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

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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 potentiate the effects of cytotoxic agents in ovarian cancer cells. Thus, a promising strategy in ovarian cancer treatment is the combination of NF-κB inhibitors with current platinum-based regimens. Equally relevant are the potential effects of NF-κB inhibition in host cells such as peritoneal macrophages, thought to play a pro-tumor role during ovarian cancer progression. We will define patterns of NF-κB activity in host cells using NF-κB reporter (NGL) transgenic mice injected with mouse ID8 ovarian cancer cells. NF-κB activity in tumor cells will be monitored through stable transfection of the NGL reporter. We have begun to characterize these syngeneic models to determine reliable end-points measuring tumor burden and to establish markers of macrophage function. We have detected substantially increased NF-κB reporter activity in tumor cells in late stages of progression. Finally, we have shown that the NF-κB inhibitor Thymoquinone (TQ) induces anti-tumor effects in ovarian cancer cells alone and in combination with cisplatin, but induces an unexpected promotion of progression in vivo.
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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-kappaB (NF-κB) signaling pathway is known to play an important role in several malignancies, including ovarian cancer [3-8]. Constitutive activation of NF-κB 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-κB are known to suppress angiogenesis and progressive tumor growth [9], and potentiate the anti-tumor activity of cytotoxic agents [10], in ovarian cancer cells. Thus, a promising strategy in ovarian cancer treatment is the combination of NF-κB inhibitors with current platinum-based regimens. Equally relevant, but far less understood are the potential effects of NF-κB 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-κB signaling activity [11] 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-κB inhibition [11]. However, major gaps in knowledge still remain regarding the specific influence of NF-κB, 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-κB 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-κB reporter transgenic mice expressing a green fluorescent protein (GFP)/luciferase fusion product under the control of a synthetic NF-κB-dependent promoter [12, 13], allowing intra-vital mapping of NF-κB activity in the host during tumor development. In a complementary approach, ID8 cells stably transfected with the NF-κB reporter will be monitored in wild-type mice to study measure NF-κB activity in the developing tumor. Completion of the proposed work will not only provide a unique insight into the role of NF-κB in ovarian cancer progression, but also provide a powerful tool for the preclinical testing of NF-κB 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-κB activation in both malignant ovarian epithelial cells and host macrophages in the peritoneal microenvironment. Our objectives are (1) defining the pattern of NF-κB activity in the host-tumor microenvironment during ovarian cancer progression and (2) evaluating consequences of inhibiting NF-κB activity in tumor and host cells during ovarian cancer progression via treatment with NF-κB inhibitors alone and in combination with cisplatin chemotherapy.
Task 1. Define the pattern of NF-κB activity in the host-tumor microenvironment during ovarian cancer progression:

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

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

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

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

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

The parental cell line for our ID8-NGL cells, ID8, has previously been shown to model the peritoneal carcinomatosis characteristic of advanced serous ovarian cancer [14]. To confirm that the ID8-NGL cells formed a similar pattern of abdominal tumorigenesis, we conducted an initial trial experiment. Wild-type BL6 mice were injected IP with ID8-NGL cells or IP with PBS only as a non-injected control. Mice were sacrificed at 30d, 60d or 90d. Bioluminescence imaging (BLI) of the 90d groups were performed at the 30d, 60d and 90d time points to non-invasively measure activity of the NGL reporter in the tumor cells (see Fig 2 for a schematic representation of our experimental design).

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

Bioluminescence analysis was performed in the Vanderbilt University Small Animal Imaging Core using the Xenogen IVIS 200 bioluminescent image system with Living Image acquisition software (Caliper Life Science). Quantification of bioluminescence in the peritoneal cavity was performed through region of interest analyses of a standardized area based on anatomical landmarks. Representative images of injected versus non-injected mice are shown in Fig 4A. Levels of NF-κB reporter activity in tumor cells increased over the duration of the experimental period (Fig 4B). In contrast, non-injected mice displayed no measurable bioluminescence above background. Because we are imaging tumor cells with a NF-κB-dependent reporter, these BLI analyses are unable to differentiate between increases in reporter activity due to overall increased tumor cell number and a specific increase in NF-κB activity in the cells. Therefore, to address this issue, we performed luciferase assays of snap-frozen tumor tissue. As shown in Fig 4C, when corrected for cellular protein, there was a specific increase in NF-κB activity in later stages of tumor progression.
We confirmed that the abdominal distension observed in living mice was due to accumulation of ascites fluid at sacrifice. Characteristic of tumors from ID8 cells, the ascites contained significant amounts of blood. We were able to reliably collect the ascites fluid with a hypodermic needle. Therefore, volume of ascites collected at sacrifice will be another primary end-point of measuring extent of disease. Besides blood cells, we anticipated that ascites would contain inflammatory cells from the host and tumor cells. To assess effects during early stages of tumor progression, we performed peritoneal lavages with sterile PBS to collect peritoneal cell populations in injected mice prior to the development of ascites or in control non-injected mice.

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

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

Our cytospin analyses revealed that macrophages were the predominant inflammatory cell population present in the peritoneal cavity of either non-injected or injected mice. Macrophages made up consistently 90% or greater of the inflammatory cells harvested in ascites fluid or in peritoneal lavages irrespective of duration of exposure to tumor cells (Fig 5A). In contrast, the overall number of macrophages harvested did increase with the presence of tumor cells per se and with increasing duration of tumor progression (Fig 5B). These data are suggestive of an elevated inflammatory response due to the presence of tumor cells. Currently, the precise contributions to this inflammatory response by host-initiated defenses against the tumor cells or by tumor-directed mechanisms promoting tumorigenesis are unknown. We will begin to address this issue in this proposal, through examining expression of markers of cytotoxic, anti-tumor M1 and pro-tumor M2 macrophages (see below).

During these cytospin counts, large cell clumps without definable borders were also detected (see arrows in Fig 5C). These were likely tumor cells, consistent with abdominal tumor dissemination through the peritoneal fluid. This was confirmed with luciferase activity assays of the tumor cell-specific NF-κB reporter in cell pellets collected from ascites fluid or peritoneal lavages. Quantification of raw NF-κB reporter activity indicated that there was a time-dependent increase (Fig 5D). In contrast, when the values were normalized for corresponding cell protein levels, there was no overall difference between the time points (Fig 5E). These data suggest that the overall number of tumor cells in peritoneal fluid increases with ovarian cancer progression, which we would anticipate. However, to make definitive conclusions about tumor cell burden in ascites fluid and NF-κB activity in these disseminating tumor cells, we will need to efficiently separate them from macrophages (see following paragraph for strategies to do this). Increases in macrophage numbers that we have demonstrated at later time points of tumorigenesis will contribute to total cellular protein levels, “diluting” our ability to detect specific effects in the tumor cells when luciferase activity is normalized to protein.

We conducted preliminary quantitative RT-PCR (QPCR) analyses to determine the expression of marks of M1 (cytotoxic) and M2 (tumor-associated) macrophages from RNA extracted from ascites or peritoneal lavage fluid. The markers chosen, CCL3 and Mannose-receptor (Mann-R), were well-established marks of M1 and M2 macrophages, respectively [15]. We anticipate that the presence of tumor cells in ascites fluid would be a potential confounding factor in these analyses. Therefore, we first quantified mRNA expression of the epithelial marker, CK-18. As shown in Fig 6A, RNA extracted from ascites collected from injected mice showed approximately 5-fold and 9-fold enrichment of the epithelial mark, cytokeratin-18 (CK-18) at 30d and 90d after tumor cell injection, respectively. Results were expressed relative to corresponding levels of the internal reference gene, GAPDH. Subsequent analyses quantifying mRNA expression of CCL3 and Mann-R in injected versus non-injected mice were therefore normalized to corresponding CK-18 levels. As shown in Fig 6B, there was a relative increase in Mann-R mRNA expression in injected mice compared to non-injected controls, with higher relative levels at 90d. A similar pattern of effect was observed for CCL3, except the overall magnitude of the increase was less than Mann-R (Fig 6C). These preliminary analyses revealed that there is an overall increase in expression of M1 and M2 macrophage marks in response to the presence of tumor cells in the
peritoneal cavity. However, it is unclear from our data whether the relative proportion of M2 to M1 macrophages changes in response to tumor cells. We would anticipate such an increase, but are currently limited by our ability to harvest pure populations of tumor cells or peritoneal macrophages, as described in the preceding paragraph. We will pursue more sophisticated methods, such as differential plating adhesion or column-based methods to separate macrophages from tumor cells before extracting protein for luciferase assays or RNA for QPCR. We will also perform experiments utilizing flow cytometry to separate macrophages on the basis of M1 and M2 marks to quantify specific macrophage populations.

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

We have experienced slower than expected breeding of our NGL reporter transgenics. Consequently, we have not been able to conduct an experiment with as many mice as originally planned (3 time points x 10 injected mice + 5 non-injected mice = 45). To address this issue, we have initiated a breeding program with homozygous NGL mice to increase yields of NGL-positive progeny.

We performed an initial trial with 4 NGL reporter mice injected with ID8 cells and measured BLI at baseline (prior to tumor cell injection) and 30, 60 and 69d after injection (Fig 7). In NGL mice, BLI measures NF-κB reporter in host tissue. Our underlying hypothesis is that increases in NF-κB activity in both tumor and host cells contribute to ovarian cancer progression. As described above, we have identified markedly increased NF-κB activity in tumor cells at later stages of progression and increased infiltration of inflammatory cells into the peritoneal cavity. Therefore, we anticipated that we would observe increased reporter activity in the peritoneal cavity in NGL reporter mice during progression. In contrast, there was a trend towards decreased NGL reporter activity at later time points following ID8 cell injection, which reached statistical significance at day 60 (Fig 8A-C). Similar results were obtained for raw abdominal luciferase activity (Fig 8B) and when abdominal activity was normalized to corresponding brain signal (Fig 8C).

We acknowledge that this is a small group of mice, and we are very cautious in our interpretation. In fact, results presented in Task 2c shows no statistically significant difference between any time points in mice receiving only Vehicle treatment. It is possible that the sensitivity of detection of reporter activity in individual cells in the peritoneal cavity is below the limits of the imaging device, particularly when the signal may also be “damped” by the presence of ascites fluid. This contrasts to signal from the ID8-NGL tumor cells, which form a solid mass. It is clear, therefore, that more sophisticated analyses at a single cell level, for example flow cytometry, will be required to produce a definite conclusion about NF-κB reporter activity in host cell populations such as macrophages.

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

Our experiments using liposomal clodronate to inhibit peritoneal macrophages will be started imminently. We are receiving 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].

Milestone #1: We have confirmed that there is specific upregulation of NF-κB reporter activity in ID8-NGL cells in response to multiple activators of NF-κB. We have also confirmed that ID8-NGL cells form tumors in the abdominal cavity of mice that are reproducible and have identified two indices measuring direct tumor burden: number of peritoneal implants and mesenteric tumor mass. We have validated various methods of analyzing tumor cells and components of the host response from the collection of ascites fluid or from peritoneal lavages. Collectively, the result of subtasks 1a and 1c indicate the robustness of our ID8-NGL cells as a model for monitoring NF-κB activity in tumor cells during ovarian cancer progression and regression following drug treatment (Tasks 2 and 3). These aims include sufficient controls to enable preparation of a
manuscript for peer-reviewed publication in an ovarian cancer journal. Results from subtasks 1d and 1b indicate that we have established the model of growing ID8 cells in NGL reporter mice, and that BMDM-NGL cells harvested from these mice respond as expected to TNF-α stimulation, respectively. As discussed above, however, we are limited in making specific conclusions about NF-κB activity in host cells by our current methods. The remainder of Task 1 will be undertaken in Year 2 of funding.

**Task 2. To determine the consequences of reducing NF-κB response in tumor and host cells by treatment with NF-κB inhibitors (thymoquinone (TQ) and curcumin).**

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

Effects of the NF-κB inhibitor Thymoquinone (TQ) in cells cultured *in vitro* are summarized in Fig 9. We have validated that short-term treatment with TQ induces decreases the stimulatory effects of TNF-α on NF-κB reporter activity in ID8-NGL cells (Fig 9A&B). When cells are treated for longer periods with TQ, we observe reduced NF-κB reporter activity after 24h (Fig 9C) and a concentration-dependent reduction in cell growth after 72h (Fig 9D). We have also validated that TQ inhibits TNF-α-induced NF-κB reporter activity in BMDM-NGL cells (Fig 9E).

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

We have preliminary data showing that the NF-κB inhibitor Thymoquinone (TQ) inhibits cell growth and survival in ID8-NGL cells cultured in vitro (Fig 9). We conducted an initial experiment using TQ in WT mice injected with ID8-NGL cells as described in Task 1c. Mice were treated with Vehicle or TQ from day 30-60 following tumor cell injection, and sacrificed at day 70. 8 mice per group were used. BLI was performed at 30d, 60d and 70d in a subset of the mice (Fig 10A).

Treatment of tumors with TQ produced several unanticipated results:

First, levels of NF-κB reporter activity in tumor cells from TQ-treated mice was significantly higher compared to Vehicle-treated mice (Fig 10B). We confirmed increased NF-κB reporter activity in TQ-treated tumors by luciferase activity assays in snap-frozen tumors harvested at sacrifice (Fig 10C). Representative BLI images are shown in Fig 10D.

Second, TQ treatment resulted in significantly greater ascites volume at sacrifice (Fig 11A&B). In contrast, in counts directly measuring tumor burden, there were no significant differences in the number of peritoneal implants and mesenteric tumor mass between Vehicle and TQ-treated mice. Furthermore, as shown in Fig 12A&B, TQ treatment resulted in a significant reduction in the percentage of cells positive for the proliferation mark, Ki67, consistent with its cytostatic effects in ID8-NGL cells cultured *in vitro*.

Therefore, these results suggest that TQ has complex effects *in vivo* compared to *in vitro*. There does appear to be a component of direct inhibition of tumor cell growth, but it is possible that inhibition of NF-κB activity in host cells is having a pro-tumorigenic, possibly pro-inflammatory effect. This would be consistent with a recent study in a lung cancer model showing that prolonged treatment with the NF-κB inhibitor bortezomib has pro-inflammatory effects and promotes tumor progression [17]. In contrast, short exposure to bortezomib produces the anticipated effects of inhibiting tumor cell growth in that study. A possible immunological mechanism for promotion of tumor progression through systemic NF-κB inhibition is that anti-tumor cytotoxic macrophages
may require NF-κB signaling for normal function, and NF-κB inhibitors impair their ability to efficiently target tumor cells [15].

Cytospin analyses revealed that macrophages were the predominant inflammatory cell population present in the peritoneal cavity of either Vehicle or TQ-treated mice (Fig 13A). In contrast, the overall number of macrophages harvested did increase with TQ treatment (Fig 13B), 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-κB reporter in cell pellets from ascites fluid. As shown in Fig 13C, there was increased NF-κB reporter activity in tumor cells in the ascites from TQ-treated mice. This result was confirmed by QPCR analysis of CK18 mRNA expression (Fig 14A), which showed that TQ reduced the relative epithelial content of ascites fluid.

Collectively, these data suggest that TQ has both anti- and pro-tumorigenic effects, likely mediated through effects on tumor cells and host inflammatory cells, respectively. However, the overall effect of prolonged treatment is deleterious, as evidenced by increased accumulation of ascites. An increased pro-tumorigenic inflammatory response mediated by TQ in our model is also supported by the upregulation of the tumor-associated macrophage M2 mark, Mann-R (Fig 14B).

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

We also performed an experiment evaluating the effects of TQ on NF-κB 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. 7 mice per group were used. BLI was performed at baseline, 30d, and 60d in a subset of the mice (Fig 15A).

Minimal overall changes were observed between Vehicle and TQ-treated groups when NF-κB reporter activity was quantified (Fig 15B). In contrast to the experiment described in Task 1d, there was no significant reduction in NF-κB reporter activity during ovarian cancer progression under basal conditions (that is, Vehicle-treated mice). However, consistent with the results from our ID8-NGL cells, ascites volume at sacrifice was significantly higher with TQ treatment (Fig 16A), but the number of peritoneal implants and mesenteric tumor mass were not significantly different between Vehicle and TQ-treated mice.

Milestone #2: We have confirmed that the NF-κB inhibitor TQ produces similar inhibitory effects on NF-κB signaling and on cell growth and viability in our mouse ovarian cancer cell models as has previously reported in human ovarian cancer cell lines and other cancer cell types. We have evidence by IHC detection of Ki67 suggesting that TQ does exert an expected direct anti-proliferative effect on the cancer cells, but paradoxically also observed a stimulatory effect of prolonged TQ treatment on ascites accumulation in vivo. Like other NF-κB inhibitors utilized clinically, such as curcumin and bortezomib, TQ is not a specific inhibitor of NF-κB. This may potentially underlie, at least in part, the augmented inflammatory response observed in our Task 2 studies. Therefore, we will repeat our experiments with the specific NF-κB inhibitor listed as an alternative in our original grant application, BMS-345541. This drug has been previously utilized by the Yull/Blackwell laboratories [12, 18]. The results of Task 2 suggest we may need to reconsider the experimental design of our proposed experiments in Task 3 combining an NF-κB inhibitor with cisplatin in vivo. These aims include sufficient controls to enable preparation of a manuscript for peer-reviewed publication in an ovarian cancer journal. The remainder of Task 2 will be undertaken in Year 2 of funding.
Task 3. To evaluate NF-κB activity in host and tumor cells and efficacy of treatment with NF-κB inhibitors in combination with cisplatin (Months 6-24).

3a. Treat ID8-NGL or BMDM-NGL cells with NF-κB inhibitor in the presence or absence of cisplatin. We have conducted preliminary experiments with the combination of TQ and cisplatin in SRB growth assays and have observed evidence of synergistic anti-tumor effects (Fig 17).

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

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

Finally, the studies listed under Tasks 3b-c were scheduled for year 2 of this funding and have not yet commenced.

Milestone #3: We have confirmed the potential of combination NF-κB inhibitor and cisplatin in inducing synergistic anti-tumor effects in vitro. The remainder of Task 3 will be undertaken in Year 2 of funding.
KEY RESEARCH ACCOMPLISHMENTS

During this reporting period:

1) We have established a new collaboration between the Khabele/Wilson and Yull laboratories, taking advantage of each group’s expertise to perform studies that would be unlikely to be done by either group alone. We have regular joint lab meetings for data sharing and interpretation.

2) We have developed and begun to characterize syngeneic models of ovarian cancer progression permissive for non-invasive monitoring of NF-κB activity in tumor cells (via ID8-NGL cells grown in WT mice) and in host cells (via ID8 cells grown in NGL reporter mice).

3) We have identified several robust indices for analyzing tumor burden in an intra-peritoneal cancer model (volume of ascites, number of peritoneal implants, mesenteric tumor mass).

4) We have obtained data suggesting that activity of NF-κB is increased in ovarian cancer cells, particularly in the late stages of progression.

5) We have obtained data demonstrating that the NF-κB inhibitor TQ induces anti-tumor effects in ovarian cancer cells in vitro alone and in combination with cisplatin, an established chemotherapeutic agent in ovarian cancer treatment.

6) However, our in vivo data indicate that TQ treatment has more complex effects on ovarian cancer progression, with an increased inflammatory response being a possible mechanism by which prolonged NF-κB inhibition can lead to augmented progression. These data offer caution for the use of NF-κB inhibitors in ovarian cancer patients.
REPORTABLE OUTCOMES

Manuscripts, Abstracts, Presentations


Funding applied for based on work supported by this award

Yull F: Targeted activation of macrophages to limit ovarian cancer progression, $125,000/yr. Dr. Yull will use this award to explore the effects of targeted activation or inhibition in peritoneal macrophages on ovarian cancer progression in the ID8-NGL model of tumorigenesis. OCRP Pilot Award 2012. [no appendix entry]
CONCLUSIONS

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

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

APPENDICES

Appendix materials start with Supporting Data [pages 16-32]

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 I Lianyi Chen are also supported in part by this grant.

1. Wilson Andrew J., Biographical Sketch [pages 33-35]
2. Yull, Fiona E., Biographical Sketch [36-39]

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

Fig 1. A) Similar to TNF-α (10ng/ml), the established activator of NF-κB signaling IL-1β (10ng/ml) also significantly increased NF-κB reporter activity in ID8-NGL cells grown in vitro (4h exposure). B) TNF-α (10ng/ml) also stimulates NF-κB reporter activity in BMDM-NGL cells (6h treatment). Values are mean ± SD of 3 independent experiments. * p < 0.01 relative to Control, Student’s T test.
**Fig 2.** Schematic representation of the timeline for our initial trial experiment in WT BL/6 mice injected with ID8-NGL cells or mock-injected (PBS) at Day 0. BLI: bioluminescence imaging; Sac: sacrifice.
**Fig 3.** A) Mice injected with ID8-NGL cells show distended abdomen indicative of ascites formation at 90d after injection. B) Mice injected with ID8-NGL cells display abdominal dissemination of tumor cells. The main sites of tumor implantation are the peritoneal (yellow arrows) and in the mesentery (white arrow). C) H&E staining example of tumor implantation in the peritoneal wall.
Fig 4. A) Representative BLI of WT mice injected with ID8-NGL cells or PBS (non) over 90d following injection showing specific NF-κB reporter signal in tumor cells. B) Quantification of BLI in the peritoneal cavity shows an increase in NF-κB reporter activity with increasing time of progression. C) Luciferase activity was measured in harvested tumors and expressed relative to cellular protein. Data show a specific increase in NF-κB reporter in tumor cells in later stages of progression. ** p < 0.01 relative to 30d or 60d, Student’s T test. Data are mean + SD from 5 mice/group.
Fig 5. A) No significant differences in the proportion of macrophages counted in cytospins from ascites fluid or peritoneal lavages collected from injected or non-injected mice at sacrifice were seen. B) Overall number of macrophages collected increased in mice with tumors and with duration of progression. C) Representative images of cytospins showing the presence of tumor cells (yellow arrows). D) Raw luciferase activity in cell pellets from ascites fluid or peritoneal lavages show an increase with duration of progression. E) However, no differences were seen when luciferase activity was normalized to cellular protein in the samples described in D). ** p < 0.01 relative to 30d or 60d, Student’s T test. Values are mean + SD for at least 2 mice per group.
Fig 6. A) QPCR analysis of the mRNA expression of the following genes in RNA samples extracted from ascites fluid or peritoneal lavages collected at sacrifice: A) the epithelial marker, CK-18; B) the marker of tumor-associated M2 macrophages, mannose-receptor; C) the marker of cytotoxic anti-tumor M1 macrophages, CCL3. Values were normalized to GAPDH. * p < 0.01 relative to corresponding 90d non-injected control, Student’s T test.
**Fig 7.** Schematic representation of the timeline for our initial trial experiment in NGL reporter mice injected with ID8 cells at Day 0. Baseline BLI were taken prior to injection.
Fig 8. A) Representative BLI of NGL reporter mice injected with ID8 cells showing specific NF-κB reporter signal in host cells. B) Quantification of raw BLI in the peritoneal cavity shows decreased NF-κB 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, Student’s T test.
Fig 9. Effects of NF-κB inhibitor TQ in ID8-NGL cells grown in vitro. A) 2h pre-treatment with 50 μM TQ inhibited the stimulatory effect of 10 ng/ml TNF-α on NF-κB reporter activity in protein extracts. B) Western Blot showing the stimulatory effect of TNF-α on GFP and phospho-IκB expression after 4h, again inhibited by TQ. Representative BLI of ID8-NGL cells cultured in vitro showing similar effects is also shown. C) Reduction in NF-κB reporter activity following 24h treatment with 50 μM TQ. D) Results from SRB assays showing that TQ inhibits growth in a dose-dependent manner (72h treatment). E) 2h pre-treatment with 50 μM TQ also inhibited the stimulatory effect of 10 ng/ml TNF-α on NF-κB reporter activity in BMDM-NGL cells. * 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 10. A) Schematic diagram showing treatment schedule for Vehicle or TQ in WT mice injected with ID8-NGL cells. B) Quantification of BLI in the peritoneal cavity shows increased NF-κB reporter activity in TQ-treated mice. C) Luciferase activity was measured in harvested tumors and expressed relative to cellular protein. Data show a specific increase in NF-κB reporter in tumors from TQ-treated mice. D) Representative BLI images comparing NF-κB reporter signal in Vehicle and TQ-treated cells. * p < 0.01 relative to Vehicle, Student’s T test.
Fig 11. A) Representative images of Vehicle and TQ-treated mice shows greater abdominal distension in TQ-treated mice, indicative of ascites. B) Quantification of ascites fluid volume at sacrifice confirmed increased ascites in TQ-treated mice. In contrast, there were no significant differences in tumor burden between Vehicle and TQ-treated mice when C) peritoneal implants or D) mesenteric tumor mass were quantified. P values generated by Mann-Whitney test.
Fig 12. A) Representative images of IHC detection of the proliferation mark, Ki67, in ID8-NGL tumors detected from Vehicle or TQ-treated mice. B) TQ induced approximately 25% reduction in the percentage of nuclei positive for Ki67 staining. Values are mean ± SD for 5 independent fields from each of 2 Vehicle and 2 TQ-treated tumors. * P < 0.05 compared to Vehicle, Student’s T test.
Fig 13. A) No significant differences in the proportion of macrophages counted in cytospins from ascites fluid or peritoneal lavages collected from Vehicle or TQ-treated mice were seen. B) Overall number of macrophages collected increased in TQ-treated mice. C) TQ treatment significantly increased NF-κB reporter activity when luciferase activity was normalized to corresponding cellular protein in the samples described in C). * p < 0.01 relative to Veh, Student’s T test. Values are mean + SD for 3 mice per group.
Fig 14. A) QPCR analysis of the mRNA expression of the following genes in RNA samples extracted from ascites fluid or peritoneal lavages from Vehicle or TQ-treated mice: A) the epithelial marker, CK-18; B) the marker of tumor-associated M2 macrophages, mannose-receptor; C) the marker of cytotoxic anti-tumor M1 macrophages, CCL3. Values were normalized to GAPDH. p < 0.02 relative to corresponding 90d non-injected control, Mann-Whitney test.
Fig 15. 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. C) Representative BLI images comparing NF-κB reporter signal in Vehicle and TQ-treated cells.
Fig 16. A) Quantification of ascites fluid volume at sacrifice confirmed increased ascites in TQ-treated NGL reporter mice. In contrast, there were no significant differences in tumor burden between Vehicle and TQ-treated mice when C) peritoneal implants or D) mesenteric tumor mass were quantified. P values generated by Mann-Whitney test.
Fig 17. SRB assays showing that the combination of TQ (10µM) and cisplatin (1µM) produces an inhibitory effect on cell growth greater than the sum of the individual drug effects, suggestive of synergism. Values are mean + SD for 3 experiments; # p < 0.01 relative to the individual effect of each drug alone.
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.**

<table>
<thead>
<tr>
<th>NAME</th>
<th>POSITION TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrew James Wilson, Ph.D</td>
<td>Research Assistant Professor</td>
</tr>
</tbody>
</table>

**eRA COMMONS USER NAME** (credential, e.g., agency login)

WILSONAJ

**EDUCATION/TRaining** (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>MM/YY</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melbourne University, Australia</td>
<td>B.Sc (Hons)</td>
<td>1992-1994</td>
<td>Immunology/Pathology</td>
</tr>
<tr>
<td>Department of Medicine, Melbourne University, Australia</td>
<td>Ph.D.</td>
<td>1995-1998</td>
<td>Medicine</td>
</tr>
<tr>
<td>Department of Medicine, Melbourne University, Australia</td>
<td>Postdoctoral</td>
<td>1998-1999</td>
<td>Colon biology</td>
</tr>
<tr>
<td>Albert Einstein Cancer Center, Bronx, NY</td>
<td>Postdoctoral</td>
<td>2001-2003</td>
<td>Colon cancer</td>
</tr>
</tbody>
</table>

**A. Personal Statement**

I have a long-standing 11 year experience in cancer research including dissecting signaling pathways driving the tumorigenic process in cancer cells, generation and characterization of stable cell lines in tissue culture, and investigating efficacy of anti-cancer drugs in preclinical studies in vitro and in mouse models in vivo. At Vanderbilt, I have been able to apply this knowledge to discovering new treatment strategies for ovarian cancer in 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-κB function during cancer progression. Thus, I am well-placed to study the role of NF-κB 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 current project is to characterize the effects of established and novel NF-κB inhibitors on both tumor and, as can often be overlooked in cancer therapy, host cells.

**B. Positions and Honors**

**Positions and Appointments**

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


D. Research Support

**Active Support**

W81XWH-11-1-0509 Wilson (PI) 07/01/11-06/30/13
Department of Defense
Nuclear factor-kappa B in the host-tumor microenvironment of ovarian cancer
The major goal of this project is to determine the role of NF-κB in the progression of ovarian cancer in an innovative murine model, which will permit the evaluation of the anti-tumor effects of inhibitors of NF-κB alone and in combination with established chemotherapeutic drugs in ovarian cancer treatment.
Role: PI

K08CA148887  Khabele (PI)  5/1/10-4/30/15
NCI
Targeting Histone Deacetylases with Small Molecule Inhibitors in Ovarian Cancer
The major preclinical goal of this project is to determine the efficacy of Class I histone deacetylases, particularly HDAC3, as targets for therapy in ovarian cancer cells, as single agents and in combination with DNA damaging agents such as cisplatin. This project aims to translate these preclinical findings in murine models of ovarian cancer and ultimately in human ovarian cancer patients.
Role: Technical support

Completed Support

Marsha Rivkin Cancer Foundation Pilot Project Award  Wilson (PI)  04/01/10-03/31/11
The Role of the Nuclear Orphan Receptor TR3/Nur77 in Ovarian Cancer
The goals of this research are to determine the role of TR3 in apoptosis mediated by HDAC inhibitors and DNA damaging agents in ovarian cancer cells, and to determine whether there is an association between TR3 expression, response to therapy and/or tumor stage in a tissue microarray comprising tumors from over 200 ovarian cancer patients.
Role: PI
Yull, Fiona Elizabeth, D.Phil.

NAME

Yull, Fiona Elizabeth, D.Phil.

eRA COMMONS USER NAME (credential, e.g., agency login)

FIONA_YULL

POSITION TITLE

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.)

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<th>DEGREE</th>
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<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of St. Andrews, St. Andrews, UK</td>
<td>B.Sc. (Hons)</td>
<td>1985</td>
<td>Biochemistry w/Microbiology</td>
</tr>
</tbody>
</table>

A. Personal Statement

I have a longstanding 21-year 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-κB family of transcription factors in disease, particularly cancer. We have successfully used these in a large number of collaborative studies relating to the contribution of NF-κB signaling in several types of cancer including lung, breast, prostate and skin. My main interests have been on mammary cancer progression from primary tumor, through metastasis in the circulation to the establishment of final metastatic tumors in a secondary site and on how inflammation contributes to lung cancer. To address the roles of NF-κB in these processes we have developed a modular inducible “tool kit” of transgenics. These enable up and down-regulation of NF-κB signaling directed to specific cell types (mammary epithelium, macrophages and lung epithelium) providing the opportunity to address the roles of NF-κB in specific cells in the tumor microenvironment and defined stages of progression. Given the potential for macrophages to play a critical role in cancer progression, I now want to utilize our expertise with analysis of effects of modulation of NF-kappaB specifically within macrophages to attempt to develop new therapeutic approaches.

B. Positions and Honors

Positions and Employment

1985-1989 Predoctoral Fellow under the direction of Drs. A. and S. Kingsman, Oxford University, UK
1989-1995 Postdoctoral Fellow under the direction of Dr. J. Clark, The Roslin Institute, Roslin, Edinburgh, UK
1995-1998 Postdoctoral Fellow, Dept. of Microbiology and Immunology, under the direction of Dr. L. Kerr, Vanderbilt University Medical Center, Nashville, Tennessee
2000-2004 Research Assistant Professor, Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee
2004-2010 Assistant Professor of Cancer Biology, Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee
2010-Pres. Associate Professor, Dept. of Cancer Biology, VUMC, Nashville, TN

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
2010-2011 Ad hoc member, DOD BCRP FY10 Programmatic Review Panel

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

Honors
2000-2004 Aventis Leadership Development Program Fellow

C. Selected Peer-reviewed Publications (from 60 peer-reviewed publications)


D. Research Support

Ongoing Research Support

5 R01 CA 113734-04 (Yull) 07/01/07 - 05/31/12 (No cost extension – 05/31/13)
NIH/NCI
“Epithelial NF-κB Signaling in Mammary Tumorigenesis”

Goal: The proposed project will define the role of NF-κB signaling as a master regulator of these processes, integrating multiple processes and thereby determining ultimate physiological outcome.

Aims: Evaluate the effects of modulation of NF-κB activity within mammary epithelium on mammary development. We will utilize 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.

W81XWH-11-1-0242 (Yull) 07/01/11-06/30-13
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.

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

W81XWH-11-1-0509 (Wilson) 07/25/11-08/24/13
Department of Defense
Nuclear factor-kappaB Activity in the Host-tumor Microenvironment of Ovarian Cancer
Study the patterns of nuclear factor-kappa B activity in the host versus the tumor epithelium during progression of ovarian cancer in a murine model. Bioluminescent reporters in ovarian cancer cell lines or transgenic mice will determine patterns of NF-κB activity and responses to pharmacologic interventions during tumor progression.

4 R01 CA 076142-13 (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: Determine contributions of Pten and NF-κB signaling to AR action *in vitro*. Identify targets in the prostate for therapeutic intervention and to understand how NF-κB activation results in castrate resistant prostatic cells.

5 R01 HL 085317 (Blackwell)               04/10/07 - 03/31/17  
NIH/NHLBI  
“Epithelial-Fibroblast Interactions in Lung Fibrosis”  
Goal: This proposal uses novel mouse models to test the hypothesis that specific phenotypic alterations in alveolar epithelial cells affect the response to injurious stimuli, impact fibroblast activation, and determine the severity and progression of lung fibrosis.  
Aims: To define the extent of epithelial-mesenchymal transition as a source of fibroblasts in experimental pulmonary fibrosis. In these studies, we will determine the proportion of lung fibroblasts derived from epithelium via EMT and examine the phenotypic characteristics of epithelial-derived fibroblasts.

5 R01 78188-02 (McGuinness)    06/01/09 - 05/31/14  
NIH/NCI  
Impact of Inflammation on the control of muscle  
Goal: Identify the steps controlling MGU that are impacted by inflammation so that future therapies can have a more targeted approach in correcting MGU during an inflammatory stress such as sepsis  
Aims: The impact of LPS on the relative control glucose transport and glucose phosphorylation have in determining MGU. If inflammatory stress amplifies the impact LCFA and glucose availability have in modulating MGU. If modulating oxidative stress (Nitric oxide availability and NF-κB activation) will improve MGU by augmenting glucose phosphorylation and mitochondrial ATP flux.
Investigating the patterns of nuclear factor-kappa B activity in the host-tumor microenvironment during ovarian cancer progression


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-κB) is thought to be critical to the distinct clinical pattern of spread of ovarian tumors. However, the relative contribution of NF-κB 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-κB activity in these cell populations during ovarian tumorigenesis. To study the host NF-κB response, ID8 mouse ovarian cancer cells were injected intra-peritoneally into C57BL/6 mice carrying an NF-κB-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-κB 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-κB reporter activity during tumor progression. There was a modest overall decrease in abdominal NF-κB activity over time in ID8-injected NGL mice, although the possibility of “masking” effects of the ascites fluid on bioluminescence, and NF-κB activity in specific host cell populations, need to be elaborated. These models will allow us to define the patterns of NF-κB 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-κB in ovarian cancer.