- κ B activity in tumors was independently confirmed by analysis of nuclear expression of phosphorylated-p65 (serine 276) (Fig. 3G). We then demonstrated that phospho-p65 and Ki67 were co-expressed in the nuclei of a subset of tumor cells, but that there was minimal overlap between the two within the nuclei (Fig. 3H). This is consistent with the localization of Ki67 to ribosomal RNA-encoding nucleoli in proliferating cells [37].

Populations of monocytes and mature macrophages increase in association with tumor load within the peritoneal cavity. To assess the composition of peritoneal cell populations prior to the development of ascites and during early phases of peritoneal spread, we performed peritoneal lavages with sterile PBS to collect cells from injected mice or control non-injected mice. Cytospin counts of H&E-stained slides revealed large cell clusters without definable borders consistent with tumor cells in the peritoneal fluid (see arrows in Fig. 4A), which were excluded from our inflammatory cell counts. Mononuclear cells were the predominant inflammatory cell population present in the peritoneal cavities of both non-injected and injected mice. Furthermore, in injected mice mononuclear cells made up consistently 90% or greater of the inflammatory cells harvested in ascites fluid or in peritoneal lavages irrespective of duration of exposure to tumor cells (data not shown). The overall number of mononuclear cells harvested was elevated approximately 8-fold relative to non-injected controls at 90 days in the presence of tumor cells and with increasing duration of tumor spread (approximately 15-fold increase from the 30 day to 90 day time point in injected mice) (Fig. 4B). Since macrophages are a major subset of mononuclear cells, and given the established role of peritoneal macrophages in ovarian cancer progression [4], we also performed immunofluorescent analysis of ascites fluid to detect epithelial (tumor) cells and mature macrophages with antibodies against pan-cytokeratin and F4/80, respectively (Fig. 4C). These analyses confirmed that the tumor cells were a major component of ascites fluid at later time points (Fig. 4D), and that mature macrophages account for at least 60% of the non-epithelial cells present. Collectively, these results suggest an elevated inflammatory response is associated with the presence of tumor cells in the peritoneal cavity.

We also examined expression of markers of cytotoxic, anti-tumor M1 and pro-tumor M2 macrophages in RNA extracted from peritoneal lavage fluid or ascites. CC chemokine ligand 3 (CCL3) and mannose-receptor (mann-R) are well-established markers of M1 and M2 macrophages, respectively

[37]. Expression of mann-R, was significantly increased in injected mice compared to non-injected controls at both the 30 day and 90 day time points, with an approximately 20-fold increase at 90 days (Fig. 5A). There was a markedly smaller increase in CCL3 expression at 90 days in tumor-bearing mice (approximately 5-fold), with no significant difference observed at 30 days (Fig. 5B). Consistent with these observations, the ratio of mann-R to CCL3 expression was significantly higher in injected mice at both time points (Fig. 5C).

Reduced NF- κ B activity in tumors is associated with reduced expression levels of mannose-receptor in ascites fluid. To determine whether modulation of NF- κ B activity in tumors could alter the macrophage characteristics in ascites fluid, we treated ID8-NGL-injected mice with the NF- κ B inhibitor, thymoquinone (TQ). Initial validation experiments confirmed that there was a concentration-dependent reduction in NGL reporter activity in ID8-NGL treated with TQ *in vitro* (Fig. 6A). 30 days after ID8-NGL injection, mice were injected IP with TQ or PBS vehicle thrice weekly for 10 days. Because of the limited amount of macroscopic tumor observed at the time of sacrifice, we were unable to accurately quantify drug effects on tumor burden. Furthermore, as expected, no ascites was observed at this relatively early stage of tumor progression. We prioritized snap-freezing tumors for luciferase assays, and determined that TQ treatment reduced NGL reporter activity by approximately 25% in tumors in luciferase assays compared to vehicle-treated tumors (Fig. 6B).

Reduced NF- κ B activity with TQ treatment was associated with significantly reduced levels of the M2 macrophage marker mann-R ($48.3 \pm 5.1\%$ compared to vehicle, $p < 0.02$, Mann-Whitney test) (Fig. 6C) in peritoneal lavage fluid, reflected in a significant reduction in the mannR:CCL3 expression ratio (Fig. 6D). Therefore, this study provides evidence that macrophage populations may be shifted towards an “anti-tumor” M1 phenotype by reducing NF- κ B activity in tumor cells.

DISCUSSION

In this study, we have modified the ID8 syngeneic mouse model of ovarian cancer to allow intravital tracking of NF- κ B activity, known to play a critical role in development of ascites and peritoneal dissemination [4, 13-16], during tumor progression. The ID8-NGL reporter cells are an important tool for building upon our knowledge of the role of NF- κ B in ovarian cancer spread, including the link between NF- κ B activity in tumors and recruitment of host immune macrophages within the peritoneal cavity. The relationship between tumor progression and the immune system cannot be fully addressed by clinically useful xenograft models of human ovarian cancer, because those models require the use of immunocompromised mice. Therefore, the ID8-NGL immunocompetent model has potential clinical relevance because the development of ascites, peritoneal carcinomatosis and macrophage infiltrates recapitulate advanced human ovarian cancer [27, 28, 35].

Here, we show that ID8-NGL reporter cells show the appropriate response to NF- κ B activation and inhibition *in vitro*, and to the NF- κ B inhibitor, TQ, *in vivo*. We have also demonstrated that there was markedly increased NF- κ B activation in ID8-NGL cells during the later stage of tumor progression, which was associated with rapid ascites accumulation and tumor dissemination. Furthermore, NF- κ B activation was directly associated with cell proliferation in a subset of tumor cells *in vivo*. These results are consistent with previously published results [12, 39, 40]. We were unable to independently confirm our *in vivo* bioluminescence results by fluorescent imaging of ID8-NGL tumors, due to insufficiently strong GFP fluorescence of the fusion reporter protein. However, GFP expression was detectable by alternative techniques, such as western blot and indirect immunofluorescence, and these results were consistent with our bioluminescence and luciferase assay data.

While other groups have focused on immune cell infiltration into tumors [22-24], we studied the immune cell population in ascites and peritoneal lavage fluid. We found that macrophages dominate the

immune cell population in the peritoneal cavity, and the number of macrophages increases in parallel with the progression of ascites and peritoneal tumor dissemination. Furthermore, M2 and to a lesser extent M1 markers of tumor-associated and cytotoxic macrophage phenotypes, respectively, increase with peritoneal tumor spread in ovarian cancer. An area of ongoing investigation is to determine how activity of NF- κ B in tumor cells influences macrophages and macrophage phenotypes in promoting ovarian cancer spread. We believe that this ID8-NGL model serves as an important system for addressing this question. As proof-of-principle, we demonstrated that chemical reduction of NF- κ B activity in tumors was associated with reduced levels of the M2 macrophage marker, mannose-receptor, in ascites fluid. Although specific inflammatory cell populations were not dissected, these results provide evidence that macrophage populations may be shifted towards an “anti-tumor” M1 phenotype by reducing tumor NF- κ B activity with a systemic NF- κ B inhibitor. Furthermore, the ID8-NGL model could serve as a powerful tool for pre-clinical testing of agents that target NF- κ B, such as TQ, in future intervention studies.

We acknowledge that a limitation of our NF- κ B-linked reporter model is that bioluminescent imaging does not provide a direct measure of the total number of tumor cells, in contrast to imaging of tumors derived from ID8-Luc cells, which have constitutive stable expression of the luciferase reporter [4]. However, luciferase assays on harvested tumors where cellular content can be normalized confirmed strongly increased NF- κ B reporter activity at late-stage progression. This suggests that additional insight can be gained by comparison of live imaging with data from isolated tumor cell populations. Other non-invasive imaging modalities, performed in parallel to bioluminescence imaging, such as microPET/CT or intra-vital imaging of fluorescent dyes [35], are alternative options to match NF- κ B activity with tumor burden in the ID8-NGL model.

Conclusions: We have generated mouse ID8 ovarian cancer cells stably expressing the NGL NF- κ B reporter plasmid to track NF- κ B activity in a syngeneic mouse model. The strengths of this model are (i) the capacity to evaluate the role of NF- κ B activity in mediating the link between cancer progression and the immune system in ovarian cancer, and (ii) the ability to measure NF- κ B activity through a dual luciferase -GFP fusion protein, allowing multiple independent assays to confirm changes in NF- κ B activity in living animals and in harvested tumors. Additional applications for this model include pre-clinical development of drugs that target NF- κ B, such as NF- κ B inhibitors, in ovarian cancer.

ABBREVIATIONS

NF- κ B: Nuclear factor-kappaB; NGL: NF- κ B-GFP-Luciferase; WT: wild type

COMPETING INTERESTS

The authors disclose no competing interests.

AUTHORS' CONTRIBUTIONS

AJW characterized ID8 cells stably expressing the NGL reporter, oversaw the experiments, performed dissections and collection of ascites/peritoneal lavages, performed tumor cell injections, analyzed the data, and drafted the manuscript. LC was responsible for husbandry C57BL/6 mice, and performed bioluminescence imaging and cytopsin counts. WB was responsible for mouse husbandry, and performed bioluminescence imaging and cytopsin counts. JS maintained cell lines, performed bioluminescence imaging, and processed ascites/peritoneal lavage fluid for RNA and protein extraction. OT performed IHC and IF on tissue samples. HJL helped generate ID8-NGL cells. FY conceived the study, provided the NGL reporter and C57BL/6 mice, and consulted on experimental design. DK conceived the study, consulted on experimental design and data analysis, and drafted the manuscript. All of the authors have read and approved the final version.

ACKNOWLEDGMENTS

The Vanderbilt Small Animal Imaging Core, The Vanderbilt Imaging Core The Allergy/Pulmonary & Critical Care Med Division Immunohistochemistry Core. **Grant support:** DOD W81XWH-11-1-0509 (AJW); DOD W81XWH-09-1-0459, NIH R01CA113734 (FY); NIH K08CA148887 (DK)

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FIGURE LEGENDS

Figure 1. Characterization of ID8-NGL cells. (A) Schematic diagram of the pEGFP_{luc} NGL reporter plasmid. The plasmid contains 4 tandem copies of a 36 base pair enhancer of the HIV long terminal repeat, which contains 2 consensus NF- κ B binding sites (blue). Basal NF- κ B reporter activity in ID8-NGL cells compared to parental ID8 cells was measured in (B) luciferase assays from protein extracts and (C) by bioluminescence imaging of cell monolayers. (D) Western blot showing GFP expression in ID8-NGL cells not observed in parental ID8 cells. Actin was used as the loading control. (E) Stimulatory effect of a 4h treatment with TNF- α and IL-1 β (both 10 ng/ml) on luciferase activity in protein extracts from ID8-NGL cells. (F) Western blot showing the stimulatory effect of TNF- α on GFP and p-I κ B expression after 4h is inhibited by 2 hours' pre-treatment with the NF- κ B inhibitor, thymoquinone (50 μ M). Bioluminescent imaging is also shown. (G) Stimulatory effect of TNF- α on luciferase activity was also inhibited by thymoquinone (50 μ M). Values are mean+SD of 3 independent experiments. * $p < 0.01$ relative to Control, # $p < 0.01$ relative to TNF alone, both Student's t test.

Figure 2. NF- κ B reporter activity increases during ovarian cancer progression. (A) Representative BLI of WT mice injected with ID8-NGL cells or PBS (non-inject) over 90d following injection showing NF- κ B reporter signal in tumor cells. Quantification of BLI in the abdominal cavity is shown in (B). BLI was significantly correlated with (C) mesenteric tumor mass and (D) volume of ascites at 90d (Spearman). (E) Luciferase activity of the NF- κ B reporter was measured in harvested tumors and expressed relative to cellular protein. (F) Western blot analysis of p65 in nuclear extracts from harvested tumors. Equal loading was shown by probing for the nuclear-specific protein, histone H3. (G) Nuclear p65 expression relative to corresponding histone H3 levels was measured by densitometry. Values shown are mean + SD from 5 mice per group. ** $p < 0.01$ relative to 30d or 60d, Mann-Whitney test.

Figure 3. Molecular characterization of ID8-NGL tumors. (A) H&E stain of a representative area of ID8-NGL tumors. (B) Immunohistochemical detection of the marker of high-grade serous ovarian tumors, PAX8. (C) Fluorescent microscopy showing expression of the epithelial marker pan-cytokeratin (CK) expression relative to TO-PRO-3-stained nuclei. (D) Immunohistochemical detection of the mature macrophage marker, F4/80, in tumor sections. High power (x100) confocal images of the representative tumor section showing immunofluorescent detection of (E) the proliferation marker Ki67/mib-1, (F) the GFP reporter and (G) overlap of Ki67/mib-1 and phospho-p65 (serine 276) (red) in the epithelial component of the tumor. TO-PRO-3-stained nuclei are in blue. (H) Co-expression of phospho-p65 (red) and Ki67/mib-1 (green) is observed in the nuclei of a subset of cells. The panels on the right show merged images of the corresponding boxed areas for two representative nuclei.

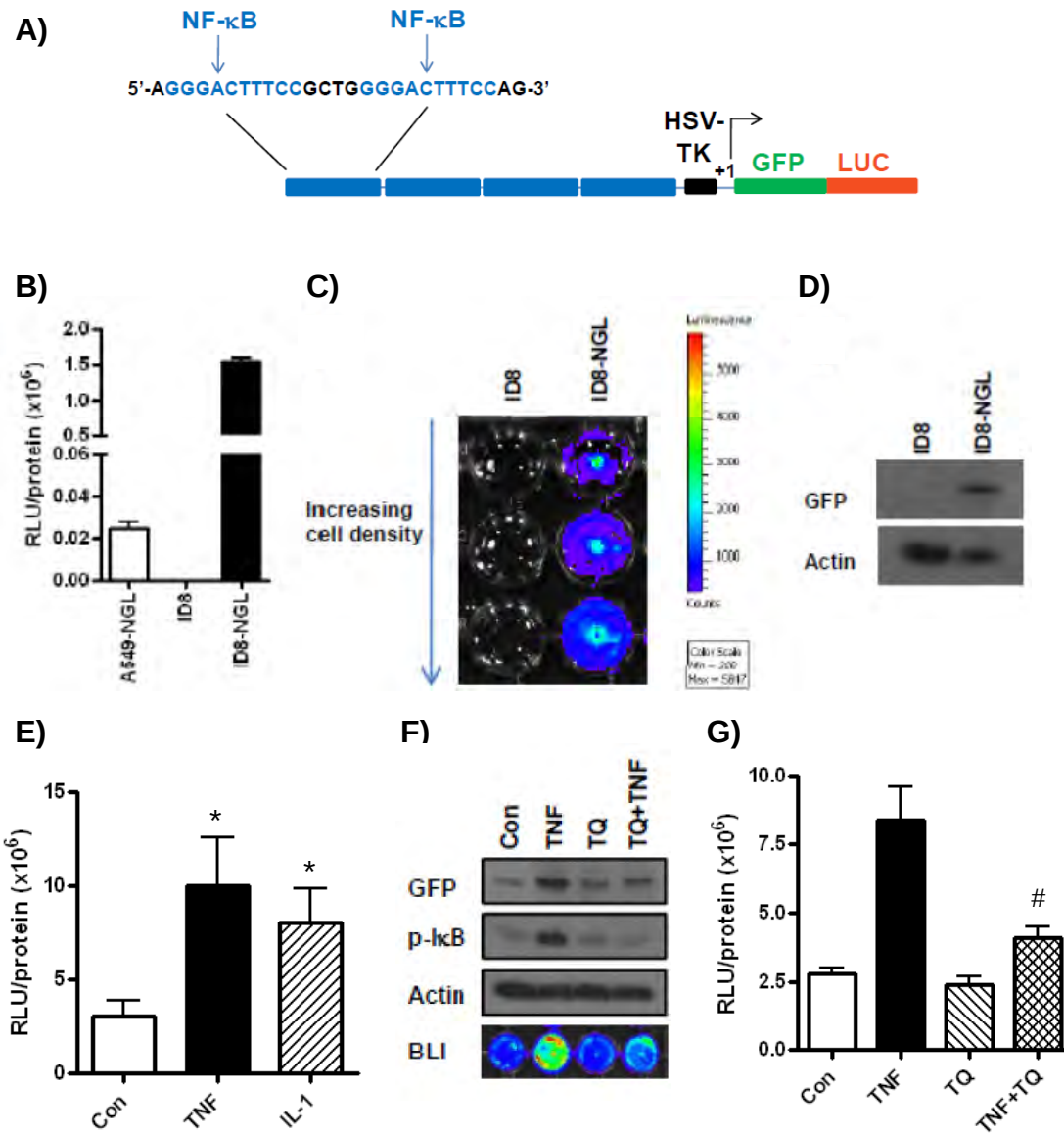
Figure 4. Analysis of fluid collected from the peritoneal cavity. (A) Representative images of cytopsin analyses of peritoneal lavage fluid/ascites collected from mice injected with tumor cells (Inject) or mock-PBS-injected mice (Non). Tumor cell clumps are shown (yellow arrow). (B) Overall number of mononuclear cells collected increased in mice with tumors and with duration of progression. (C) Expression of the epithelial marker, pan-cytokeratin (CK; red), and the mature macrophage marker, F4/80 (green), in ascites fluid on cytopsin slides. Percentage of CK-positive cells is shown in (D). Values are mean+SD of counts from 200 cells from each of 3 mice per group. * $p < 0.01$ relative to non-inject, Mann-Whitney test.

Figure 5. Elevation of M2 macrophage marker in tumor-bearing mice. QPCR analysis of the mRNA expression of the markers of (A) M2 macrophages, mannose-receptor (mann-R) and (B) M1

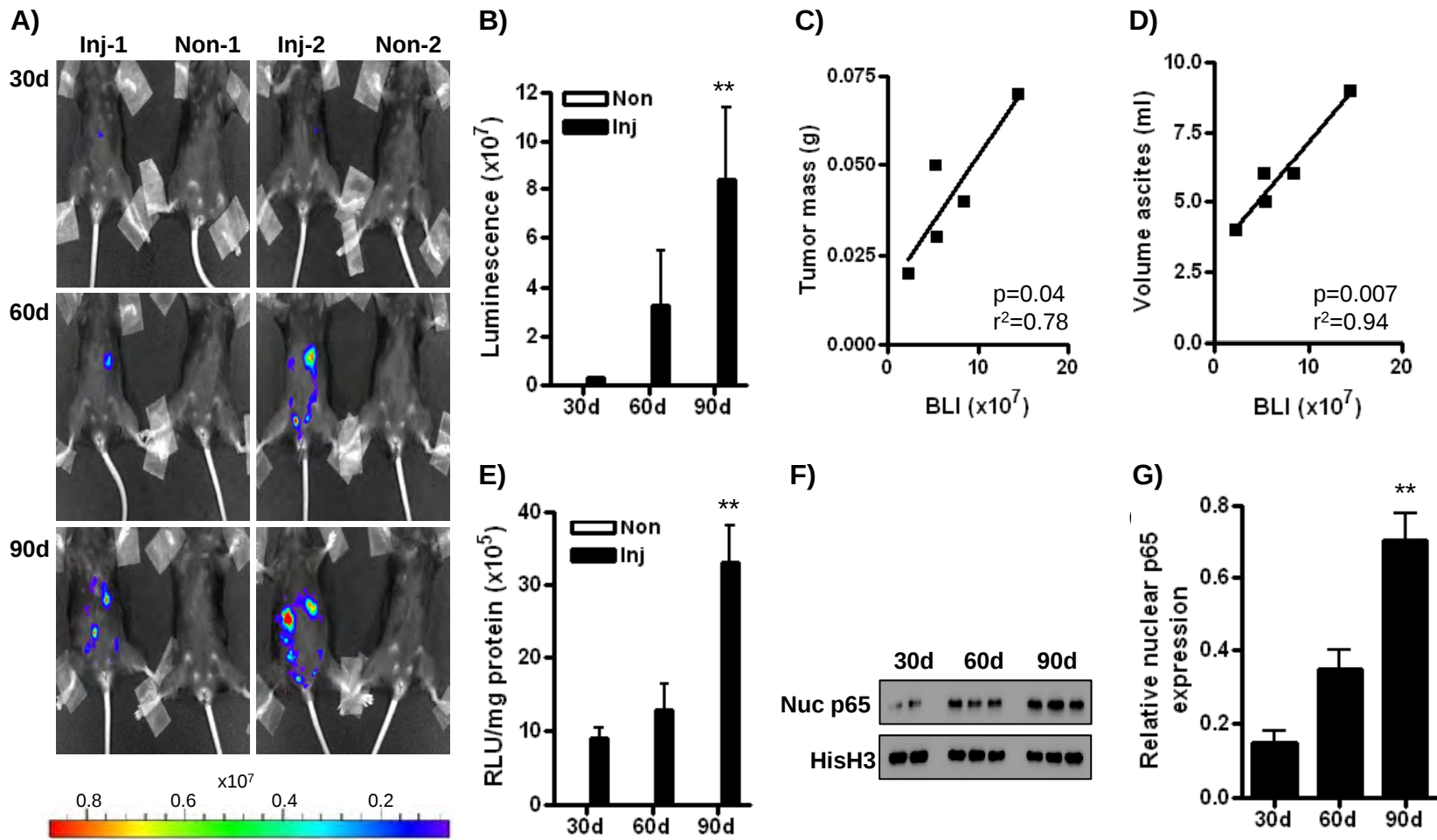
macrophages, CCL3, in RNA extracted from peritoneal lavages or ascites fluid. Values were normalized to corresponding levels of GAPDH mRNA expression and also CK18 mRNA levels, to account for epithelial content. The expression ratio of mann-R to CCL3 is shown in **(C)**. Values are mean+SD for 3 mice per group. * $p < 0.01$ relative to non-injected mice, Mann-Whitney test.

Figure 6. Thymoquinone reduces NF- κ B activity in tumors and expression of M2 macrophage marker in ascites fluid. **(A)** Effects of increasing concentrations of TQ on NF- κ B reporter activity in ID8-NGL cells after 24 hours' treatment as measured in luciferase assays. **(B)** Effect of 10 days treatment with 20 mg/kg TQ or PBS vehicle (thrice weekly IP injections) on NF- κ B reporter activity in harvested ID8-NGL tumors. **(C)** QPCR analysis of the mRNA expression of the markers of M2 macrophages, mannose-receptor (mann-R) and M1 macrophages, CCL3, in RNA extracted from peritoneal lavages. Values were normalized to corresponding levels of GAPDH mRNA expression and also CK18 mRNA levels, to account for epithelial content. The expression ratio of mann-R to CCL3 is shown in **(D)**. Values are mean+SD for 5 mice per group. * $p < 0.01$ relative to vehicle, Mann-Whitney test.

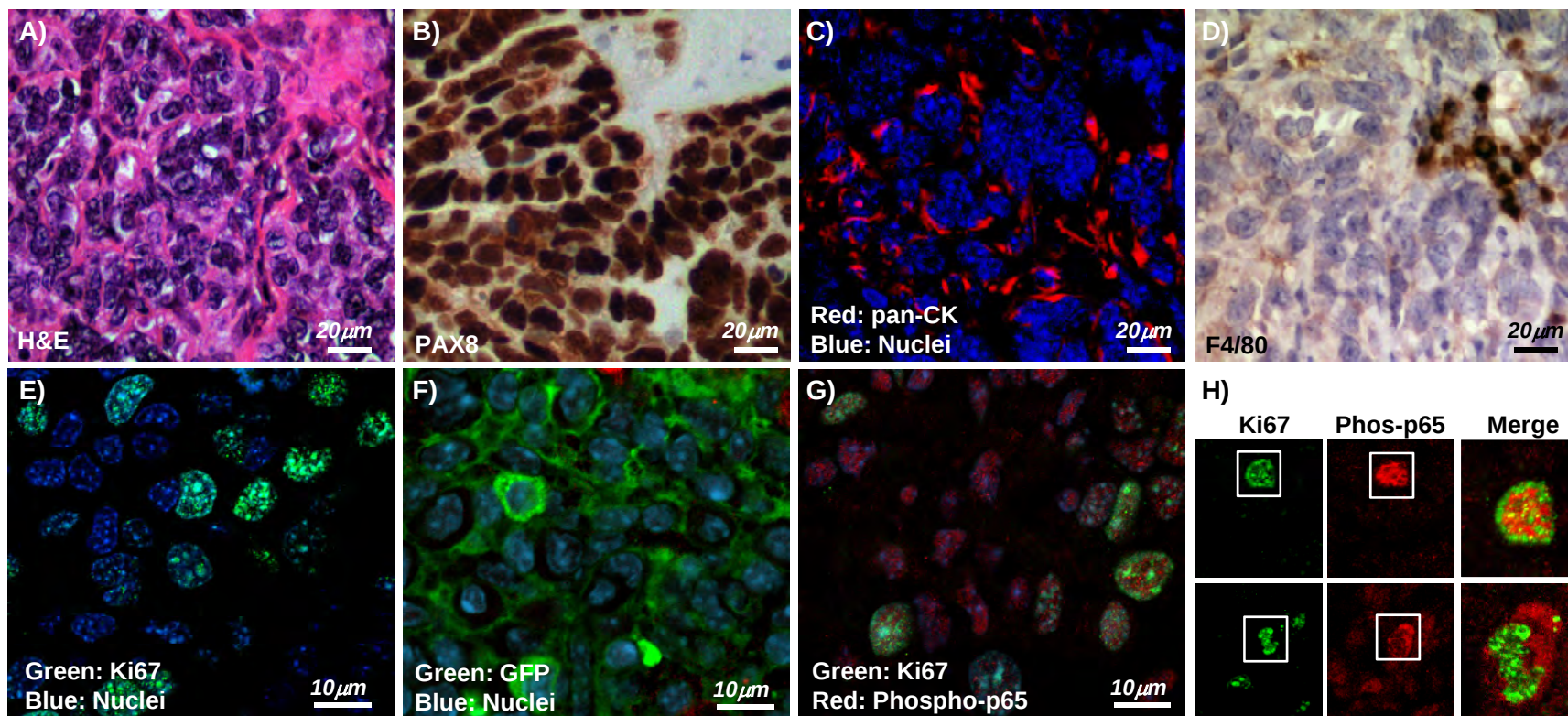
Wilson et al: Figure 1



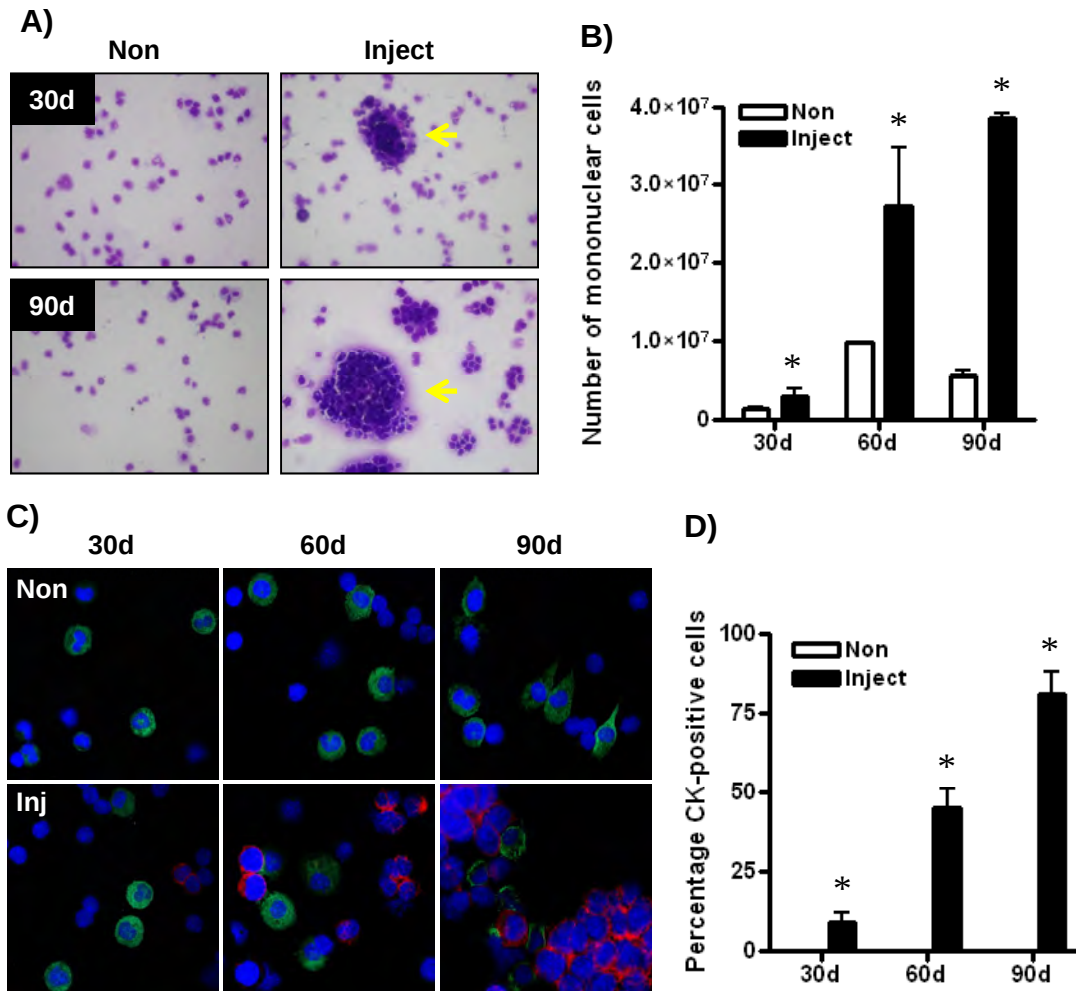
Wilson et al: Figure 2



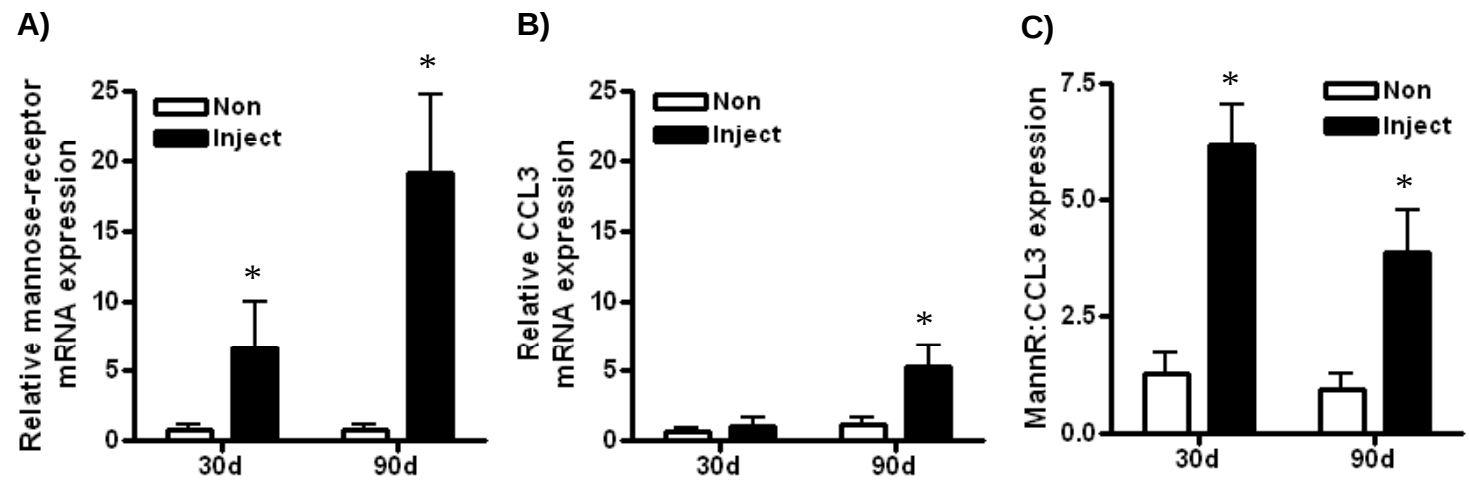
Wilson et al: Figure 3



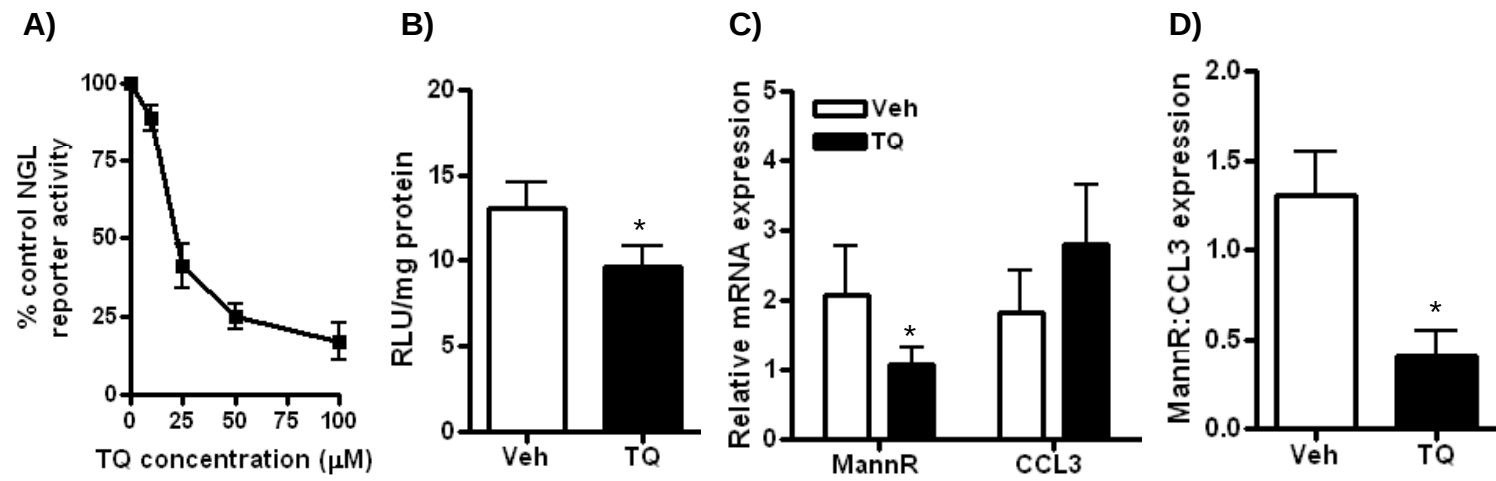
Wilson et al: Figure 4



Wilson et al: Figure 5



Wilson et al: Figure 6



Opposing effects of the NF-kappaB inhibitor thymoquinone in a syngeneic mouse model of ovarian cancer

Andrew J. Wilson*, Jeanette Saskowski, Whitney J Barham, Lianyi Chen, Dineo Khabele, Fiona Yull

Ovarian cancer is the most lethal gynecologic malignancy. Most women are diagnosed with advanced disease characterized by widespread peritoneal carcinomatosis and abdominal ascites. Activation of inflammatory processes via nuclear factor-kappa B (NF- κ B) is involved in ovarian cancer progression and linked to chemotherapy resistance. The potential of NF- κ B inhibitors to suppress tumor progression and sensitize tumor cells to platinum chemotherapy has led to their successful use in preclinical tumor models. One such inhibitor is thymoquinone (TQ), a component of black seed oil, widely used in traditional medicine. However, most NF- κ B inhibitors including bortezomib have had disappointing clinical results in the treatment of solid tumors. It is possible that NF- κ B inhibition in inflammatory cells such as macrophages in the peritoneal cavity may underlie the relatively poor efficacy and toxicity observed in patients. Therefore, this study aimed to determine the effects of TQ on ovarian cancer progression in the immunocompetent host. ID8 mouse ovarian cancer cells expressing the NF- κ B-dependent GFP/luciferase (NGL) fusion reporter transgene (ID8-NGL) were injected intraperitoneally (IP) into C57BL/6 mice. Reporter activity was visualized by bioluminescence imaging. TQ or PBS vehicle (VEH) were injected IP thrice weekly from day 30-60 after tumor cell injection. Mice were then sacrificed, ascites collected and tumors harvested. *In vitro* studies confirmed that TQ inhibited NF- κ B activity in ID8-NGL cells, inhibited cell growth, and exerted co-operative inhibitory effects with cisplatin on cell growth. Consistent with the observed *in vitro* growth inhibition, TQ induced approximately 30% reduction in tumor cells expressing the proliferation marker Ki67 compared to VEH tumors *in vivo*. However, there was no overall difference in tumor burden (peritoneal and mesenteric tumor nodules). Strikingly, the volume of ascites collected from TQ mice was 5-fold higher than VEH mice, and cytospin analysis of ascites showed that TQ induced a 4-fold increase in the number of macrophages, suggesting an elevated inflammatory response. TQ also increased NF- κ B reporter activity by 50% compared to levels in VEH mice. These data suggest that TQ exerts both anti- and pro-tumorigenic effects, likely mediated through NF- κ B inhibition in tumor cells and host inflammatory cells, respectively. However, the net effect of treatment is deleterious (increased ascites). These results caution that treating ovarian cancer patients with systemic NF- κ B inhibitors may have unanticipated adverse effects, and that a greater understanding of the effects of NF- κ B inhibition is necessary before performing combination studies with platinum agents such as cisplatin.