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

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<b>14. ABSTRACT</b> 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 defined patterns of NF-κB activity in (i) ID8 tumor cells stably expressing the NGL NF-κB reporter plasmid, and (ii) in host cells in ID8-injected NGL reporter mice. We showed increased NF-κB reporter activity in tumor cells and in host macrophages during progression, and increased markers of M2 macrophages in ascites fluid. Reducing NF-κB activity in tumor cells with TQ treatment elevated expression of M1 macrophage markers, while longer-term TQ treatment lead to increased ascites, elevated NF-κB signaling and elevated expression of M2 markers. 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, reduced M2 and induced M1 macrophage markers, and decreased levels of known pro-tumorigenic cytokines in ascites fluid.					
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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- $\kappa$ B (NF- $\kappa$ B) signaling pathway is known to play an important role in several malignancies, including ovarian cancer (3-8). Constitutive activation of NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ B inhibitors with current platinum-based regimens. Equally relevant, but far less understood are the potential effects of NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ B inhibition (12). **However, major gaps in knowledge still remain regarding the specific influence of NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ B reporter transgenic mice expressing a green fluorescent protein (GFP)/luciferase fusion product under the control of a synthetic NF- $\kappa$ B-dependent promoter (13, 14), allowing intra-vital mapping of NF- $\kappa$ B activity in the host during tumor development. In a complementary approach, ID8 cells stably transfected with the NF- $\kappa$ B reporter will be monitored in wild-type mice to study measure NF- $\kappa$ B activity in the developing tumor. Completion of the proposed work will not only provide a unique insight into the role of NF- $\kappa$ B in ovarian cancer progression, but also provide a powerful tool for the preclinical testing of NF- $\kappa$ 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- $\kappa$ B activation in both malignant ovarian epithelial cells and host macrophages in the peritoneal microenvironment.* Our ***objectives*** are (1) defining the pattern of NF- $\kappa$ B activity in the host-tumor microenvironment during ovarian cancer progression and (2) evaluating consequences of inhibiting NF- $\kappa$ B activity in tumor and host cells during ovarian cancer progression via treatment with NF- $\kappa$ B inhibitors alone and in combination with cisplatin chemotherapy.

## BODY

### **Task 1. Define the pattern of NF- $\kappa$ B activity in the host-tumor microenvironment during ovarian cancer progression.**

#### ***Sub-task 1a.***

We have published our observations characterizing the ID8 mouse ovarian cancer cells stably transfected with the NGL reporter (ID8-NGL), including response to TNF- $\alpha$  and/or IL-1 $\beta$  stimulation by increased NF- $\kappa$ B reporter activity [Figure 1 in ref (15)].

#### ***Sub-task 1b.***

We confirmed that bone marrow-derived macrophages harvested from NGL reporter mice, designated BMDM-NGL, also respond to TNF- $\alpha$  stimulation with approximately 2-fold increase in NF- $\kappa$ B reporter activity in these cells (Fig 1B). This result provides supporting evidence for our Task 1d studies by demonstrating a robust response of cells derived from NGL reporter mice to NF- $\kappa$ B stimulation.

#### ***Sub-task 1c.***

We have published our data showing that ID8-NGL cells form reproducible tumors and irreversible ascites in C57BL/6 mice over a period of up to 90 days, and that bioluminescence imaging (BLI) and luciferase assay analysis of the NGL reporter reliably tracks NF- $\kappa$ B activity in tumors, which increases in a time-dependent manner during progression. Besides measuring NF- $\kappa$ B reporter activity, we validated two independent measures of NF- $\kappa$ B function in tumors: western blot analysis of nuclear p65 expression and immunofluorescence (IF) analysis of phosphorylated p65 expression. In addition, we validated other assays measuring multiple other endpoints which will be used in Tasks 2&3: indices of tumor burden, expression of the GFP reporter and proliferation marker Ki67 in tumors, and analysis of ascites fluid to define specific cell populations through differential morphology cell counts, and quantitative real-time PCR (QPCR) and IF analysis of macrophage markers associated with anti-tumor (M1) or pro-tumor functions (M2). These results are presented in Figures 2-5 of ref (15).

#### ***Sub-task 1d.***

Our second syngeneic model utilized NGL transgenic reporter mice, which express the NF- $\kappa$ B reporter in every cell of the body, injected with wild-type ID8 cells.

We were unable to overcome the technical limitations of measuring NF- $\kappa$ B activity in host cells by BLI, which we described in previous Annual Reports. As shown in Fig 2, we detected decreased detection of NGL reporter activity at later time points following injection of ID8 cells, for either raw abdominal luminescence or when abdominal luminescence was normalized to corresponding brain signal. In contrast to ID8-NGL tumors, which form a macroscopic solid mass, host cells in the tumor microenvironment will be present as single cells or potentially small clusters of cells. The signal may also be “damped” by the presence of ascites fluid, which is a feature of our syngeneic model. Therefore, the sensitivity of detection of reporter activity in individual cells in the peritoneal cavity is likely to be below the limits of the in vivo imaging device used.

We dissected the host inflammatory cell component of ascites and peritoneal lavage fluids from ID8-injected and mock (PBS)-injected mice, respectively, following isolation of macrophages by differential adhesion (Fig 3A). We performed luciferase assays on the isolated adherent cell population, and showed that there was (i) increased NF- $\kappa$ B reporter activity in macrophages harvested from tumor-bearing mice compared to mock-

injected mice and (ii) NF- $\kappa$ 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. 3B).

Our attempts to analyze host cell populations by flow cytometry, as originally intended, were not successful, as we were unable to overcome the prohibitive background fluorescence present in ascites samples.

### ***Sub-task 1e.***

We performed an 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 (16).

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. 4A-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. 4D&E).

***Milestone #1:*** We have published results from experiments undertaken in Sub-tasks 1a and 1c. These studies demonstrated a marked increase in tumor NF- $\kappa$ B activity during late stages of tumorigenesis, validated various methods of analyzing tumor cells and peritoneal M2-like and M1-like macrophage populations from ascites fluid or peritoneal lavages, and established the robustness of our ID8-NGL cells as a model for monitoring NF- $\kappa$ B activity in tumor cells following drug treatment (Tasks 2 and 3). We also established the complementary model of growing ID8 cells in NGL reporter mice in order to track NF- $\kappa$ B activity in host cells during ovarian cancer progression. We showed increased NF- $\kappa$ B reporter activity in host macrophages during ovarian cancer progression, and that clodronate-sensitive macrophages play a role in ovarian cancer progression.

## **Task 2. To determine the consequences of reducing NF- $\kappa$ B response in tumor and host cells by treatment with NF- $\kappa$ B inhibitors.**

### ***Sub-task 2a.***

Effects of the NF- $\kappa$ B inhibitor thymoquinone (TQ) in cells cultured *in vitro* are summarized in Fig 5. We have validated that short-term treatment with TQ decreased the stimulatory effects of TNF- $\alpha$  on NF- $\kappa$ B reporter activity in ID8-NGL (Fig. 5A&B) and BMDM-NGL cells (Fig. 5C). When cells were treated for longer periods with TQ, we observed a concentration-dependent reduction in NF- $\kappa$ B reporter activity after 24h (Fig. 5D) and cell growth after 72h (Fig. 5E), and induction of cell apoptosis (Fig. 5E). A portion of these data were presented in Figure 1 in ref (15).

### ***Sub-task 2b.***

In order to determine the effects of NF- $\kappa$ B inhibition on tumor NF- $\kappa$ B activity and on ovarian cancer progression, we compared the response of ID8-NGL tumor-bearing mice to TQ and vehicle-only treatment. 30 days after tumor injection, mice were randomized to vehicle and TQ-treated groups and treated for 30 days before sacrifice at day 70, as outlined in Fig 6A.

Treatment of tumors with TQ produced several unanticipated results.

First, TQ treatment resulted in significantly greater ascites volume at sacrifice (Fig. 6B&C). 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. 6D&E). 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).

Second, levels of NF- $\kappa$ B reporter activity in tumor cells from TQ-treated mice measured *in vivo* were significantly higher compared to vehicle-treated mice (Fig. 7A). We confirmed increased NF- $\kappa$ B reporter activity in TQ-treated tumors by luciferase activity assays in snap-frozen tumors harvested at sacrifice (Fig 7B). Representative BLI images are shown in Fig 7C. Up-regulated NF- $\kappa$ B activity in tumors was also demonstrated by western blot detection of nuclear p-65 expression in tumors harvested from TQ-treated mice (Fig. 8A&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 reduction in the percentage of tumor cells positive for the proliferation mark, Ki67/mib-1 (Fig. 9A&B), and small increase in apoptosis (Fig. 10A&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 9A). However, the proportion of Ki67-positive cells also expressing phosphorylated p65 was significantly increased in TQ-treated tumors compared to vehicle (Fig. 9C), 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 11A). In contrast, the overall number of macrophages harvested increased significantly with TQ treatment (Fig 11B), 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- $\kappa$ B reporter in cell pellets from ascites fluid. As shown in Fig 11C, there was increased NF- $\kappa$ 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. 12A). In contrast, 30 day treatment with TQ resulted in upregulation of NF- $\kappa$ B reporter activity (Fig. 12A). NF- $\kappa$ B reporter activity data for the 10 day time point only were presented in Figure 6 in ref (15).

Reduced NF- $\kappa$ 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. 12B). Therefore, macrophage populations may be shifted towards an “anti-tumor” M1 phenotype by reducing NF- $\kappa$ B activity in tumor cells. In contrast, elevated NF- $\kappa$ 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. 12B). 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. 12C). NGL reporter and QPCR data for mann-R and CCL3 expression in macrophages for the 10 day time point only were presented in Figure 6 in ref (15).

### ***Sub-task 2c.***

We also performed an experiment evaluating the effects of TQ on NF- $\kappa$ 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. BLI was performed at baseline, 30d, and 60d in a subset of the mice (Fig 13A). Minimal overall changes were observed between vehicle and TQ-treated groups when NF- $\kappa$ B reporter activity was quantified (Fig 13B). In contrast to the experiment described in Task

1d, there was no significant reduction in NF- $\kappa$ 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 by differential adhesion. Luciferase assays on the isolated macrophage population showed that TQ treatment induced an approximately 2-fold overall increase in NF- $\kappa$ B reporter activity (Fig. 14A), accompanied by significant increases in mRNA expression of both M2 mann-R and IL-10 macrophage marks and the M1 CCL3 mark (Fig. 14B). Consistent with the results from our ID8-NGL cells, ascites volume at sacrifice was significantly higher with TQ treatment (Fig 14C), but the number of peritoneal implants and mesenteric tumor mass were not significantly different between vehicle and TQ-treated mice (Fig 14D&E).

It is unclear at present if NF- $\kappa$ B activity is elevated in M2 or M1 populations or both. A possible immunological mechanism for promotion of tumor progression through systemic NF- $\kappa$ B inhibition is that anti-tumor M1 cytotoxic macrophages may require NF- $\kappa$ B signaling for normal function, and NF- $\kappa$ B inhibitors impair their ability to efficiently target tumor cells (17). However, this will remain speculative until NF- $\kappa$ B activity can be tracked in specific M2 and M1 macrophage populations. Like other NF- $\kappa$ B inhibitors utilized clinically, such as curcumin and bortezomib, TQ is not a specific inhibitor of NF- $\kappa$ 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- $\kappa$ B inhibitor bortezomib has pro-inflammatory effects and promotes tumor progression (18). In contrast, short exposure to bortezomib produces the anticipated effects of inhibiting tumor cell growth in that study.

**Milestone #2:** Results of a portion of these studies have been published (15), and a majority of the remaining studies from Task 2 have been presented in scientific meetings and will form part of a manuscript that will be submitted to *Cancer Biology and Therapy* (see attached). Our results indicate that TQ has more complex effects *in vivo* than *in vitro*. There were contrasting effects of short-term (10 day) and longer-term (30 days) exposure of tumor-injected mice to TQ. Compared to vehicle, short-term treatment reduced NF- $\kappa$ B reporter activity in tumors accompanied by increased expression of the M1-like macrophage marker CCL3, suggestive of anti-tumor activity. Longer-term TQ treatment induced modest overall anti-proliferative and pro-apoptotic effects in tumors, consistent with results in cultured cells. However, there was markedly increased ascites formation with long-term TQ treatment in tumor-injected mice accompanied by increased overall NF- $\kappa$ B activity in both tumors and peritoneal macrophages, with no overall difference in tumor burden. Moreover, TQ induced a dramatic increase in expression of M2-like macrophage markers isolated from ascites fluid. Increased ascites in response to prolonged exposure to systemic NF- $\kappa$ B inhibitors has high potential relevance to the clinic, since select NF- $\kappa$ B inhibitors are being used as mono-therapy and in combination with other chemotherapeutic drugs in clinical trials in ovarian cancer patients. The interaction between TQ and established cisplatin chemotherapy was dissected in our Task 3 studies.

**Task 3. To evaluate NF- $\kappa$ B activity in host and tumor cells and efficacy of treatment with NF- $\kappa$ B inhibitors in combination with cisplatin).**

***Sub-task 3a.***

Effects of TQ in combination with cisplatin in ID8-NGL cells cultured *in vitro* are summarized in Fig 15. In sulforhodamine B (SRB) cytotoxicity assays, we showed that TQ and cisplatin act synergistically to reduce cell growth through isobologram analysis (19), as evidenced by a Combination Index significantly less than 1 at Effective Dose (ED)50, ED75 and ED90 (Fig. 15A&B). Moreover, the pro-apoptotic effects of cisplatin are markedly enhanced by combination TQ treatment (Fig. 14C), indicating apoptosis induction is an important mechanism of action of the anti-tumor effects of the combined drugs. Consistent with our previous observations (15), TQ reduced NF- $\kappa$ B reporter activity by approximately 50% in luciferase assays after 24 hour treatment.

Cisplatin induced a modest, but statistically significant, increase in NF- $\kappa$ B reporter activity ( $20.1 \pm 2.7\%$ ,  $p = 0.045$ ) (Fig 15D), which was reduced to levels comparable to TQ alone by combining TQ and cisplatin. Collectively, these results suggest a link between NF- $\kappa$ B inhibition and enhanced response to cisplatin in cultured cells.

### ***Sub-task 3b.***

In these studies, we wished to determine the effects of combined TQ and cisplatin treatment on tumor burden, tumor NF-B activity and on host macrophage profiles. Mice injected with ID8-NGL cells were treated with vehicle, TQ (20 mg/kg daily), cisplatin (2 mg/kg weekly) or the TQ/cisplatin combination from day 30-60 following tumor cell injection, and then sacrificed. As shown in Fig. 16A-C, cisplatin alone reduced established indices of tumor burden in mice with intraperitoneal tumors, volume of ascites, number of peritoneal implants and mesenteric tumor mass (15), by  $>80\%$  compared to vehicle-treated mice. Combining TQ and cisplatin resulted in enhanced reduction in peritoneal implants and mesenteric tumors compared to either drug alone, with a similar effect on ascites volume that just failed to reach statistical significance (Fig 16A-C). Surprisingly, treatment with TQ alone induced a 2-fold increase in ascites volume, with no overall effect on peritoneal or mesenteric tumors. We have confirmed these observations for TQ only treatment in multiple mouse cohorts.

Immunofluorescence analysis of tumors demonstrated that, consistent with effects on tumor burden, cisplatin reduced the percentage of cells positive for the proliferation marker, Ki67/mib-1 by  $83 \pm 6\%$  and increased cells showing expression of the apoptosis marker cleaved caspase-3, apoptosis (Fig. 16D-E). Compared to cisplatin or TQ treatment alone, the TQ/cisplatin combination significantly greater levels of apoptosis (Fig. 16D-E), consistent with our in vitro results.

Contrasting drug effects on NF- $\kappa$ B reporter activity were observed in harvested ID8-NGL tumors compared to cultured cells. As shown in Fig 17A, treatment with TQ unexpectedly led to an overall increase in NF- $\kappa$ B reporter activity in luciferase assays. However, combining TQ and cisplatin abrogated TQ-stimulation, leading to overall similar levels compared to vehicle and cisplatin alone (Fig 17A). Supporting our results, QPCR analysis of steady-state mRNA levels of the established NF- $\kappa$ B targets, TNF- $\alpha$  and IL-1 $\beta$ , in drug-treated tumors revealed a similar pattern of effect to the luciferase assays (Fig 17B). We also determined TNF- $\alpha$  and IL-1 $\beta$  mRNA expression in macrophages isolated from ascites fluid using a differential adhesion method (Fig 17C). Expression of both genes was robustly stimulated by TQ treatment, an increase that was completely abrogated in the TQ/cisplatin combination. Although cisplatin alone significantly reduced TNF- $\alpha$  mRNA levels, overall expression levels of both genes were similar following treatment with combined TQ/cisplatin and vehicle treatment. Combined with the tumor data, our results suggest that in contrast to cultured cells, enhanced anti-tumor effects of TQ/cisplatin compared to vehicle or cisplatin alone were not mediated by reduced NF- $\kappa$ B activity in tumors or macrophages.

We have previously demonstrated extensive macrophage infiltration into intraperitoneal tumors derived from ID8-NGL cells (15). In order to assess whether drug effects on tumor burden were reflected in changes in macrophage infiltration and/or macrophage populations, we measured expression of the well-established macrophage marker, F4/80, and the marker of M2-like macrophages, arginase-1, in IF assays. Representative images are shown in Fig 18A. As shown in Fig 18B, the TQ/cisplatin combination significantly reduced the percentage of cells staining positive for arginase-1 compared to vehicle, TQ and cisplatin alone. In contrast, overall macrophage infiltration, measured by the percentage of F4/80-positive cells, was not changed by cisplatin alone or in combination with TQ, but was markedly increased by TQ treatment. These results indicate that the TQ/cisplatin combination induced changes in the tumor microenvironment prohibitive for tumor progression.

We have established that the enhanced response to TQ/cisplatin in our syngeneic model was not mediated by a TQ-mediated reduction in NF- $\kappa$ B activity in tumors, but was potentially mediated by changes in the local tumor

microenvironment. In order to more completely understand drug effects on the tumor microenvironment, particularly the role of peritoneal macrophages, we analyzed the cell and soluble components of ascites fluid.

We have previously shown that mononuclear cells, particularly macrophages, are the predominant inflammatory cell population in the peritoneal cavity of ID8-NGL tumor-bearing mice (15). In harvested ascites or peritoneal lavage fluid from drug-treated mice, we first performed morphological analysis of cytopsin slides and demonstrated an overall decrease in mononuclear cells with cisplatin treatment compared to vehicle (Fig 19A). TQ alone markedly increased mononuclear cell number, consistent with an elevated inflammatory response, which was decreased by the TQ/cisplatin combination to levels comparable to those of cisplatin treatment alone (Fig 19A).

Further dissection of the specific macrophage populations present in ascites fluid was performed. First, QPCR analysis showed that cisplatin significantly reduced expression of established markers of pro-tumorigenic M2-like macrophages (mannose-receptor and IL-10) and increased expression of an anti-tumorigenic M1-like mark, CCL3 (Fig. 19C). The TQ/cisplatin combination also led to significant reduction of the stimulatory effects of TQ alone on the M2 marks (Fig. 19B) 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). Second, immunofluorescence analysis of the established M2 marker, arginase-1, in isolated F4/80-positive macrophages demonstrated a similar induction of expression by TQ alone expression to mannose-receptor or IL-10 above (Fig 19D). Cisplatin significantly reduced the number of arginase-1/F4/80 double positive cells alone and when combined with TQ.

We also analyzed the cytokine profile of ascites fluid harvested from drug-treated mice using a commercially available cytokine array plate (Signosis Inc.). As shown in Fig 20A, there was evidence of co-ordinated regulation of cytokines Both cisplatin- and TQ/cisplatin-treated mice displayed up-regulation of multiple cytokines with known anti-tumorigenic functions (CCL3, TNF- $\alpha$ , CCL5, IL-17a) and down-regulation of pro-tumorigenic cytokines (IL-10, MCP-1) [20,21] and growth factors linked to angiogenesis, increased vascular permeability and/or epithelial-mesenchymal transition (VEGF, IGF-1, FGF-2) [21-23]. Finally, in ELISA assays of ascites fluid, we confirmed that the TQ/cisplatin significantly reduced levels of VEGF compared to cisplatin alone and vehicle and completely abrogated the stimulatory effect of TQ (Fig 20B).

### ***Sub-task 3c.***

Because of the technical issues involved with bioluminescence imaging of the NGL reporter mice, we prioritized studies where ID8-NGL cells were injected into wild type mice, and have not yet completed the NGL reporter mice studies..

***Milestone #3:*** Results of Task 3 studies form a large part of a manuscript that will be submitted to the *Cancer Biology and Therapy* (see attached). 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, reduced M2 and induced M1 macrophage markers, and reduced levels of known pro-tumorigenic cytokines/growth factors. However, the mechanism of enhanced cytotoxicity of the TQ/cisplatin combination differed between cultured cells and tumors. NF- $\kappa$ B inhibition by TQ was associated with increased apoptosis in cultured ID8-NGL cells. In contrast, enhanced anti-tumor effects of the combination *in vivo* were not associated with a reduction of NF- $\kappa$ B activity in tumors or macrophages compared to vehicle or cisplatin alone. 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- $\kappa$ 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- $\kappa$ B inhibitors and emphasizes the need for therapy directly targeting NF- $\kappa$ 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.

## KEY RESEARCH ACCOMPLISHMENTS

Through the duration of this completed grant:

- 1) We further strengthened the collaborative efforts of the Khabele/Wilson and Yull laboratories. We will continue to have regular joint lab meetings for data sharing and interpretation. Our collaborative studies have provided a framework for grant applications exploring these exciting new directions in ovarian cancer therapy.
- 2) We published the results of our Sub-task 1a, 1c and 2b studies (15), presented our research at national (American Association for Cancer Research) and internal Vanderbilt Research Conferences, and will submit a manuscript to the Journal of Gynecologic Oncology (see attached).
- 3) We successfully characterized two complementary syngeneic mouse models of ovarian cancer allowing tracking of NF- $\kappa$ B activity in developing ovarian tumors, and in host cells in the peritoneal cavity during progression of ovarian tumors, through the NGL reporter.
- 4) We have shown that NF- $\kappa$ B activity is elevated in ovarian cancer cells and in host peritoneal macrophages, particularly in the late stages of progression.
- 5) We have identified contrasting effects between short-term (anti-tumorigenic) and longer-term (pro-tumorigenic, leading to increased ascites) exposure to TQ in tumor-bearing mice, which has high relevance to the clinic where systemic NF- $\kappa$ B inhibitors are in clinical trials as mono-therapy and in combination with established chemotherapy. These results urge caution for the use of systemic NF- $\kappa$ B inhibitors in patients and may underlie in part why NF- $\kappa$ B inhibitors have limited efficacy as mono-therapy and high toxicity in some patients.
- 6) We showed 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, reduced M2 and induced M1 macrophage markers, and reduced levels of known pro-tumorigenic cytokines/growth factors, such as VEGF.
- 7) We demonstrated that the macrophage-depleting drug clodronate reduced tumor burden, suggesting an important role for resident peritoneal macrophages in ovarian cancer progression.
- 8) We validated multiple new end-points for molecular and cellular analysis of tumor progression and drug effects that will be used for later studies.
- 9) Finally, we determined that BLI analysis of the peritoneal cavity was unsuccessful in the NGL reporter mice injected with ID8 cells primarily due to insufficient resolution to detect luminescence from single cells or small aggregates and/or damping effect of ascites on the luminescent signal, and instead measured NF- $\kappa$ B reporter activity in macrophages isolated from ascites fluid.

## REPORTABLE OUTCOMES

### *Manuscripts, Abstracts, Presentations*

1. **AJ Wilson**, H-J Lee, WJ Barham, L Chen, H Onishko, D Khabele, F Yull. Investigating the patterns of nuclear factor-kappa B activity in the host-tumor microenvironment during ovarian cancer progression. **Poster presentation** at the American Association for Cancer Research meeting. Mar 31-Apr 4, 2012, Chicago, IL (peer-reviewed). **[abstract on page 43]**
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 44]**
3. Draft of manuscript to be submitted to *Cancer Biology and Therapy* **[manuscript PDF pages 45-68]**

### *Funding applied for based on work supported by this award*

Yull F (PI): 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- $\kappa$ 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] – not funded**

Yull F (PI): 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- $\kappa$ B) in macrophages can limit ovarian cancer progression and synergize with chemotherapy using the ID8-NGL cell model. R21 2013. **[no appendix entry] – not funded**

Khabele (PI): Epigenetic therapy and the tumor microenvironment in ovarian cancer, \$200,000/yr. Dr Khabele will test whether histone deacetylase 3 is an effective target of therapy in ovarian tumor cells and in host peritoneal macrophages. RO1, 2013. **[no appendix entry] – not funded**

Yull F (PI): Can modulation of macrophage functions be effective for ovarian cancer therapy? \$150,000/yr. These studies were designed to provide validation in animal models of the novel concept that high level activation of NF-kappaB in macrophages could be used as therapy. In addition, they were designed to develop and test two different strategies by which these findings could be translated into clinical usage. OCRP Investigator-Initiated Research Award Pre-application 2014. **[no appendix entry] – not funded**

Wilson A (PI): Using TR3/NR4A1 activators to limit ovarian cancer progression. \$150,000/yr. These studies were designed to test the novel therapeutic strategy targeting the pro-apoptotic TR3 nuclear orphan receptor in preclinical ovarian cancer models, including the ID8-NGL syngeneic model. OCRP Pilot Project Award 2014. **[no appendix entry] - pending**

## CONCLUSIONS

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

In order to reach our ultimate goal of testing the efficacy of combination NF- $\kappa$ B inhibitor and cisplatin therapy in preclinical ovarian cancer models in the immunocompetent host, four major milestones were reached; 1) development and characterization of reproducible syngeneic *in vivo* models of ovarian cancer progression [manuscript published – ref (15)]; 2) understanding the role of NF- $\kappa$ B signaling in both host and tumor cells during ovarian cancer progression and regression following drug treatment (completed); 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 (completed), 4) precisely defining and fully understanding the effects of NF- $\kappa$ B inhibitors alone and in combination with cisplatin on both tumor and host cell populations during ovarian cancer progression (completed).

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23. An Y, Cai Y, Guan Y, et al. Inhibitory effect of small interfering RNA targeting insulin-like growth factor-I receptor in ovarian cancer OVCAR3 cells. *Cancer Biother Radiopharm* 2012; 25: 545-52.

## APPENDICES

Appendix materials start with Supporting Data [pages 16-35]

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.

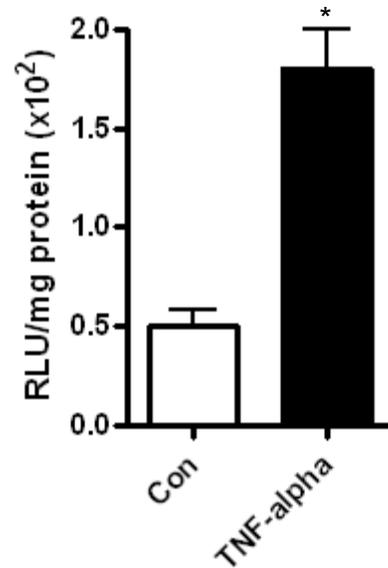
1. Wilson Andrew J., Biographical Sketch [pages 36-38]
2. Yull, Fiona E., Biographical Sketch [pages 39-42]

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

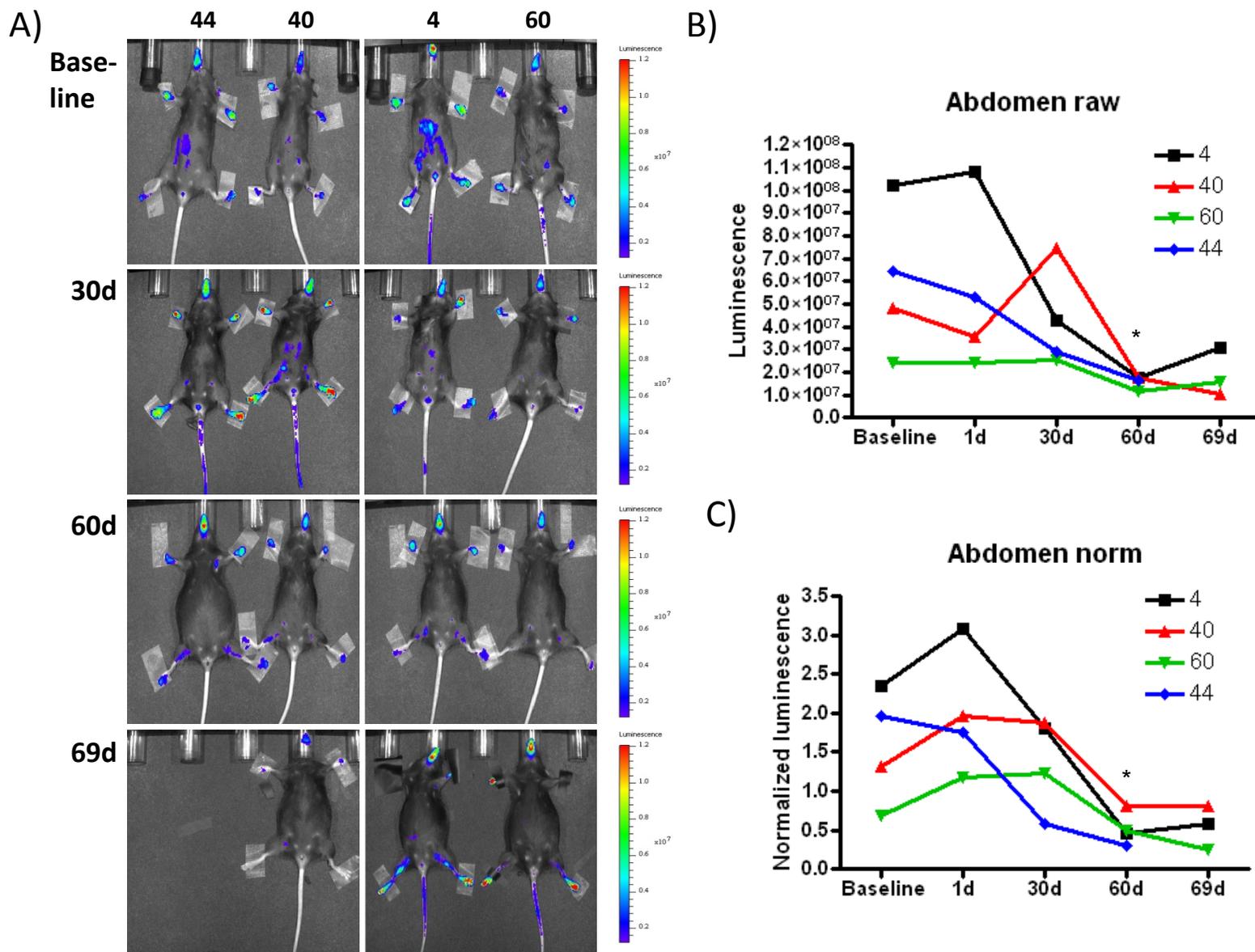
1. **AJ Wilson**, H-J Lee, WJ Barham, L Chen, H Onishko, D Khabele, F Yull. Investigating the patterns of nuclear factor-kappa B activity in the host-tumor microenvironment during ovarian cancer progression. **Poster presentation** at the American Association for Cancer Research meeting. Mar 31-Apr 4, 2012, Chicago, IL (peer-reviewed). [abstract on page 43]
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 44]
3. Draft of manuscript to be submitted to *Cancer Biology and Therapy* [manuscript PDF pages 45-68]

## APPENDICES

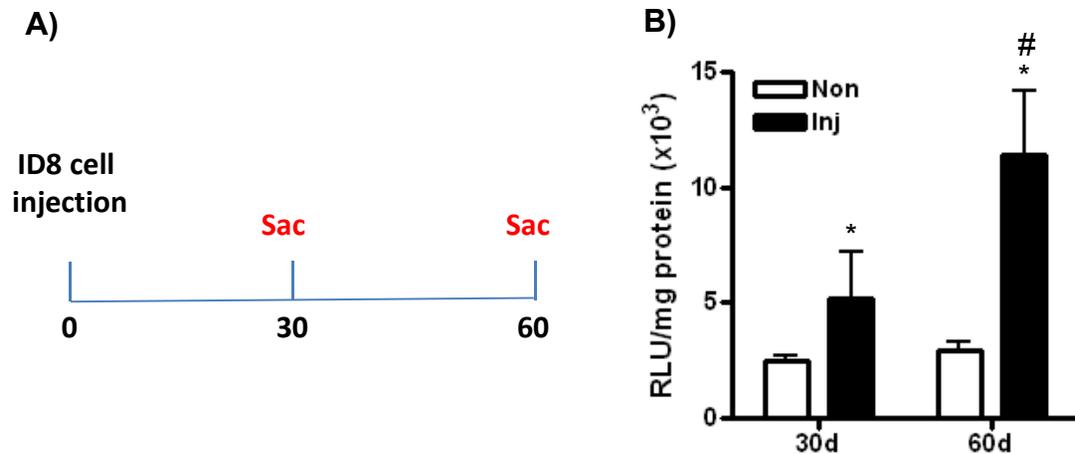
### Supporting Data



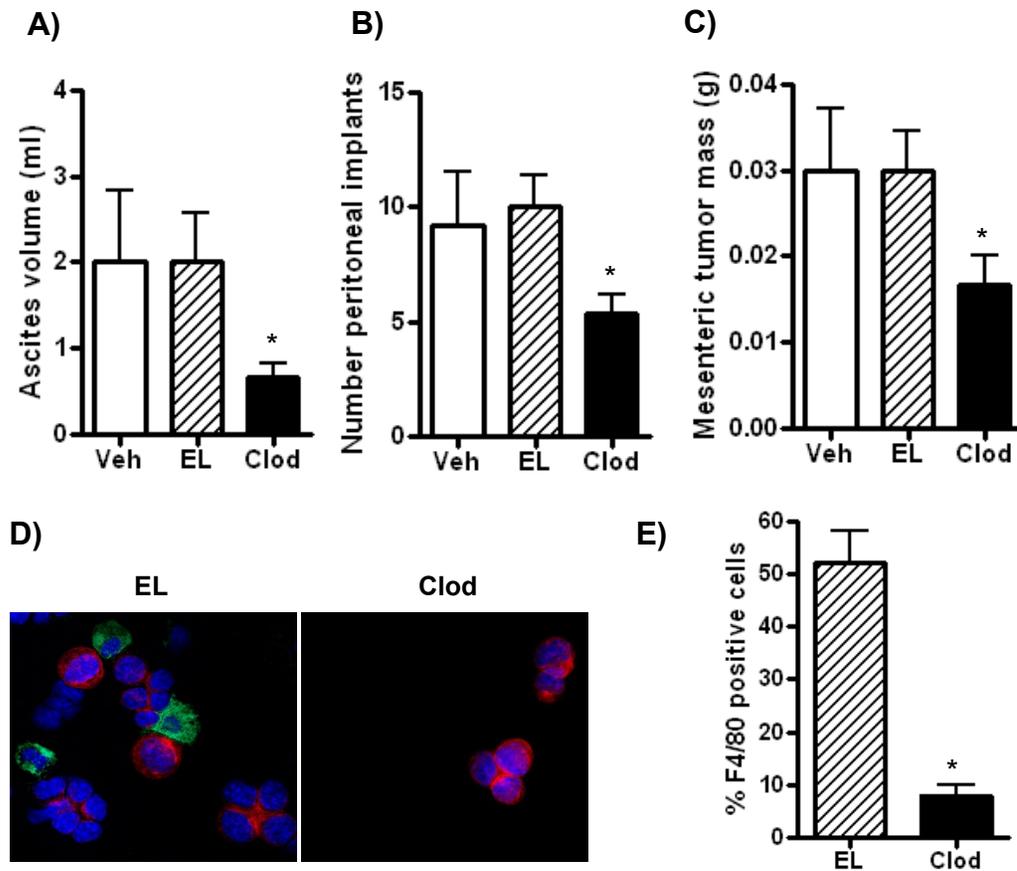
**Fig 1.** TNF- $\alpha$  (10ng/ml) stimulates NF- $\kappa$ B reporter activity in BMDM-NGL cells (6h treatment). Values are mean + SD of 3 independent experiments. \*  $p < 0.01$  relative to control, Student's T test.



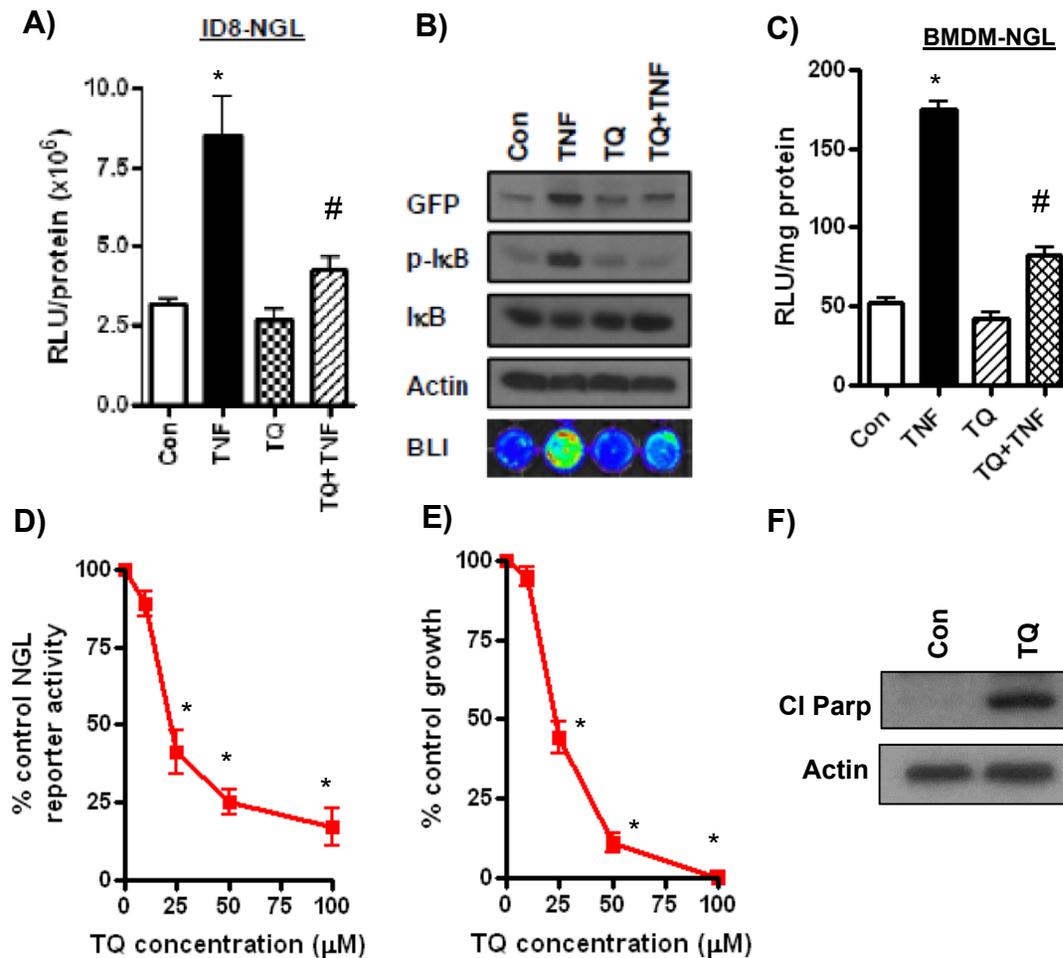
**Fig 2.** (A) Representative BLI of NGL reporter mice injected with ID8 cells showing specific NF- $\kappa$ B reporter signal in **host** cells. (B) Quantification of raw BLI in the peritoneal cavity shows decreased NF- $\kappa$ B reporter activity during ovarian cancer progression. (C) When abdominal BLI was normalized to corresponding brain BLI, similar results were observed. \*  $p < 0.05$  relative to baseline, Mann-Whitney test.



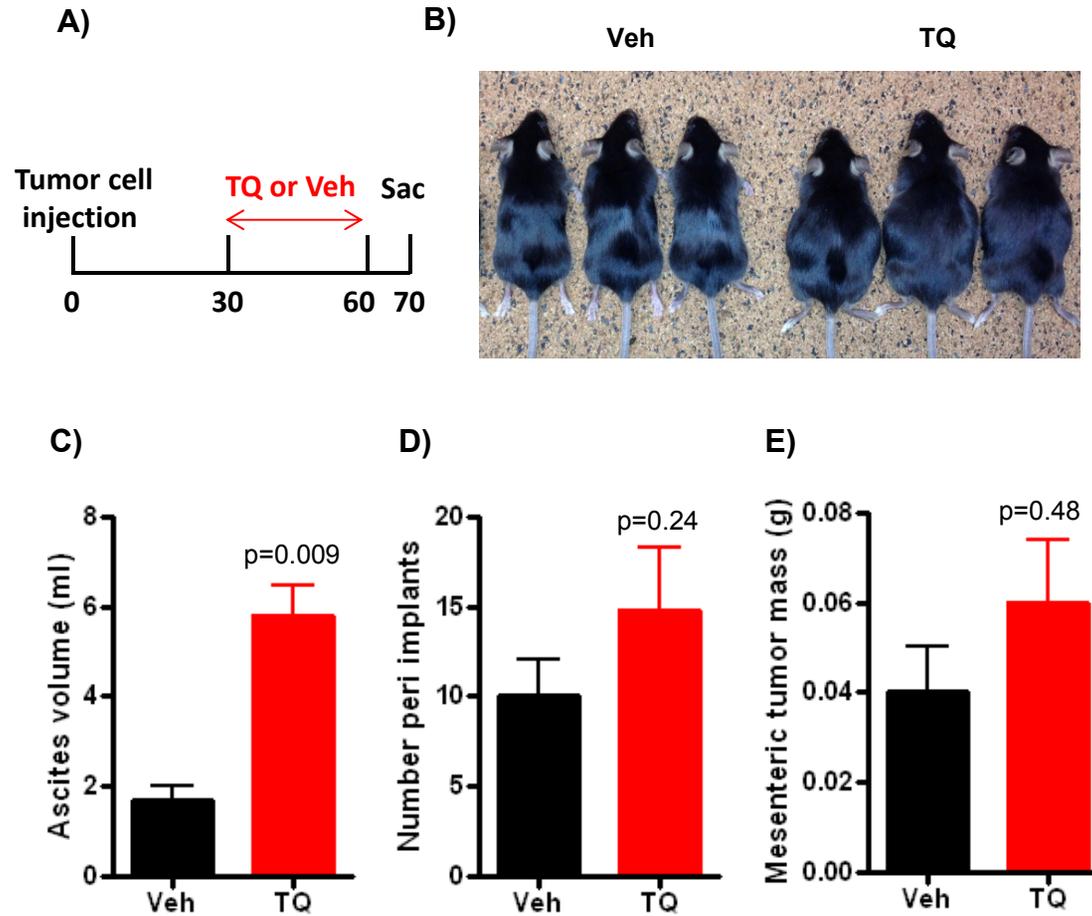
**Fig. 3.** (A) Schematic representation of the timeline for our experiment comparing NF- $\kappa$ B activity in non-injected versus ID8-injected NGL reporter mice. Mice were injected with ID8 cells at Day 0. (B) Luciferase activity of the NF- $\kappa$ B reporter was measured in isolated macrophages 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.



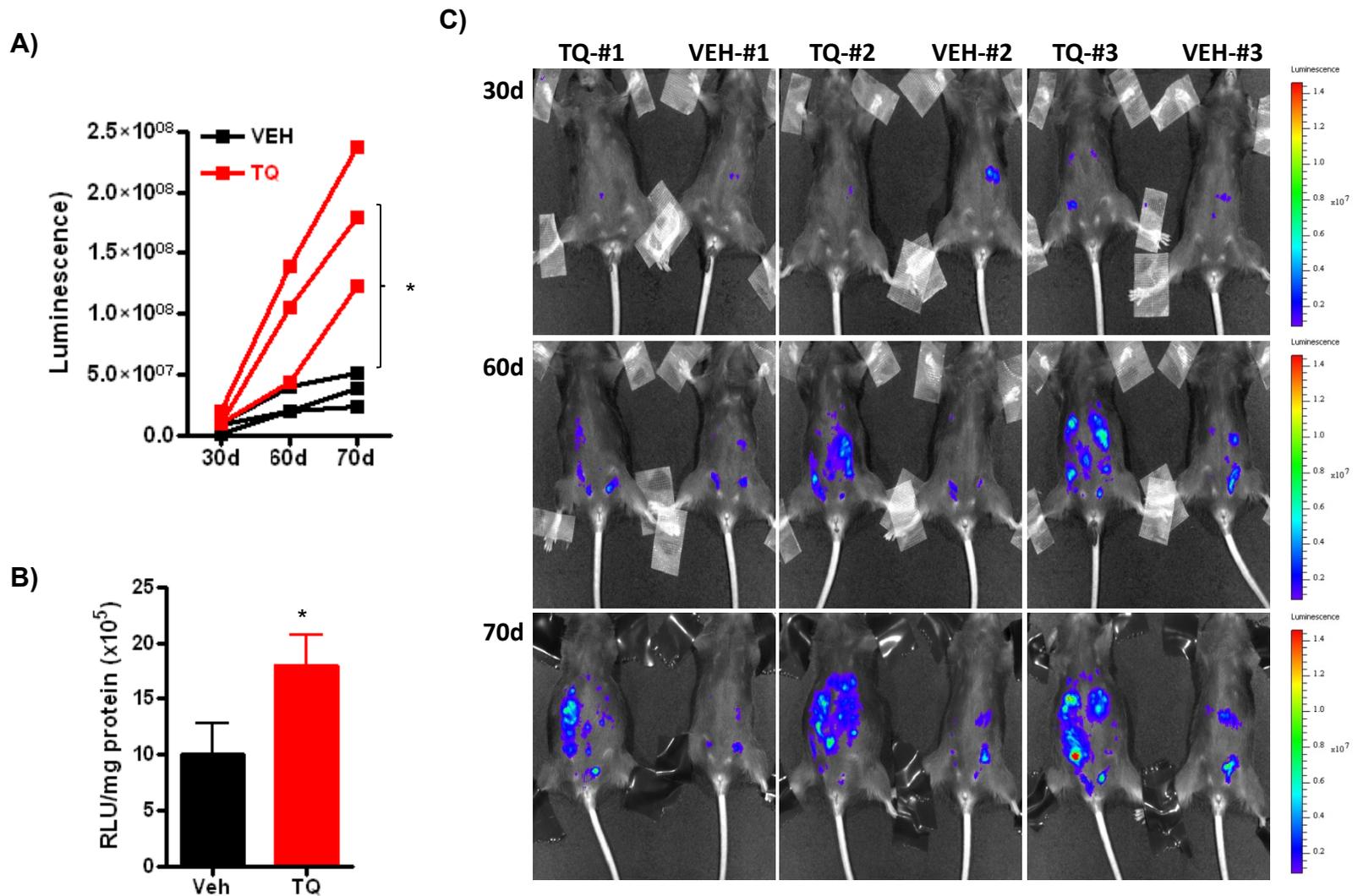
**Fig. 4.** Quantification of (A) ascites fluid volume, (B) number of peritoneal implants and (C) mesenteric tumor mass (g) at sacrifice in mice treated with Vehicle (PBS), empty liposomes (EL) or liposomal clodronate (Clod). (D) Representative fluorescent images of cytopins from EL or Clod-treated mice 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.



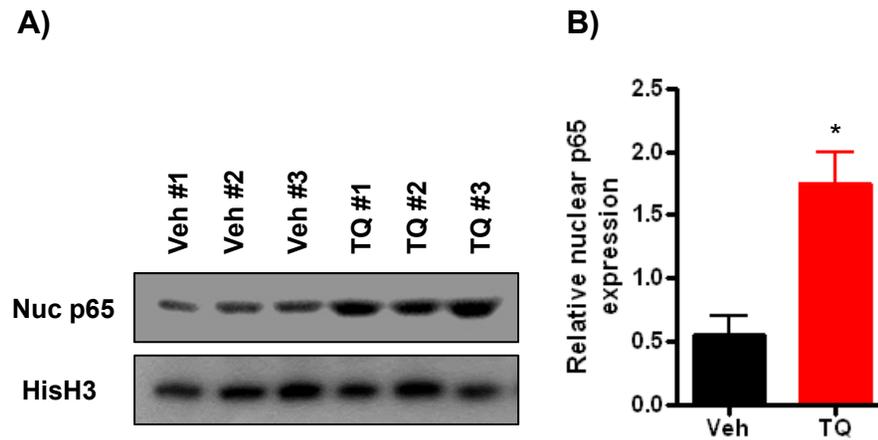
**Fig 5.** Effects of NF- $\kappa$ B inhibitor TQ in ID8-NGL cells grown *in vitro*. (A) 2h pre-treatment with 50  $\mu$ M TQ inhibited the stimulatory effect of 10 ng/ml TNF- $\alpha$  on NF- $\kappa$ B reporter activity in protein extracts. (B) Western Blot showing the stimulatory effect of TNF- $\alpha$  on GFP and phospho-I $\kappa$ 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  $\mu$ M TQ also inhibited the stimulatory effect of 10 ng/ml TNF- $\alpha$  on NF- $\kappa$ B reporter activity in BMDM-NGL cells. (D) Reduction in NF- $\kappa$ 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  $\mu$ 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.



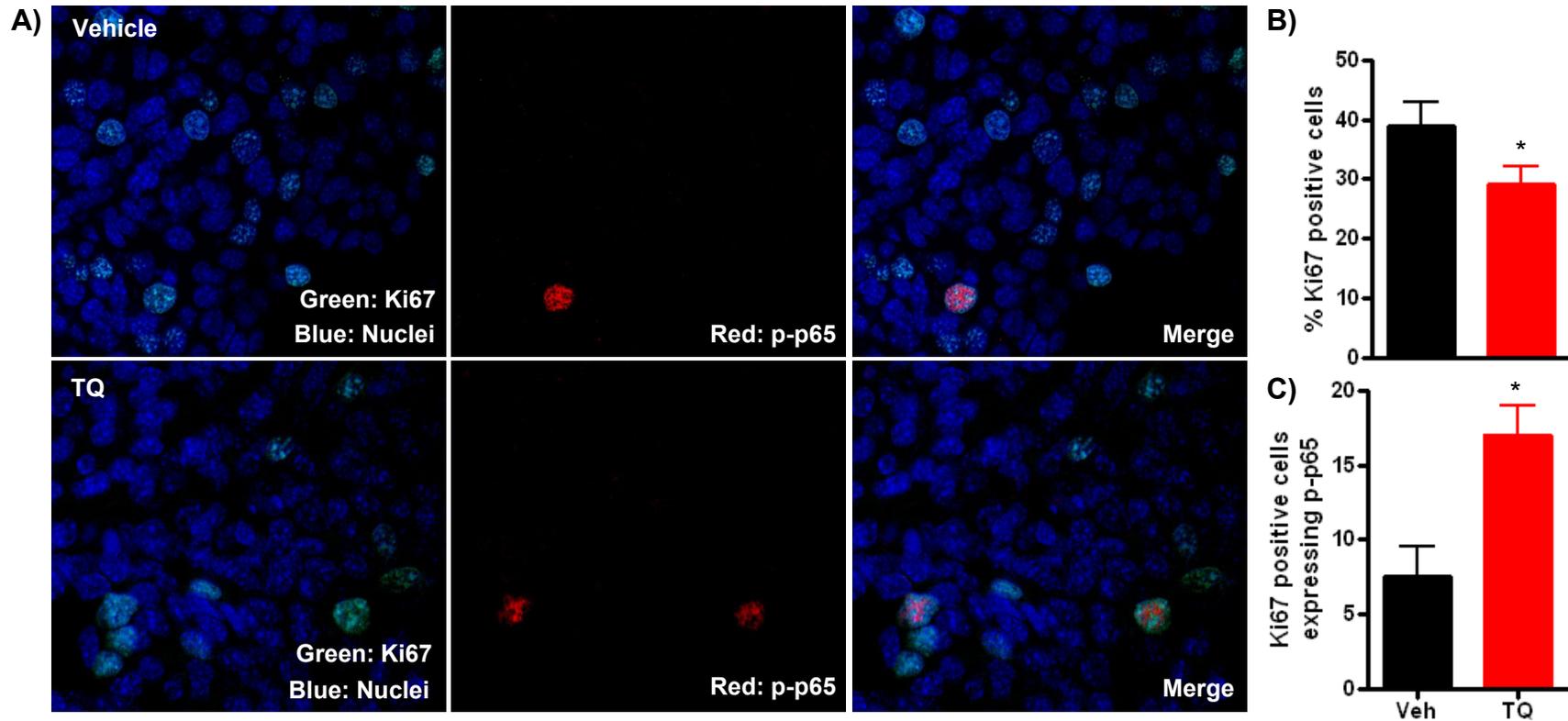
**Fig 6.** A) Schematic diagram showing treatment schedule for Vehicle or TQ in WT mice injected with ID8-NGL cells. B) Representative images of vehicle and TQ-treated mice shows greater abdominal distension in TQ-treated mice, indicative of ascites. C) Quantification of ascites fluid volume at sacrifice confirmed increased ascites in TQ-treated mice. In contrast, there were no significant differences in tumor burden between Vehicle and TQ-treated mice when D) peritoneal implants or E) mesenteric tumor mass were quantified. P values show the effect of TQ relative to vehicle and were generated by Mann-Whitney test.



**Fig 7.** A) Quantification of BLI in the peritoneal cavity shows increased NF- $\kappa$ B reporter activity in TQ-treated mice. B) Luciferase activity was measured in harvested tumors and expressed relative to cellular protein. Data show a specific increase in NF- $\kappa$ B reporter in tumors from TQ-treated mice. C) Representative BLI images comparing NF- $\kappa$ 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.

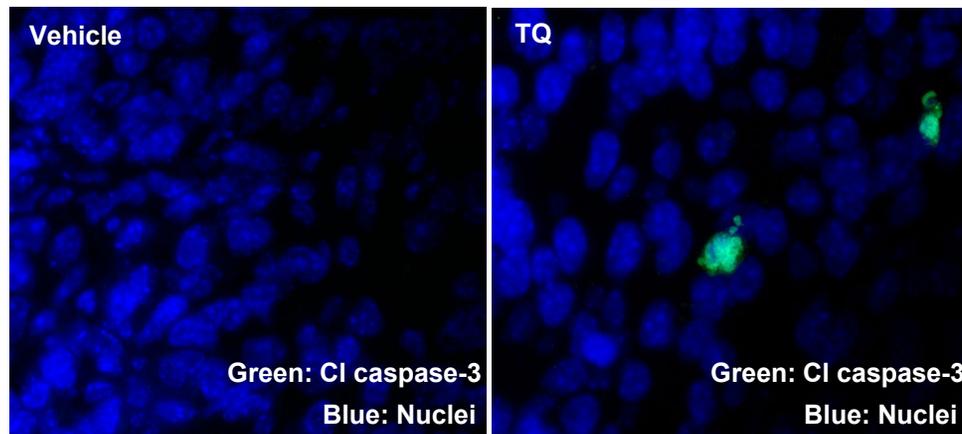


**Fig. 8** (A) Western blot analysis of p65 in nuclear extracts from harvested Vehicle or TQ-treated tumors. Equal loading was shown by probing for the nuclear-specific protein, histone H3. (B) Nuclear p65 expression relative to corresponding histone H3 levels was measured by densitometry. Values are mean+SD for 3 mice per group \*  $p < 0.01$  relative to vehicle-treated mice, Mann-Whitney test.

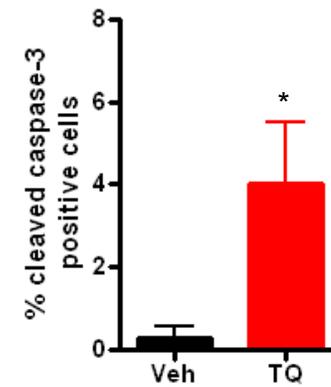


**Fig. 9** (A) Immunofluorescent detection of the proliferation marker Ki67/mib-1 and the phosphorylated p65 (p-p65) in ID8-NGL tumors harvested from vehicle or TQ-treated mice. TO-PRO-3-stained nuclei are in blue. Co-expression of phospho-p65 (red) and Ki67/mib-1 (green) is observed in the nuclei of 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.

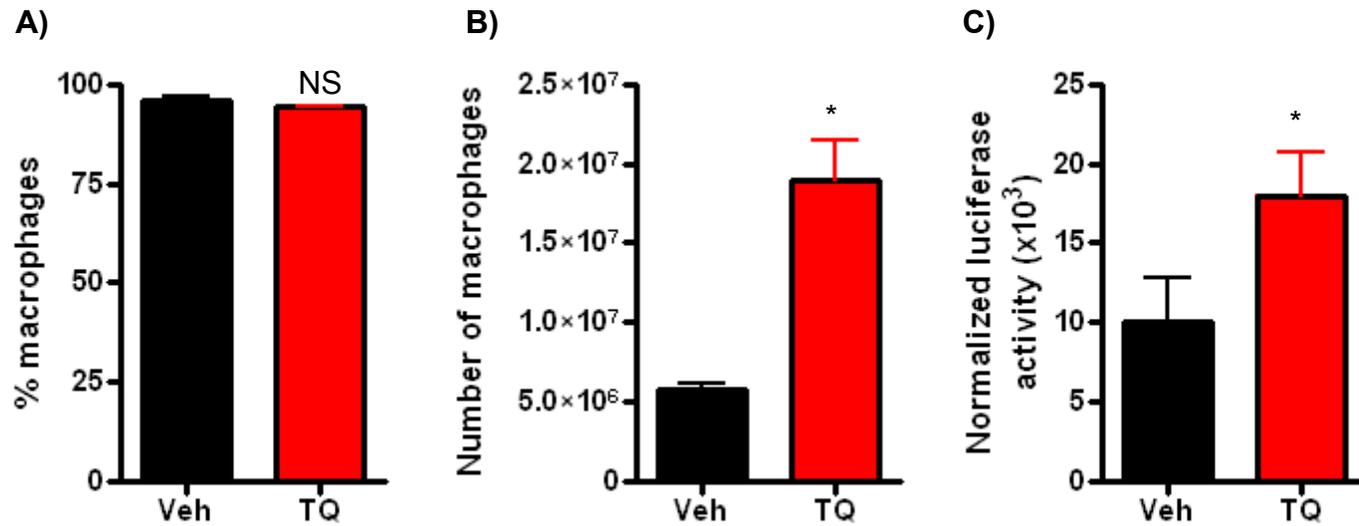
A)



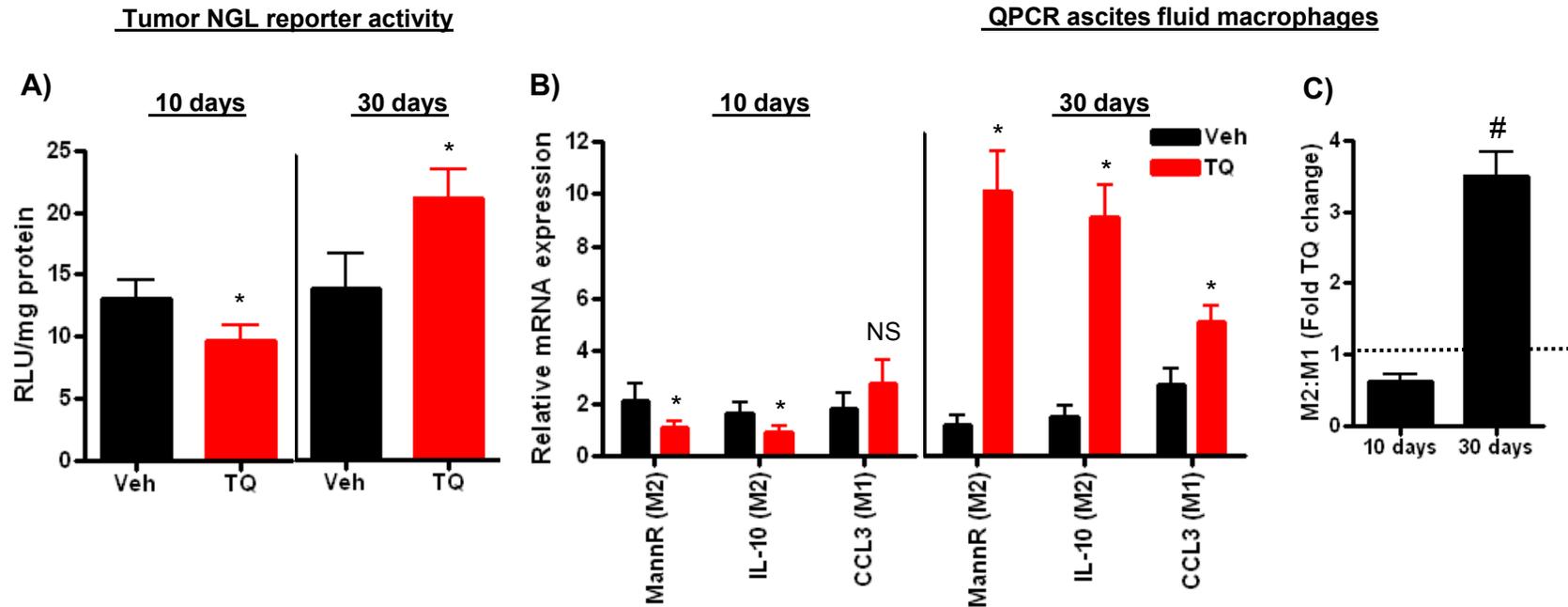
B)



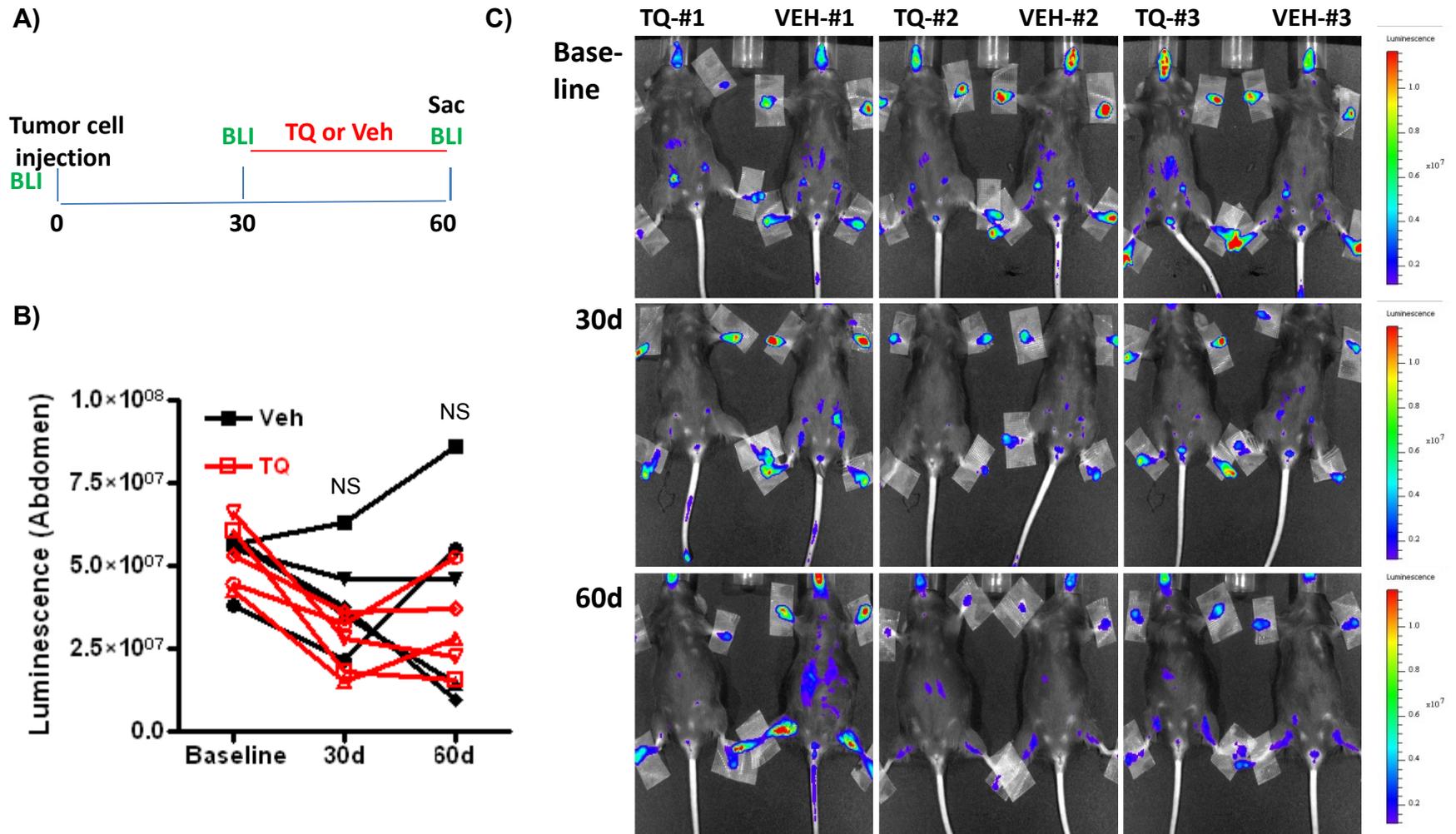
**Fig. 10** (A) Immunofluorescent detection of the apoptosis marker cleaved caspase-3 in ID8-NGL tumors harvested from vehicle or TQ-treated mice. TO-PRO-3-stained nuclei are in blue. (B) The percentage of tumor cells positive for cleaved caspase-3 staining. Values are mean+SD for 3 mice per group. \*  $p < 0.01$  relative to vehicle, Mann-Whitney test.



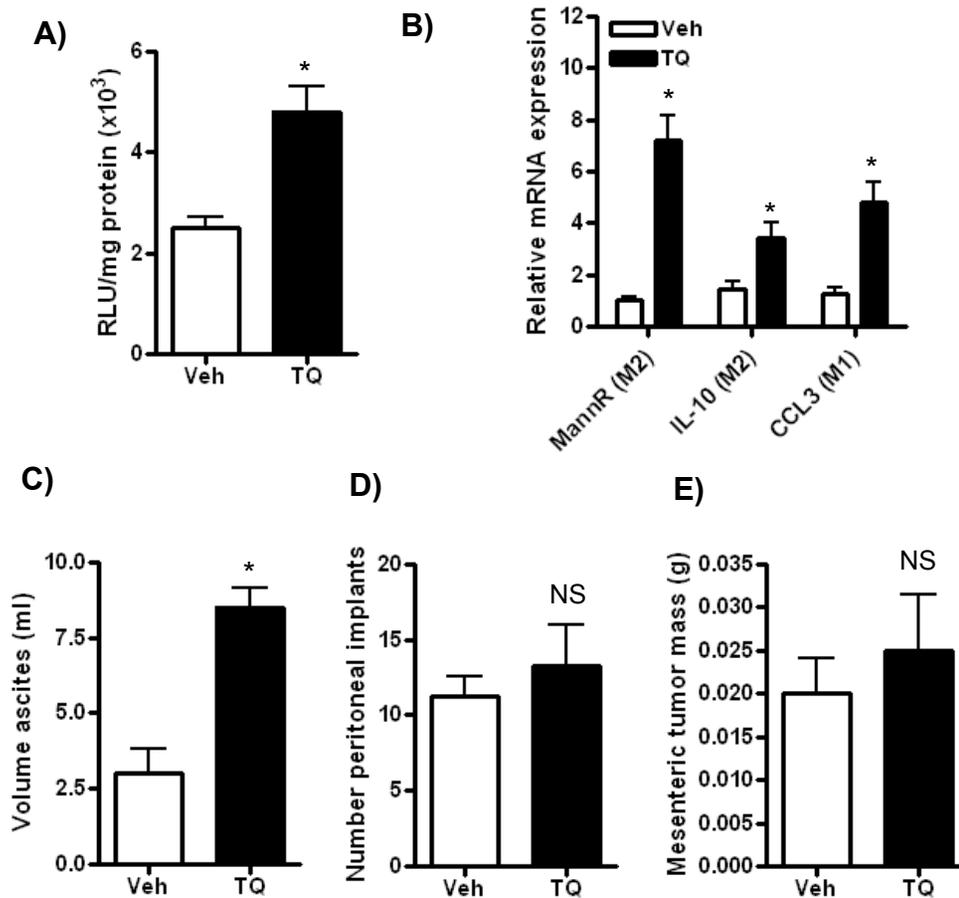
**Fig 11.** (A) No significant differences in the proportion of mononuclear cells counted in cytopins from ascites fluid or peritoneal lavages collected from vehicle or TQ-treated mice were seen. (B) Overall number of mononuclear cells increased in ascites collected from TQ-treated mice. (C) TQ treatment significantly increased NF- $\kappa$ 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.



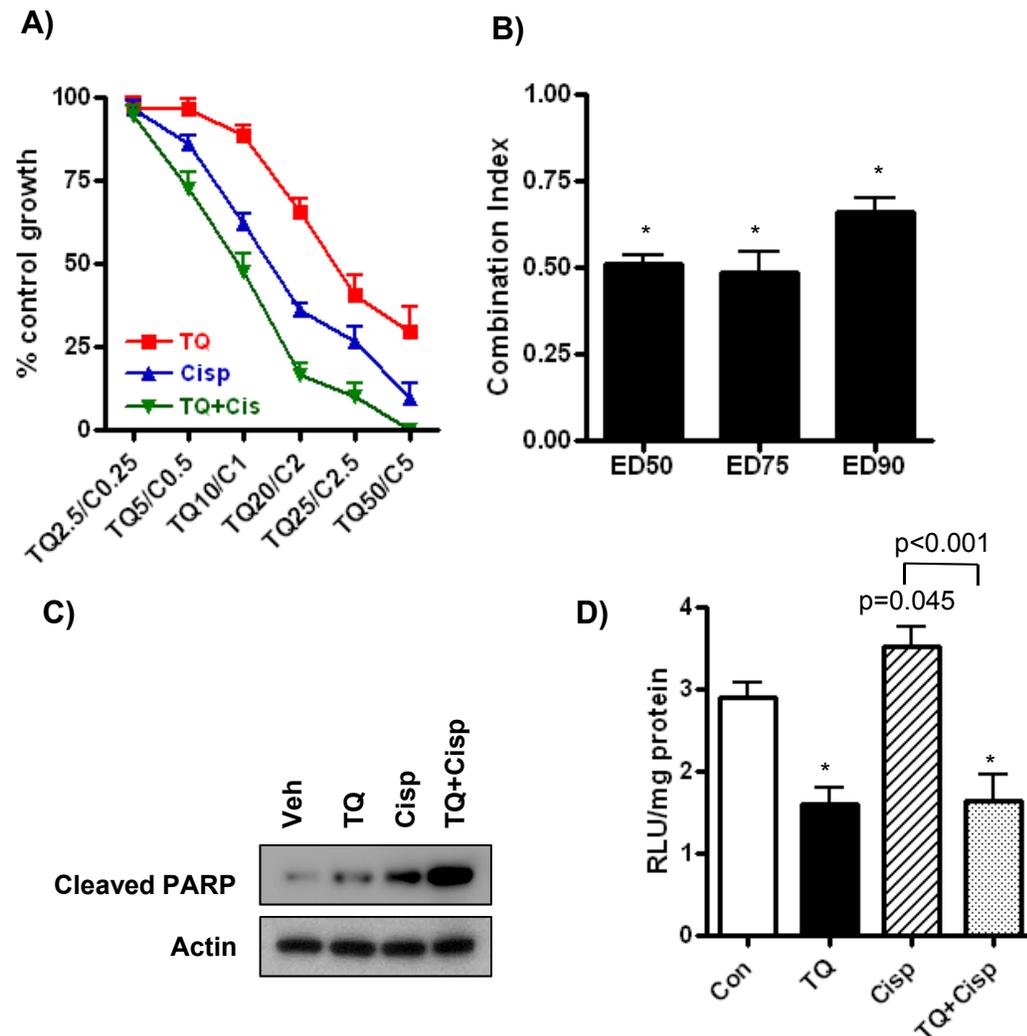
**Fig. 12.** (A) Relative luciferase activity in harvested ID8-NGL tumors following 30 days' TQ treatment. (B) QPCR analysis of the mRNA expression of the markers of M2 macrophages, mannose-receptor (mann-R) and interleukin-10 (IL-10) and M1 macrophages, CCL3, in RNA extracted from peritoneal lavages 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.



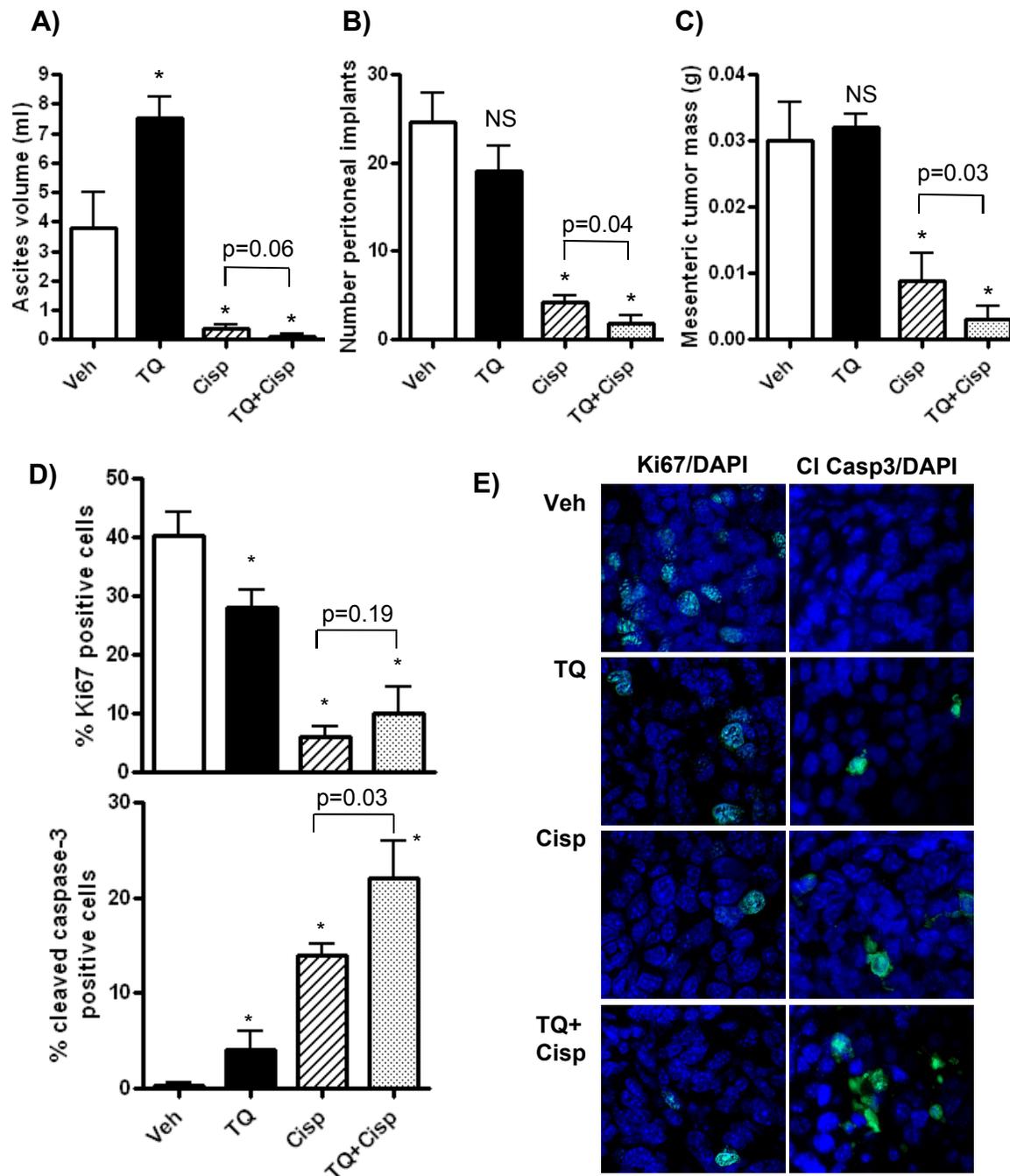
**Fig 13.** (A) Schematic diagram showing treatment schedule for vehicle or TQ in NGL reporter mice injected with ID8 cells. (B) Quantification of BLI in the peritoneal cavity shows no differences between NF-κB reporter activity in Vehicle or TQ-treated mice. Values are mean+SD from 5 mice per group. 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-κB reporter signal in Vehicle and TQ-treated cells.



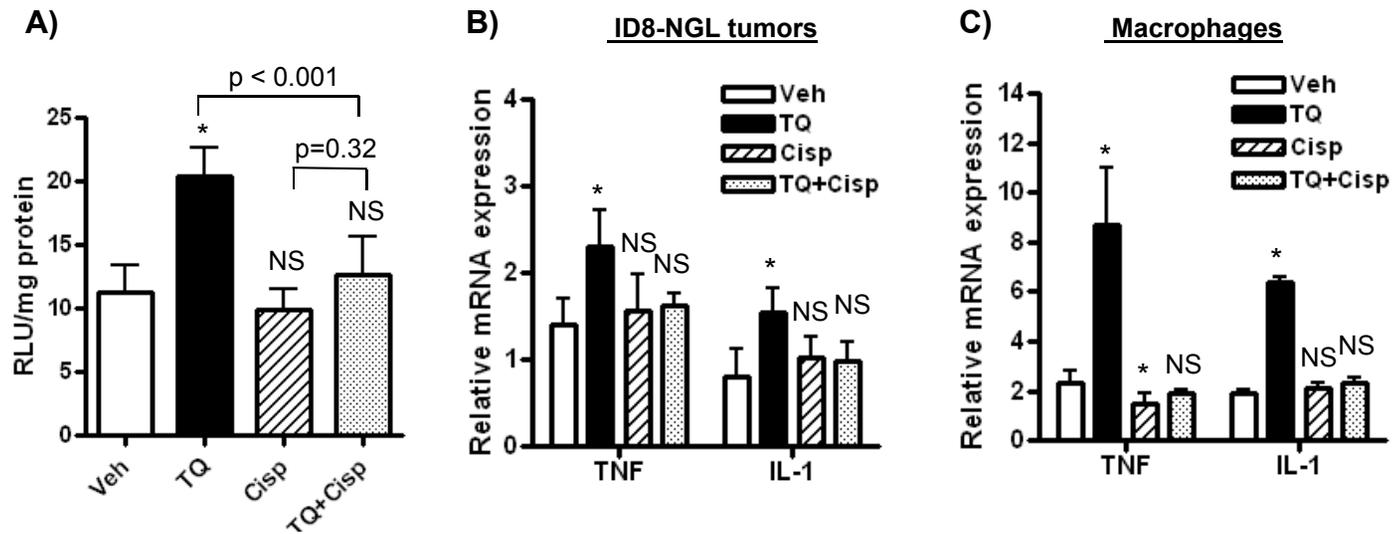
**Fig 14.** (A) Luciferase activity of the NF- $\kappa$ B reporter was measured in isolated macrophages from ascites or peritoneal lavage fluid collected from vehicle or TQ-treated NGL reporter mice injected with ID8 cells, and expressed relative to cellular protein. (B) QPCR analysis of the mRNA expression 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.



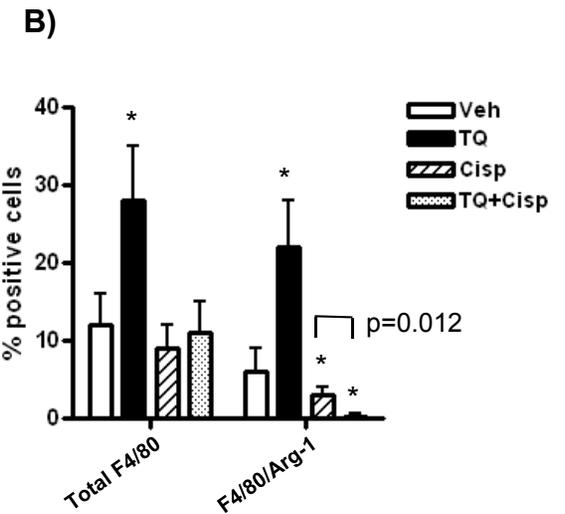
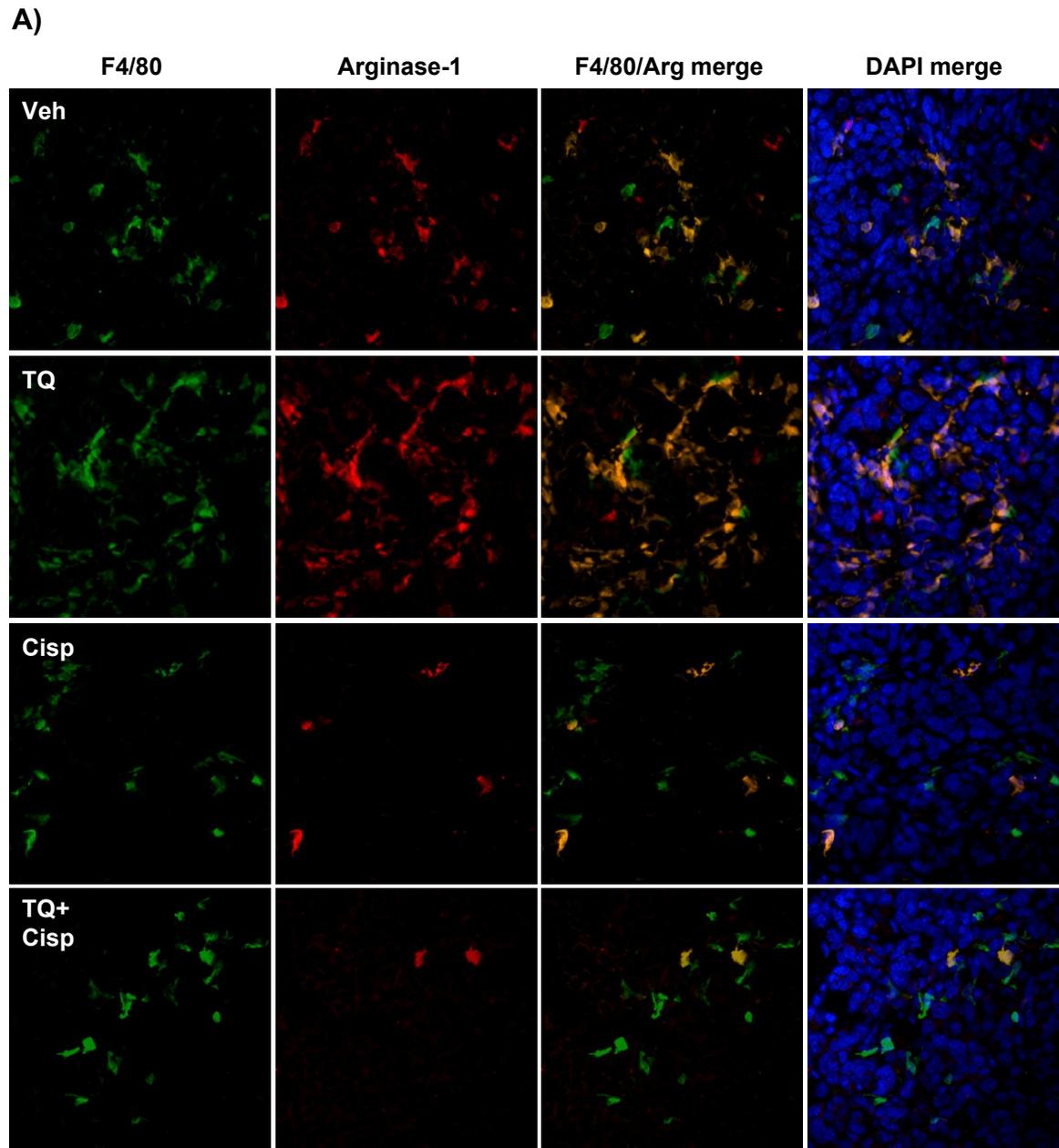
**Fig 15.** (A) SRB assays showing drug effects of TQ and/or cisplatin (concentrations in mM, 72 hours) in ID8-NGL cells. (B) Isobologram analysis for NCI/ADR-RES, OVCAR3 and SKOV-3 cells demonstrating that the Combination Index was significantly less than 1 for Effective Doses (ED) ED50, ED75 and ED90, indicating synergistic drug interaction between TQ and cisplatin. Values are mean  $\pm$  SE for 3 experiments; \*  $p < 0.01$ , Student's t test. (C) Western Blot analysis of cleaved PARP following 24 hour treatment of ID8-NGL cells with TQ (25 $\mu$ M), cisplatin (1 $\mu$ M) or the combination. Actin was used as a loading control. (D) Luciferase assay of ID8-NGL cells following 24 hour treatment with TQ (25 $\mu$ M), cisplatin (1 $\mu$ M) or the combination. Values are mean  $\pm$  SE for 3 experiments; \*  $p < 0.01$  compared to control, Student's t test.



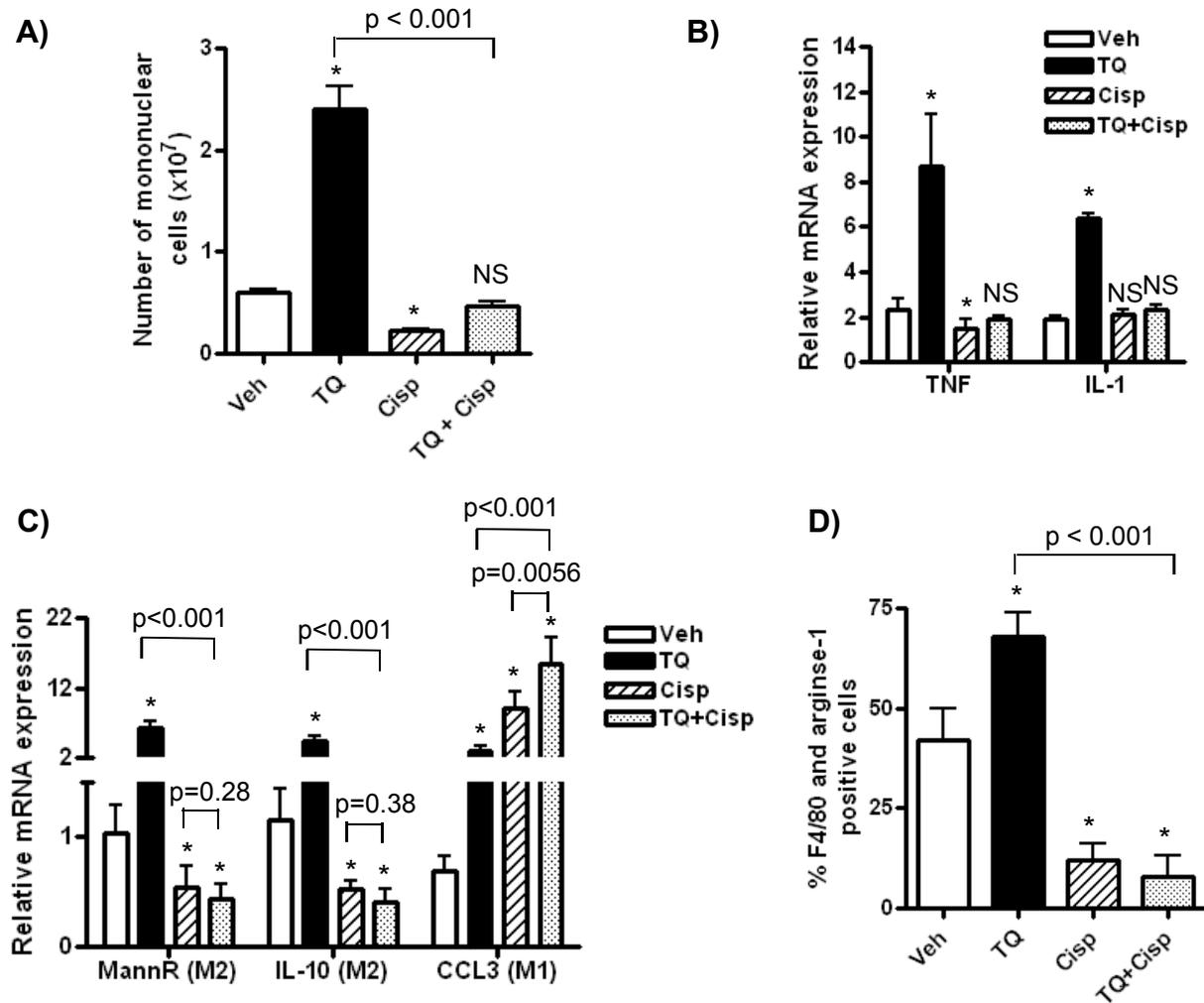
**Fig 16.** In BL/6 mice injected with ID8-NGL cells and treated with vehicle, TQ, cisplatin or the combination. **(A)** ascites fluid volume, **(B)** number of peritoneal implants and **(C)** mesenteric tumor mass was measured at sacrifice. Values are mean+SD for 5 mice per group. **(D)** The percentage of tumor cells positive for the proliferation marker Ki67/mib-1 and cleaved caspase-3 staining in ID8-NGL tumors harvested from mice treated with vehicle, TQ, cisplatin or the combination. **(E)** Representative fields of drug-treated tumors. Ki67/mib-1 or cleaved caspase-3 are in green. DAPI-stained nuclei are in blue. \*  $p < 0.01$  relative to vehicle; NS: not significant relative to vehicle; all  $p$  values determined by Mann-Whitney test.



**Fig 17.** BL/6 mice were injected with ID8-NGL cells and treated with vehicle, TQ (20 mg/kg), cisplatin (2 mg/kg) or the combination. **(A)** Luciferase assays of NF-κB reporter activity in harvested ID8-NGL tumors. QPCR analysis of the established NF-κB target genes, TNF-α and IL-1β, in **(B)** harvested tumors and **(C)** isolated macrophages from ascites fluid. Relative expression was calculated using corresponding GAPDH levels as the internal control. \* p < 0.01 relative to vehicle; NS: not significant relative to vehicle; all p values determined by Mann-Whitney test.

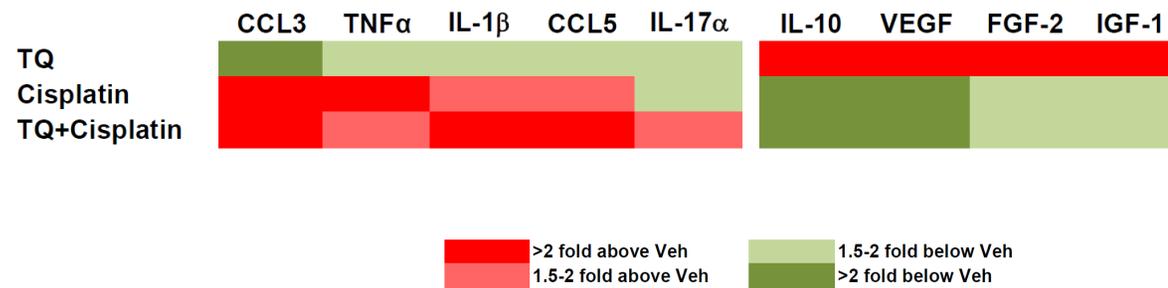


**Fig 18.** (BL/6 mice were injected with ID8-NGL cells and treated with vehicle, TQ (20 mg/kg), cisplatin (2 mg/kg) or the combination. **(A)** IF analysis of expression of the macrophage markers F4/80 (green) and arginase-1 (red). DAPI-stained nuclei are in blue. **(B)** Quantification of the percentage of total cells positive for F4/80 and cells co-staining for F4/80 and arginase-1. Values were determined from 5 representative fields at high power (x40). \*  $p < 0.01$  relative to vehicle; NS: not significant relative to vehicle; all  $p$  values determined by Mann-Whitney test. .

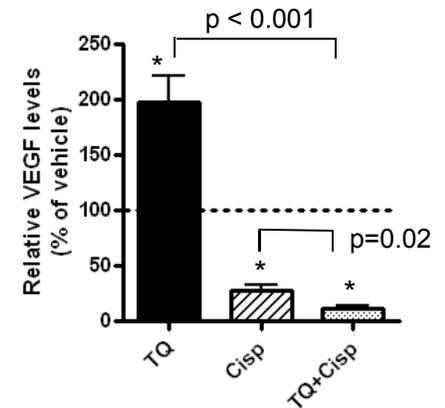


**Fig 19.** (A) Overall number of mononuclear cells increased in ascites collected from TQ-treated mice, as quantified in differential cell counts of H&E-stained cytopsin slides. QPCR analysis of (B) the established NF- $\kappa$ B target genes, TNF- $\alpha$  and IL-1 $\beta$ , and (C) markers of M2 macrophages, mannose-receptor (mann-R) and interleukin-10 (IL-10) and M1 macrophages, CCL3, in isolated macrophages isolated from peritoneal lavages or ascites fluid. Relative expression was calculated using corresponding GAPDH levels as the internal control. (D) Immunofluorescent detection of the M2 macrophage marker, arginase-1, in F4/80 positive macrophages isolated from ascites fluid. Values are mean+SE 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 20. (A)** Cytokine/growth factor profiles in ascites from drug-treated mice. Values are mean+SE for 5 mice per group. \*  $p < 0.01$  relative to vehicle; all  $p$  values determined by Mann-Whitney test. **(B)** VEGF levels in ascites fluid was measured by ELISA and expressed relative to corresponding protein levels. Values are mean+SE for 3 mice per group \*  $p < 0.01$  relative to vehicle; NS: not significant relative to vehicle; all  $p$  values determined by Mann-Whitney test.

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## BIOGRAPHICAL SKETCH

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NAME Wilson, Andrew		POSITION TITLE Research Assistant Professor of Obstetrics and Gynecology	
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)	1992-94	Immunology/Pathology
Department of Medicine, Melbourne University, Australia	Ph.D.	1995-98	Medicine
Department of Medicine, Melbourne University, Australia	Postdoctoral	1998-99	Colon biology
Albert Einstein Cancer Center, Bronx, NY	Postdoctoral	2001-03	Colon cancer

### A. Personal Statement

I have a long-standing 13 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 laboratory of Assistant Professor Dineo Khabele, a physician-scientist and practicing gynecologic oncologist. We have developed a fruitful collaboration with Dr Fiona Yull of Vanderbilt, an expert on generating mouse models for *in vivo* studies of NF- $\kappa$ B function during cancer progression. Thus, I am well-placed to study the role of NF- $\kappa$ B signaling in ovarian cancer using highly innovative syngeneic models, allowing its role in both tumor and host cells to be examined. With the assistance of Dr Khabele, we also have a pipeline to the clinic of drugs that show promising preclinical efficacy. A major goal of the recently completed project was to characterize the effects of established and novel NF- $\kappa$ B inhibitors on both tumor and, as can often be overlooked in cancer therapy, host cells. We successfully characterized a new syngeneic mouse model of ovarian cancer, showed that NF- $\kappa$ B activity is increased during tumor progression, and tested the combination of the NF- $\kappa$ B inhibitor, thymoquinone, and cisplatin in this model and showed enhanced anti-tumor effects compared to either drug alone. We also discovered an unexpected finding that prolonged treatment with thymoquinone increased ascites through an enhanced inflammatory response, which urges caution for the use of systemic NF- $\kappa$ B inhibitors in patients and may underlie why NF- $\kappa$ B inhibitor as single agent therapy for ovarian cancer have limited efficacy and high toxicity.

### B. Positions and Honors

#### Positions/ Appointments and Memberships

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  
 American Association for Cancer Research: Associate member 2001-2011; Active Member 2011-Present

### C. Selected peer-reviewed publications (15 out of 39 total publications)

1. Konstantinopoulos PA, **Wilson AJ**, Saskowski J, Wass E, Khabele D. Suberoylanilide Hydroxamic Acid (SAHA) enhances olaparib activity by targeting homologous recombination DNA repair in ovarian cancer. *Gynecol Oncol.* 2014 Jun;133:599-606. PMID: 24631446
2. **Wilson AJ**, Barham W, Saskowski J, Tikhomirov O, Chen L, Lee HJ, Yull F, Khabele D. Tracking NF-kappaB activity in tumor cells during ovarian cancer progression in a syngeneic mouse model. *J Ovarian Res.* 6: 63, 2013. PMID: PMC3846584
3. Basu A, Bodycombe NE, Cheah JH, Price EV, Liu K, Schaefer GI, Ebright RY, Stewart ML, Ito D, Wang S, Bracha AL, Liefeld T, Wawer M, Gilbert JC, **Wilson AJ**, Stransky N, Kryukov GV, Dancik V, Barretina J, Garraway LA, Hon CS, Munoz B, Bittker JA, Stockwell BR, Khabele D, Stern AM, Clemons PA, Shamji AF, Schreiber SL. An interactive resource to identify cancer genetic and lineage dependencies targeted by small molecules. *Cell* 154: 1151-1161, 2013. PMID: PMC3954635
4. **Wilson AJ**, Liu AY, Roland J, Adebayo OB, Fletcher SA, Slaughter JC, Saskowski J, Crispens MA, Jones HW 3rd, James S, Fadare O, Khabele D. TR3 modulates platinum resistance in ovarian cancer. *Cancer Res.* 73: 4758-69, 2013. PMID: PMC3944084
5. **Wilson AJ**, Lalani AS, Wass E, Saskowski J, Khabele D. Romidepsin (FK228) combined with cisplatin stimulates DNA damage-induced cell death in ovarian cancer. *Gynecol Oncol.* 127:579-86, 2012. PMID: PMC3541411.
6. **Wilson AJ**, Cheng YQ, Khabele D. 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. 5:12, 2012. *J Ovarian Res.* PMID: PMC3394212
7. **Wilson AJ**, Holson E, Wagner F, Zhang YL, Fass DM, Haggarty SJ, Bhaskara S, Hiebert SW, Schreiber SL, Khabele D. 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: PMC3218590
8. Khabele D, Fadare O, Liu AY, **Wilson AJ**, Wass E, Osteen K, Crispens MA. An orthotopic model of platinum-sensitive high grade serous fallopian tube carcinoma. *Int. J. Clin. Exp. Pathol.* 5: 37-45, 2012. PMID: 22295145; PMC3267484
9. 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: PMC3004468
10. 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
11. **Wilson AJ**, Chueh AC, Tögel L, Corner GA, Ahmed N, Goel S, Byun DS, Nasser S, Houston MA, Jhaver M, Smartt HJ, Murray LB, Nicholas C, Heerdt BG, Arango D, Augenlicht LH, Mariadason JM. 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: PMC2939837
12. **Wilson AJ**, Byun DS, Nasser S, Murray LB, Ayyanar K, Arango D, Figueroa M, Melnick A, Kao GD, Augenlicht LH, Mariadason JM. HDAC4 promotes growth of colon cancer cells by repression of p21. *Mol. Biol. Cell* 19:4062-475, 2008. PMID: PMC2555950
13. Jhaver M, Goel S, **Wilson AJ**, Montagna C, Ling YH, Byun DS, Nasser S, Arango D, Shin J, Klampfer L, Augenlicht LH, Perez-Soler R, Mariadason JM. PIK3CA/PTEN expression status predicts response of colon cancer cells to the EGFR inhibitor cetuximab. *Cancer Res.* 68: 1953-1961, 2008. PMID: PMC3972216
14. **Wilson AJ**, Arango D, Mariadason JM, Heerdt BG, Augenlicht LH. TR3/Nur77 in colon cancer cell apoptosis. *Cancer Res.* 63: 5401-5407, 2003. PMID: 14500374
15. **Wilson AJ**, Gibson PR. Short-chain fatty acids promote the migration of colonic epithelial cells in vitro. *Gastroenterology.* 113: 487-496, 1997. PMID: 9247468.



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## 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.**

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NAME Yull, Fiona Elizabeth, D.Phil.	POSITION TITLE		
eRA COMMONS USER NAME (credential, e.g., agency login) fiona_yull	Associate Professor of Cancer Biology		
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- $\kappa$ 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- $\kappa$ B signaling in several types of cancer including lung, breast, prostate and skin. To address the roles of NF- $\kappa$ 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- $\kappa$ B signaling directed to specific cell types (mammary epithelium, macrophages and lung epithelium). These models provide the opportunity to address the roles of NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ 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- $\kappa$ 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 Predoctoral 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

- 2000-2007 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 Research Safety Subcommittee  
 2009-Pres. Member of Vanderbilt University IACUC Committee  
 2010,11,13,14 Ad hoc member, DOD BCRP FY10 Programmatic Review Panel  
 2011,13 Ad hoc reviewer NIH MESH study section  
 2012-2014 Member of Editorial Board of ISRN Inflammation  
 2013,14 Ad hoc reviewer NIH SEP Cancer Health Disparities and Diversity in Basic Cancer Research

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  
 2012-Pres. Member of Metastasis Research Society  
 2014 Member of International Society for Transgenic Technologies

#### Honors

2000-2004 Aventis Leadership Development Program Fellow

#### **C. Selected Peer-reviewed Publications** (from 71 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- $\kappa$ B alternative pathway (p100/p52) in mammary development and links to tumorigenesis. *J Biol Chem.* 282: 10028-35, 2007. PMID: 17261585.
2. 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.
3. 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
4. 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- $\kappa$ B signaling promotes lung carcinogenesis via recruitment of regulatory T lymphocytes. *Oncogene.* 2012; 31: 3164-76. PMID: 22002309. PMCID: PMC3266969.
5. 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.* 2011; 187:5703-11. PMID: 22048774. PMCID: PMC3221921.
6. 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.* 2011; 187:2740-7. PMID: 21775686. PMCID: PMC3159744.
7. 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. PMCID: PMC3236346.
8. 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.* 2012; 72: 2473-80. PMID: 22414581. PMCID: PMC3653596.
9. 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- $\kappa$ B Reporter Transgenic. *J Vis Exp.* (67). pii: 4030. 2012. PMID:22971993. PMCID: PMC3490260.
10. 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; 10:975-87. PMID: 23331322 PMCID: PMC3595119.

11. 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- $\kappa$ B Activity. *J Immunol* 190:4786-94. PMID:23530143 PMCID: PMC3633681.
12. 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; 3:1286-301. PMID: 23921231. PMCID: PMC3823757.
13. Wilson AJ, Barham W, Saskowski J, Tikhomirov O, Chen L, Lee HJ, Yull F, Khabele D. Tracking NF- $\kappa$ B activity in tumor cells during ovarian cancer progression in a syngeneic mouse model. *J Ovarian Res.* 2013 6:63. PMID:24020521. PMCID: PMC3846584.
14. Jin R, Yi Y, Yull FE, Blackwell TS, Clark PE, Koyama T, Smith JA Jr, Matusik RJ. NF- $\kappa$ B gene signature predicts prostate cancer progression. *Cancer Res.* 2014; 74:2763-72. PMID: 24686169. PMCID: PMC4024337.
15. Stouch AN, Zaynagetdinov R, Barham WJ, Stinnett AM, Slaughter JC, Yull FE, Hoffman HM, Blackwell TS, Prince LS. I $\kappa$ B kinase activity drives fetal lung macrophage maturation along a non-M1/M2 paradigm. *J Immunol.* 2014; 193:1184-93. PMID: 24981452. PMCID: PMC4108541.

## D. Research Support

### Ongoing Research Support

1 R01 HL 116358-01 (Blackwell/Prince, Co-PI's)  
NIH/NHLBI

09/25/12 - 06/30/15

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. We hypothesize that developing molecular imaging techniques to identify functional subsets of activated macrophages will advance understanding of inflammatory lung diseases leading to novel, macrophage-targeted therapies. We will optimize imaging probes based on FR $\beta$  expression exploring 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 085317 (Blackwell)  
NIH/NHLBI

04/01/12 - 02/28/16

"Epithelial-Fibroblast Interactions in Lung Fibrosis"

Goal: Use 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. We will determine the proportion of lung fibroblasts derived from epithelium via EMT and examine the phenotypic characteristics of epithelial-derived fibroblasts.

VA MERIT (Richmond)  
Department of Veterans Affairs

10/01/13-9/30/2017

"Modeling New Therapeutic Approaches for Malignant Melanoma". Specific aims: 1) Characterize effectiveness of combined therapy with the MDM2 antagonist, Nutlin-3a, and the AURKA inhibitor, MLN8237, for treatment of BRAF<sub>WT</sub>/p53<sub>WT</sub>, NRas mutant/p53<sub>WT</sub>, NRas<sub>WT</sub>/p53<sub>WT</sub> Melanoma and examine the effect of the Nutlin-3a and MLN8237 combination treatment on the growth of BRAF<sub>V600</sub> mutant /p53<sub>WT</sub> melanoma tumor implants from patients with resistance to BRAF inhibitor. 2) Characterize effects of combined treatment with Nutlin-3a and MLN8237 on tumor microenvironment and on melanoma metastasis and to evaluate the potential for boosting the antitumor response of myeloid cells to enhance melanoma tumor cell death in association with MLN8237 and Nutlin-3a. Express constitutively active IKK $\beta$  in myeloid cells to induce an antitumor phenotype and evaluate the response of melanoma tumors to therapy with MLN8237 and Nutlin-3a. 3) To characterize the therapeutic effectiveness of combining agonist for death receptors DR4 and DR5 with MLN8237 for treatment of BRAF<sub>WT</sub>, NRAS<sub>WT</sub> or NRAS<sub>mutant</sub> melanoma tumors and to evaluate the effectiveness of combined DR5/DR4 agonists with MLN-8237 for BRAF inhibitor resistant BRAF<sub>V600</sub> melanoma.

## Recently Completed

W81XWH-11-1-0242 (Yull Partnering PI) 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-κB 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-κB activity.

W81XWH-11-1-0509 (Wilson) 07/25/11-08/24/13 (No cost extension – 08/24/14)  
Department of Defense  
Nuclear factor-kappaB Activity in the Host-tumor Microenvironment of Ovarian Cancer  
Study the patterns of NF-κB 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-κB activity and responses to pharmacologic interventions during tumor progression.

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-κB 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: To identify role of NF-κB in inhibition of lung development following innate immune activation. Determine if early activation of fetal lung macrophages alters the macrophage phenotype as lungs mature.

5 R01 CA 113734-05 (Yull) 07/01/07 - 05/31/12 (No cost extension – 05/31/13)  
NIH/NCI  
Epithelial NF-KappaB Signaling in Mammary Tumorigenesis  
Goal: The proposed project will define the role of NF-kappaB signaling as a master regulator of these processes, integrating multiple processes and thereby determining ultimate physiological outcome. Aims: Evaluate effects of modulation of NF-κB activity in mammary epithelium on mammary development. Use a number of existing and novel transgenic and knockout mice to investigate the differential effects of increasing or decreasing NF-κB activity in the mammary epithelium or of over-expressing p100/p52 a family member correlated with human mammary adenocarcinoma on mammary development.

5 R01 CA 076142-14 (Matusik) 08/11/09 – 06/30/13  
NIH/NCI  
Transgenic Animal Models for Prostate Cancer  
Goal: The overall goal of this proposal is to study the pathways that contribute to prostate tumor progression to castrate resistant prostate cancer (CRPC). Aims: To determine the contribution of Pten and NF-κB signaling to AR action *in vitro*. The proposed research of this aim is to identify targets in the prostate for therapeutic intervention and to understand the mechanism whereby NF-κB activation results in castrate resistant prostatic cells.

Overlap: There is no overlap between the currently funded grants listed above and this application. If all of Dr. Yull's pending proposals are approved and funded, Dr. Yull will adjust her effort so as not to exceed the maximum allowed.

## **Investigating the patterns of nuclear factor-kappa B activity in the host-tumor microenvironment during ovarian cancer progression**

**Andrew J. Wilson\*, Hye-Jeong Lee, Whitney J Barham, Lianyi Chen, Halina Onishko, Dineo Khabele, Fiona Yull**

Most women diagnosed with ovarian cancer initially present with metastatic disease characterized by peritoneal implants and ascites. Activation of inflammatory processes mediated by nuclear factor-kappa B (NF- $\kappa$ B) is thought to be critical to the distinct clinical pattern of spread of ovarian tumors. However, the relative contribution of NF- $\kappa$ B activity in tumor cells and host inflammatory cells to ovarian cancer progression remains unknown. Therefore, our goal was to develop and characterize model systems to investigate NF- $\kappa$ B activity in these cell populations during ovarian tumorigenesis. To study the host NF- $\kappa$ B response, ID8 mouse ovarian cancer cells were injected intra-peritoneally into C57BL/6 mice carrying an NF- $\kappa$ B-dependent green fluorescent protein (GFP)/luciferase fusion transgene (NGL). In a reciprocal approach, ID8 cells stably expressing the NGL reporter (ID8-NGL) were injected into wild-type C57BL/6 mice to investigate NF- $\kappa$ B activation in the developing tumor. Non-invasive imaging of the mice was performed by bioluminescence imaging (BLI) of the NGL reporter. Preliminary experiments confirmed these models were highly reproducible, with peritoneal-wide tumor dissemination accompanied by the onset of late-stage, irreversible ascites. Mice injected with ID8-NGL cells showed greater than 100-fold increase in NF- $\kappa$ B reporter activity during tumor progression. There was a modest overall decrease in abdominal NF- $\kappa$ B activity over time in ID8-injected NGL mice, although the possibility of “masking” effects of the ascites fluid on bioluminescence, and NF- $\kappa$ B activity in specific host cell populations, need to be elaborated. These models will allow us to define the patterns of NF- $\kappa$ B activity in the host-tumor microenvironment during ovarian cancer progression, and will provide a powerful platform for future preclinical investigation of novel therapeutic agents targeting NF- $\kappa$ B in ovarian cancer.

## **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- $\kappa$ B) is involved in ovarian cancer progression and linked to chemotherapy resistance. The potential of NF- $\kappa$ 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- $\kappa$ B inhibitors including bortezomib have had disappointing clinical results in the treatment of solid tumors. It is possible that NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ 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- $\kappa$ 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- $\kappa$ 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- $\kappa$ B inhibitors may have unanticipated adverse effects, and that a greater understanding of the effects of NF- $\kappa$ B inhibition is necessary before performing combination studies with platinum agents such as cisplatin.

# **NF- $\kappa$ B inhibitor thymoquinone enhances cisplatin-response in a syngeneic mouse model of ovarian cancer**

Andrew J. Wilson<sup>1\*</sup>, Whitney Barham<sup>2</sup>, Jeanette Saskowski<sup>1</sup>, Oleg Tikhomirov<sup>2</sup>, Dineo Khabele<sup>1,3</sup>, and  
Fiona Yull<sup>2,3</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Vanderbilt University School of Medicine, Nashville, TN; <sup>2</sup>Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN; <sup>3</sup>Vanderbilt-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](mailto:andrew.j.wilson@vanderbilt.edu)

## **KEY WORDS:**

NF- $\kappa$ B activity; ovarian cancer; syngeneic mouse model; macrophages; thymoquinone

## **ABBREVIATIONS**

NF- $\kappa$ B: Nuclear factor-kappaB; NGL: NF- $\kappa$ B-GFP-Luciferase; TQ: thymoquinone;

## **COMPETING INTERESTS**

The authors disclose no competing interests.

## **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)

## **ABSTRACT**

Ovarian cancer is the most lethal gynecologic malignancy. Activation of inflammatory processes via nuclear factor-kappa B (NF- $\kappa$ B) is involved in ovarian cancer progression and linked to chemotherapy resistance. The potential of NF- $\kappa$ 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. Here, we aimed to determine whether TQ enhances the cytotoxic effects of cisplatin in ovarian cancer cell lines and in an established murine syngeneic model of ovarian cancer. In sulforhodamine B cytotoxicity assays, TQ and cisplatin synergized in OVCAR3 and NCI/ADR-RES human ovarian cancer cells, and in ID8 mouse ovarian cancer cell stably expressing the NGL NF- $\kappa$ B reporter plasmid (ID8-NGL). In ID8-NGL-derived intraperitoneal tumors in immunocompetent C57BL/6 mice, TQ alone unexpectedly increased ascites. However, TQ and cisplatin combined treatment significantly reduced the following compared to cisplatin alone: ascites formation and tumor burden; tumor proliferation and apoptosis; tumor infiltration of macrophages positive for the M2-like pro-tumorigenic mark, arginase-1; expression of the M2-like marks, and mannose-receptor and IL-10 in isolated peritoneal macrophages. While NF- $\kappa$ B reporter activity was not further reduced by the combination compared to cisplatin, the combination markedly inhibited expression of specific pro-tumorigenic NF- $\kappa$ B targets such as VEGF. We have established a strong rationale for targeting NF- $\kappa$ B to enhance cytotoxicity of the established ovarian cancer chemotherapeutic, cisplatin, which has the potential to improve response of a significant number of patients with recalcitrant disease.

## BACKGROUND

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-kappaB (NF- $\kappa$ B) signaling pathway is known to play an important role in several malignancies, including ovarian cancer (3-8). Constitutive activation of NF- $\kappa$ 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- $\kappa$ B are known to suppress angiogenesis and progressive tumor growth (9), and potentiate the anti-tumor activity of cytotoxic agents (10), in preclinical models of ovarian cancer. A common mechanism of resistance to the anti-tumor effects of the established chemotherapeutic drug cisplatin in ovarian cancer cells is induction of NF- $\kappa$ B (11). A promising NF- $\kappa$ B inhibitor used in preclinical models is thymoquinone, a product of the medicinal plant *Nigella sativa* (12). Thus, an evolving strategy in ovarian cancer treatment is the combination of NF- $\kappa$ B inhibitors with current platinum-based regimens.

Equally relevant, but less understood, are the potential effects of systemic NF- $\kappa$ B inhibition in host cells. Ovarian tumors are known to polarize macrophages in the tumor microenvironment to display pro-tumorigenic characteristics via aberrant NF- $\kappa$ B signaling activity (13, 19). 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 (20). 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 (21-23). Targeting M2-like, tumor-associated macrophages for “re-education” towards a cytotoxic (M1), anti-tumor function by NF- $\kappa$ B inhibition is a promising therapeutic strategy (13). However, major gaps in knowledge still remain regarding the specific influence of NF- $\kappa$ B, and the consequences of inhibiting its activity, in cancer cells and host cells during ovarian cancer progression. We have shown that short-term TQ treatment induces a shift towards M1 markers in peritoneal macrophages harvested from ascites fluid (14), but the effects of long-term treatment with NF- $\kappa$ B is not known.

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*. For this reason, we generated a mouse syngeneic model that simulates the intraperitoneal abdominal carcinomatosis accompanied by ascites formation characteristic of human ovarian cancer. ID8 mouse ovarian cancer cells were stably transfected with an NF- $\kappa$ B reporter plasmid, which allows us to track changes in patterns of NF- $\kappa$ B activity during tumor progression and in response to drug treatment (14).

In cultured ID8-NGL cells and in ID8-NGL-derived intraperitoneal tumors in immunocompetent C57BL/6 mice, we show that combined TQ and cisplatin treatment lead to synergistic anti-tumor effects *in vitro*, and reduced tumor burden proliferative and apoptotic marks in tumors. Consistent with these effects, analysis of ascites fluid showed that the TQ/cisplatin combination reduced tumor infiltration of arginase-1-positive (M2-like) macrophages, reduced expression of key pro-tumorigenic cytokines or growth factors (ex IL-10, VEGF) in the soluble fraction and M2-like markers (ex mannose receptor, IL-10). Since improving the response to cisplatin is a critical question in the clinical management of

ovarian cancer, our results provide strong preclinical evidence for the considerable potential of NF- $\kappa$ B inhibition in sensitizing tumor cells to cisplatin-induced apoptosis and growth arrest. Our studies also suggest that caution needs to be employed in using systemic NF- $\kappa$ B inhibitors, particularly as monotherapy, since prolonged TQ treatment induced an unexpected and robust increase in ascites formation.

## **MATERIALS AND METHODS**

### **Cell culture**

Mouse ovarian cancer cells stably expressing a NF- $\kappa$ B reporter plasmid, ID8-NGL (14, 27, 28), were cultured in 10% FBS-supplemented DMEM High-Glucose medium with 400  $\mu$ g/ml G418, and passaged by standard techniques (14). The human ovarian cancer cell lines, OVCAR3, NCI/ADR-RES and SKOV-3 were cultured as previously described (29). Cultured ID8-NGL cells were treated with increasing concentrations of cisplatin (Sigma Chemical Co., St Louis, MO) and/or the (NF- $\kappa$ B inhibitor, thymoquinone (Sigma Chemical Co., St Louis, MO).

### **Cell viability assays**

Sulforhodamine B (SRB) assays were used to determine cell proliferation and cytotoxicity in response to TQ and/or cisplatin as previously described (29). Absorbance was measured at 510nm using a Spectramax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA) in the High-Throughput Screening Core of the Vanderbilt Institute of Chemical Biology. The interaction between fixed ratios of SAHA and olaparib was measured with the Combination Index (CI) method (30). A CI level of  $< 0.9$ ,  $CI = 0.9-1.1$  and  $CI > 1.1$  indicates synergy, additivity and antagonism respectively, between drug combinations.

### **Animal model and drug treatment**

Wild-type C57BL/6 mice were injected intra-peritoneally (IP) with  $1 \times 10^7$  ID8-NGL cells in 200 $\mu$ l sterile PBS (14). 30 days after ID8-NGL injection, mice were randomized into the following treatment groups: vehicle (PBS), TQ (20 mg/kg thrice weekly), cisplatin (2 mg/kg weekly) and the

TQ/cisplatin for 30 days (n = 5). No signs of drug toxicity were observed in the single or combination treatment mice.

Tumor progression was 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 (14). 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).

### **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 Cyto centrifuge (500 rpm for 10 minutes) for differential counts of inflammatory cells in hematoxylin and eosin-stained cells or immunofluorescence analysis. Cytokine/growth factor composition in the soluble fraction of ascites harvested from drug-treated mice was analyzed by a mouse cytokine array plate and VEGF ELISA (Signosis Inc., La Jolla, CA).

### **RNA extraction and quantitative RT-PCR (QPCR)**

RNA from snap-frozen tumors, ascites fluid or peritoneal lavages was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) and QPCR performed as described (14). Steady-state mRNA levels of the M1 macrophage mark, CC chemokine ligand 3 (CCL3), the M2 macrophage marks, mannose receptor (mann-R) and IL-10, and the established NF- $\kappa$ B targets, TNF- $\alpha$  and IL-1 $\beta$ , 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 as previously described (14, 27, 28).

### **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 (14, 34). Immunofluorescent analysis of formalin-fixed paraffin-embedded tumor tissue or in cytospin slides of ascites fluid or isolated macrophages was performed using standard techniques (14, 32, 33). The following primary antibodies were used: rabbit polyclonal anti-Ki67/Mib-1 (Abcam, Cambridge, UK; 1:200 dilution), rabbit

polyclonal anti-cleaved caspase-3 (Cell Signaling Technology, Beverley, MA), mouse monoclonal anti-pan-cytokeratin (Abcam, 1:100), rat polyclonal anti-F4/80 (AbD, Serotec; Raleigh, North Carolina, 1:200), and rabbit polyclonal anti-arginase-1 (Santa Cruz, La Jolla, CA). Secondary antibodies used were goat anti-rat or -rabbit or -mouse conjugated with Molecular Probes Alexa488 or Alexa594 (Invitrogen). Images were acquired and analyzed as previously described (32). For quantifying the percentage of, where applicable, tumor cells or macrophages positive for these proteins, at least 5 independent fields were counted with at least 200 cells were counted per sample.

### **Western blotting**

In ID8-NGL cells treated with TQ (25 $\mu$ M) and/or cisplatin (1 $\mu$ M), whole cell protein isolation, subcellular fractionation, Western Blotting and signal detection were performed as described (35, 36). Primary antibodies used were rabbit polyclonal anti-PARP (Cell Signaling Technology; 1:1000), rabbit polyclonal anti-p65 (Cell Signaling Technology; 1:1000), mouse monoclonal anti-histone H3 (Millipore;1:1000) and anti- $\beta$ -actin (Sigma; 1:10000). Loading controls were anti-histone H3 and  $\beta$ -actin (Sigma) for nuclear fractions and whole cell lysates, respectively.

### **Statistical analysis**

Unless otherwise indicated, values shown for *in vitro* experiments were the mean + SE 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.

## RESULTS

### **TQ enhances anti-tumor effects of cisplatin in cultured ovarian cancer cells.**

Since a potential mechanism of resistance to the anti-tumor effects of cisplatin in ovarian cancer cells is cisplatin-induction of NF- $\kappa$ B (11), we tested the ability of the NF- $\kappa$ B inhibitor, thymoquinone, to sensitize a panel of ovarian cancer cell lines to cisplatin. We chose three human ovarian cancer cell lines, OVCAR3, SKOV-3 and NCI/ADR-RES, which are relatively resistant to the cytotoxic effects of cisplatin. As shown in Fig 1A, isobologram analysis of SRB cytotoxicity assays demonstrated a synergistic decrease in viability of OVCAR3 and NCI/ADR-RES cells treated with the NF- $\kappa$ B inhibitor, thymoquinone (TQ) and cisplatin combination. These findings are consistent with a recent report that in ovarian cancer cell lines with epithelial morphology (OVCAR3, NCI/ADR-RES), cisplatin preferentially induces NF- $\kappa$ B activity compared to cell lines with a more mesenchymal phenotype (SKOV-3) (11).

We have previously shown that ID8 mouse ovarian cancer cells stably transfected with the NGL reporter (ID8-NGL) are suitable for tracking NF- $\kappa$ B activity in cultured cells and in a syngeneic mouse model of ovarian cancer (14). Parental ID8 cells are known to be relatively sensitive to cisplatin (37), and we confirmed that ID8-NGL cells were more sensitive to cisplatin than any of the human cell lines tested (Fig 1C). The combination of TQ and cisplatin displayed synergy in SRB assays in ID8-NGL cells, as evidenced by a Combination Index significantly less than 1 at Effective Doses (ED) ED50, ED75 and ED90. Moreover, the pro-apoptotic effects of cisplatin were markedly enhanced by combination TQ treatment (Fig. 1D), indicating apoptosis induction was an important mechanism of action of the anti-tumor effects of the combined drugs. Consistent with our previous observations (14), TQ reduced NF- $\kappa$ B reporter activity by approximately 50% in luciferase assays after 24 hour treatment.

Cisplatin induced a modest, but statistically significant, increase in NF- $\kappa$ B reporter activity ( $20.1 \pm 2.7\%$ ,  $p = 0.045$ ) (Fig 1E), which was reduced to levels comparable to TQ alone by combining TQ and cisplatin. Collectively, these results suggest a link between NF- $\kappa$ B inhibition and enhanced response to cisplatin in cultured ovarian cancer cells.

### **TQ enhances anti-tumor effects of cisplatin in a syngeneic mouse model of ovarian cancer.**

For studies in our established ID8-NGL syngeneic mouse model (14), C57BL/6 mice injected with ID8-NGL cells were treated with vehicle, TQ and/or cisplatin from day 30-60 following tumor cell injection, and then sacrificed. As shown in Fig. 2A-C, cisplatin alone reduced established indices of tumor burden in mice with intraperitoneal tumors, volume of ascites, number of peritoneal implants and mesenteric tumor mass (14), by  $>80\%$  compared to vehicle-treated mice. Combining TQ and cisplatin resulted in enhanced reduction in peritoneal implants and mesenteric tumors compared to either drug alone, with a similar effect on ascites volume that just failed to reach statistical significance (Fig 2A-C). Surprisingly, treatment with TQ alone induced a 2-fold increase in ascites volume, with no overall effect on peritoneal or mesenteric tumors. We have confirmed these observations for TQ-only treatment in several additional experiments (data not shown).

Immunofluorescence analysis of tumors demonstrated that, consistent with effects on tumor burden, cisplatin reduced the percentage of cells positive for the proliferation marker, Ki67/mib-1 by  $83 \pm 6\%$  and increased cells showing expression of the apoptosis marker cleaved caspase-3, apoptosis (Fig. 2D-E). Compared to cisplatin or TQ treatment alone, the TQ/cisplatin combination significantly greater levels of apoptosis (Fig. 2D-E), consistent with our in vitro results.

Contrasting drug effects on NF- $\kappa$ B reporter activity were observed in harvested ID8-NGL tumors compared to cultured cells. As shown in Fig 3A, treatment with TQ unexpectedly led to an overall increase in NF- $\kappa$ B reporter activity in luciferase assays. However, combining TQ and cisplatin abrogated TQ-stimulation, leading to overall similar levels compared to vehicle and cisplatin alone (Fig 3A). Supporting our results, QPCR analysis of steady-state mRNA levels of the established NF- $\kappa$ B targets, TNF- $\alpha$  and IL-1 $\beta$ , in drug-treated tumors revealed a similar pattern of effect to the luciferase assays (Fig 3B). We also determined TNF- $\alpha$  and IL-1 $\beta$  mRNA expression in macrophages isolated from ascites fluid using a differential adhesion method (Fig 3C). Expression of both genes was robustly stimulated by TQ treatment, an increase that was completely abrogated in the TQ/cisplatin combination. Although cisplatin alone significantly reduced TNF- $\alpha$  mRNA levels, overall expression levels of both genes were similar following treatment with combined TQ/cisplatin and vehicle treatment. Combined with the tumor data above (Fig 17B), our results suggest that in contrast to cultured cells, enhanced anti-tumor effects of TQ/cisplatin compared to vehicle or cisplatin alone were not mediated by reduced NF- $\kappa$ B activity in tumors or macrophages.

### **Analysis of macrophage infiltration into tumors**

We have previously demonstrated extensive macrophage infiltration into intraperitoneal tumors derived from ID8-NGL cells (14). In order to assess whether drug effects on tumor burden were reflected in changes in macrophage infiltration and/or macrophage populations, we measured expression of the well-established macrophage marker, F4/80, and the marker of M2-like macrophages, arginase-1, in IF assays. Representative images are shown in Fig 4A. As shown in Fig 4B, the TQ/cisplatin combination significantly reduced the percentage of cells staining positive for arginase-1 compared to vehicle, TQ and cisplatin alone. In contrast, overall macrophage infiltration, measured by the percentage

of F4/80-positive cells, was not changed by cisplatin alone or in combination with TQ, but was markedly increased by TQ treatment. These results indicate that the TQ/cisplatin combination induced changes in the tumor microenvironment prohibitive for tumor progression.

**Analysis of ascites fluid demonstrated reduced levels of M2 macrophage markers and pro-tumorigenic cytokines with combined TQ/cisplatin treatment.**

We have established that the enhanced response to TQ/cisplatin in our syngeneic model was not mediated by a TQ-mediated reduction in NF- $\kappa$ B activity in tumors, but was potentially mediated by changes in the local tumor microenvironment. In order to more completely understand drug effects on the tumor microenvironment, particularly the role of peritoneal macrophages, we analyzed the cell and soluble components of ascites fluid.

We have previously shown that mononuclear cells, particularly macrophages, are the predominant inflammatory cell population in the peritoneal cavity of ID8-NGL tumor-bearing mice (14). In harvested ascites or peritoneal lavage fluid from drug-treated mice, we first performed morphological analysis of cytopsin slides and demonstrated an overall decrease in mononuclear cells with cisplatin treatment compared to vehicle (Fig 5A). TQ alone markedly increased mononuclear cell number, consistent with an elevated inflammatory response, which was decreased by the TQ/cisplatin combination to levels comparable to those of cisplatin treatment alone (Fig 5A). Further dissection of the specific macrophage populations present was performed in isolated macrophages. First, QPCR analysis showed that cisplatin significantly reduced expression of established markers of pro-tumorigenic M2-like macrophages (mannose-receptor and IL-10) and increased expression of an anti-tumorigenic M1-like mark, CCL3 (Fig. 5B). The TQ/cisplatin combination also led to significant

reduction of the stimulatory effects of TQ alone on the M2 marks (Fig. 5B) 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). Second, immunofluorescence analysis of the established M2 marker, arginase-1, in isolated F4/80-positive macrophages demonstrated a similar induction of expression by TQ alone expression to mannose-receptor or IL-10 above (Fig 5C). Cisplatin significantly reduced the number of arginase-1/F4/80 double positive cells alone and when combined with TQ.

We analyzed the cytokine profile of ascites fluid harvested from drug-treated mice using a commercially available cytokine array plate (Signosis Inc.). As shown in Fig 5D, there was evidence of co-ordinated regulation of cytokines Both cisplatin- and TQ/cisplatin-treated mice displayed up-regulation of multiple cytokines with known anti-tumorigenic functions (CCL3, TNF- $\alpha$ , CCL5, IL-17a) and down-regulation of pro-tumorigenic cytokines (IL-10, MCP-1) (38-39) and growth factors linked to angiogenesis, increased vascular permeability and/or epithelial-mesenchymal transition (VEGF, IGF-1, FGF-2) (39-41). Finally, in ELISA assays of ascites fluid, we confirmed that the TQ/cisplatin significantly reduced levels of VEGF compared to cisplatin alone and vehicle and completely abrogated the stimulatory effect of TQ (Fig 5E).

## DISCUSSION

Cisplatin resistance is common in ovarian cancer, and a key mechanism of resistance to cisplatin-mediated cytotoxicity is induction of NF- $\kappa$ B (11). Therefore, we tested the ability of the promising NF- $\kappa$ B inhibitor, TQ, to sensitize ovarian cancer cells to cisplatin in established preclinical models. We demonstrated that the combination of the NF- $\kappa$ B TQ and established cisplatin chemotherapy has synergistic anti-tumor effects in cultured human and mouse ovarian cancer cells. Enhanced anti-tumor effects compared to either drug alone were also observed in a syngeneic mouse model that recapitulates the peritoneal carcinomatosis, ascites formation and inflammatory cell infiltrates, particularly peritoneal macrophages, of human disease (14). The combination reduced ascites formation and tumor burden; tumor proliferation and apoptosis; expression of the M2-like marks, mannose-receptor and IL-10, in isolated peritoneal macrophages; and expression of pro-tumorigenic cytokines and growth factors in ascites fluid, such as IL-10 and VEGF (38-41). We also showed that infiltration of tumors by macrophages was sensitive to combined TQ and cisplatin treatment, since a strong reduction in macrophages positive for the M2-like pro-tumorigenic mark, arginase-1, was observed compared to cisplatin alone. Our observations are consistent with studies demonstrating a key role for immune cell infiltration in both progression and regression of ovarian tumors following drug treatment (21-23).

There were contrasting effects on NF- $\kappa$ B reporter activity between cultured ID8-NGL cells and in tumors. TQ-mediated inhibition of basal and cisplatin-induced NF- $\kappa$ B activity was associated with cytotoxicity in vitro. In contrast, there was no overall difference in NF- $\kappa$ B reporter activity in tumors treated with TQ/cisplatin or cisplatin alone, although the combination markedly inhibited expression of specific pro-tumorigenic NF- $\kappa$ B targets such as VEGF. There were also clearly contrasting effects short-term (10 day) and longer-term (30 days) exposure of tumor-injected mice to TQ. We have previously

published that short-term TQ treatment reduces NF- $\kappa$ B reporter activity in tumors accompanied by increased expression of the M1-like macrophage marker CCL3, suggestive of anti-tumor activity. By contrast, 30 day TQ treatment unexpectedly resulted in stimulation of NF- $\kappa$ B reporter activity in tumors accompanied by markedly increased ascites formation, expression of M2 macrophage marks in tumor-infiltrating macrophages and those isolated from ascites fluid, and of VEGF levels in ascites. These observations are suggestive of an elevated inflammatory response with prolonged exposure to a systemic NF- $\kappa$ B inhibitor in our model. It is possible that this effect is mediated by off-target actions of TQ, since it is not a specific NF- $\kappa$ B inhibitor. These observations are consistent with a recent study in a lung cancer model showing that prolonged treatment with the NF- $\kappa$ B inhibitor bortezomib has pro-inflammatory effects and promotes tumor progression (42). In contrast, short exposure to bortezomib produces the anticipated effects of inhibiting tumor cell growth in that study. Increased ascites in response to prolonged exposure to systemic NF- $\kappa$ B inhibitors has high potential relevance to the clinic, since select NF- $\kappa$ B inhibitors are being used as mono-therapy and in combination with other chemotherapeutic drugs in clinical trials in ovarian cancer patients.

In conclusion, our studies suggest caution needs to be employed in the use of systemic NF- $\kappa$ B inhibitors and emphasizes the need for therapy specifically targeting NF- $\kappa$ B signaling in tumor cells and directly modulating its activity in specific inflammatory populations, such as macrophages. Since improving the response to cisplatin is a critical question in the clinical management of ovarian cancer, our results provide strong rationale for future clinical studies of the role of targeting NF- $\kappa$ B in sensitizing resistant tumors to cisplatin in a significant number of patients.

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## FIGURE LEGENDS

### **Figure 1. TQ and cisplatin synergistically decrease cell viability in cultured ovarian cancer cells.**

(A) SRB assays showing drug effects of TQ and/or cisplatin (concentrations in mM, 72 hours) in 3 human ovarian cancer cell lines (NCI/ADR-RES, OVCAR3, SKOV-3) and the mouse ovarian cancer cell line, ID8-NGL. Isobologram analysis for (B) NCI/ADR-RES, OVCAR3 and SKOV-3 cells and (C) ID8-NGL cells showing Combination Index for, where applicable, Effective Doses (ED) ED50, ED75 and ED90. A combination  $< 1$  indicates a synergistic drug interaction between TQ and cisplatin. Values are mean  $\pm$  SE for 3 experiments; \*  $p < 0.01$ , Student's t test. N/A; not applicable. (D) Western Blot analysis of cleaved PARP following 24 hour treatment of ID8-NGL cells with TQ (25 $\mu$ M), cisplatin (1 $\mu$ M) or the combination. Actin was used as a loading control. (E) Luciferase assay of ID8-NGL cells following 24 hour treatment with TQ (25 $\mu$ M), cisplatin (1 $\mu$ M) or the combination. Values are mean  $\pm$  SE for 3 experiments; \*  $p < 0.01$  compared to control, Student's t test.

### **Figure 2. TQ and cisplatin combine to decrease tumor burden and tumor cell proliferation and apoptosis.**

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. Values are mean+SD for 5 mice per group. (D) The percentage of tumor cells positive for the (D) proliferation marker Ki67/mib-1 and cleaved caspase-3 staining in ID8-NGL tumors harvested from mice treated with vehicle, TQ, cisplatin or the combination. (E) Representative fields of drug-treated tumors. Ki67/mib-1 or cleaved caspase-3 are in green. DAPI-stained nuclei are in

blue. \*  $p < 0.01$  relative to vehicle; NS: not significant relative to vehicle; all  $p$  values determined by Mann-Whitney test.

**Figure 3. NF- $\kappa$ B activity in ID8-NGL tumors treated with TQ and/or cisplatin.** BL/6 mice were injected with ID8-NGL cells and treated with vehicle, TQ (20 mg/kg), cisplatin (2 mg/kg) or the combination. (A) Luciferase assays of NF- $\kappa$ B reporter activity in harvested ID8-NGL tumors. QPCR analysis of the established NF- $\kappa$ B target genes, TNF- $\alpha$  and IL-1 $\beta$ , in (B) harvested tumors and (C) isolated macrophages from ascites fluid. Relative expression was calculated using corresponding GAPDH levels as the internal control. \*  $p < 0.01$  relative to vehicle; NS: not significant relative to vehicle; all  $p$  values determined by Mann-Whitney test.

**Figure 4. Macrophage infiltration in tumors treated with TQ and/or cisplatin.** BL/6 mice were injected with ID8-NGL cells and treated with vehicle, TQ (20 mg/kg), cisplatin (2 mg/kg) or the combination. (A) IF analysis of expression of the macrophage markers F4/80 (green) and arginase-1 (red). DAPI-stained nuclei are in blue. (B) Quantification of the percentage of total cells positive for F4/80 and cells co-staining for F4/80 and arginase-1. Values were determined from 5 representative fields at high power (x40). \*  $p < 0.01$  relative to vehicle; NS: not significant relative to vehicle; all  $p$  values determined by Mann-Whitney test.

**Figure 5. Macrophage and cytokine profile analysis in ascites from drug-treated mice.** (A) Overall number of mononuclear cells increased in ascites collected from TQ-treated mice, as quantified in differential cell counts of H&E-stained cytospin slides. (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) Immunofluorescent detection of the M2 macrophage marker, arginase-1, in F4/80 positive macrophages isolated from ascites fluid. (D) Cytokine/growth factor profiles in ascites from drug-treated mice. (E) VEGF levels in ascites fluid was measured by ELISA and expressed relative to corresponding protein levels. Values are mean+SE for 3-5 mice per group. \*  $p < 0.01$  relative to vehicle; NS: not significant relative to vehicle; all p values determined by Mann-Whitney test.