AWARD NUMBER: W81XWH-22-1-0308

TITLE: Inhibition of Noncanonical NF-kappaB for Treatment of Ovarian Cancer

PRINCIPAL INVESTIGATOR: Fiona Yull

CONTRACTING ORGANIZATION: Vanderbilt University

REPORT DATE: JUNE 2023

TYPE OF REPORT: Annual Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUNE 2023	Annual	15MAY2022 - 14MAY2023

4. TITLE AND SUBTITLE

Inhibition of Noncanonical NF-kappaB for Treatment of Ovarian Cancer

5a. CONTRACT NUMBER
W81XWH-22-1-0308

5b. GRANT NUMBER
W81XWH-22-1-0308

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Fiona Yull

E-Mail: Fiona.yull@vanderbilt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Vanderbilt University
110 21st Ave. South, Suite 800
Nashville, Tn 37203-2408

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Inhibition of non-canonical NF-kappaB for treatment of ovarian cancer Background. Whether the **non-canonical NF-kB signaling pathway (nc-NF-kB)** represents a novel, distinct and improved target for therapy in ovarian cancer is unknown. In nc-NF-kB signaling**,** the p100/RelB complex in the cytoplasm, p100 is processed to truncated p52 and the complex translocates to the nucleus and mediates signaling. We have preliminary data showing that human and mouse ovarian cancer cells express nuclear p52, indicative of active nc-NF-kB signaling. Additional preliminary data shows inhibited growth of ovarian cancer cells via treatment with SN52 peptide that specifically inhibits nuclear import of p52 and RelB. Preliminary data also shows that mouse bone marrow-derived macrophages (BMDMs) polarized towards the M2 pro-tumor phenotype expresses higher levels of nuclear p52 than M1 or un-polarized cells. Moreover, high expression of p52 in high-grade serous ovarian tumors is associated with worse patient prognosis. This cumulative evidence suggests that nc-NF-kB represents an unrecognized therapeutic target.

15. SUBJECT TERMS

NF-kappaB, non-canonical pathway, doxycycline inducible transgenics, therapeutic peptide

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	19	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. Introduction

Ovarian cancer is the deadliest gynecologic malignancy in the United States. The prognosis is poor for patients with advanced, chemoresistant disease, and finding new treatment strategies to prolong life in these women is a key clinical challenge. Although much is known about the contributions of canonical NF-kappaB signaling to cancer, the non-canonical pathway remains relatively unexplored. Non-canonical NF-kappaB signaling (nc-NF-κB) may be a novel and important therapeutic target in ovarian cancer. Our preliminary data suggests that non-canonical NF-kappaB (nc-NF-κB) signaling is important for the growth and survival of tumor epithelial cells and also that its activity within macrophages may produce pro-tumor functions. This suggests that the under-investigated nc-NF-κB makes major contributions to disease progression. We have developed **unique** inducible transgenic mice that selectively increase nc-NF-κB signaling in specific cells (macrophages) when doxycycline is provided in drinking water. In combination with established models of tumor progression, these mice are powerful tools to elucidate roles of nc-NF-κB within macrophages in ovarian cancer progression. We will demonstrate that elevated nc-NF-κB in macrophages is sufficient to promote tumor progression, in part by generating an immune-suppressed environment but also, potentially by supporting angiogenesis. Because canonical signaling is vital for acute immune responses and its inhibition impacts immunity this has proven to be an ineffective target. However, it may be feasible to achieve therapeutic benefit without immune suppression by specific inhibition of nc-NF-κB. Options to target the non-canonical pathway are limited, due in part to lack of awareness that this represents a valid target. However, pilot studies of two interventions, a peptide inhibitor and nanoparticle-mediated inhibition in tumor-associated macrophages will be performed. Results will highlight the critical contributions of this signaling pathway, provide breakthroughs in understanding its contributions to progression of ovarian cancer, and test two different new treatments. As the potential contributions of this pathway are broad-ranging, the research findings will address multiple aspects of the OCRP overarching challenges. Studies will provide evidence that this pathway is a therapeutic target and thus, focus attention on this approach to support the development of future interventional strategies. Successful completion of our goals thus has the strong potential to benefit a large number of women with this devastating disease.

2. Keywords

NF-kappaB, non-canonical NF-kappaB pathway, doxycycline inducible transgenics, nanoparticle–based therapy, therapeutic siRNA, therapeutic peptide

3. Accomplishments

In support of successful completion of our Goals, we have obtained administrative approval from Vanderbilt IACUC and ACURO for mouse experiments [1/17/23]. A submission to Vanderbilt IRB for the use of previously archived human ovarian cancer patient-derived xenograft (PDX) grown in mice is pending.

Major Task 1: Investigate effects of stimulating nc-NF-κB signaling in ALFM mice during ovarian cancer progression

The subtasks proposed in the approved Statement of Work are:

> Subtask 1: Increase nc-NF-κB activity in macrophages using the ALFM inducible transgenics (and littermate controls) during specific time periods of ovarian cancer progression in mice (Months 1-12).

Outcomes during 2022-23:

S1 (Yull and Wilson): At the time of final ACURO approval, we commenced breeding of the appropriate mouse strains on the FVB background, ALT *(Tet-O-p52-FLAG)* and FMR *(cfms-rtTA)*, to generate **ALFM** double transgenics that express a p52-FLAG transgene specifically in macrophages when mice are exposed in doxycycline (dox) in their drinking water. Examples of the DNA-based PCR used for genotyping mice shows the generation of double positive transgenics (ALT +ve /FMR +ve, designated */*) or littermate controls are shown in **Fig 1**.

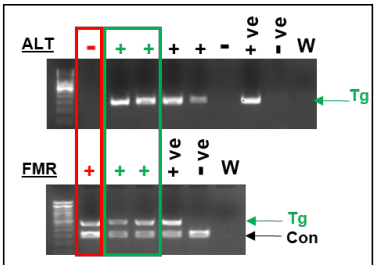


Figure 1. Genotyping of ALFM mice. PCR-based DNA genotyping showed that ALFM mice simultaneously harbored both ALT and FMR transgenic DNA (green box, designated */*). For FMR, the transgenic band corresponds to the larger, upper band of the doublet; the lower band is an internal control band. The example in the red box shows a littermate positive for FMR but negative for ALT (designated -/*, which will act as a littermate control for dox experiments). Water (W) controls showed the specificity of the PCR reactions.

To increase efficiency of generation of double transgenic mice, we previously generated a homozygous FMR mouse. A mating pair of a heterozygous positive ALT and homozygous positive FMR should generate 50% ALT +ve /FMR +ve (*/*) and 50% ALT -ve /FMR +ve littermate controls (designated -/*). However, we experienced an unexpected delay in generating our female experimental mice. Inadvertently, homozygous ALT mice were used for the initial matings with the homozygous FMR mice. As a consequence, both the ALT and FMR DNA were transmitted to all progeny, with no littermate controls generated in parallel. That error has been recognized and we have now generated sufficient mice for an initial experiment (see below).

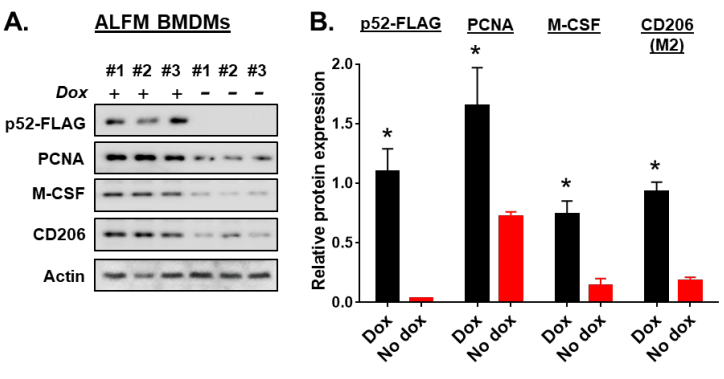


Figure 2. Expression of p52-FLAG transgene in ALFM mice increases macrophage proliferation and an M2 phenotype. A) Primary BMDMs harvested from 3 confirmed double positive ALFM mice were treated and without 1μg/ml doxycycline for 48h to induce expression of the p52-FLAG transgene. Increases were also observed in markers of cell proliferation (PCNA), macrophage mitogenic factor M-CSF and the M2 marker CD206. **B)** Densitometry of western blots shown in A). Values are mean+SEM relative to corresponding actin content. *p<0.05, Student's T test..

We then performed pilot experiments to characterize functional differences in macrophages from ALFM and control mice. First, we showed that macrophages harvested from double-positive ALFM mice can be induced by dox treatment to express the p52-FLAG transgene. As shown in **Fig 2A&B**, dox treatment induced selective increases in protein expression of the p52-FLAG transgene in bone marrow-derived macrophages (BMDMs) generated from 3 ALFM mice, compared to no expression in dox-naïve cells cultured in parallel. Increased nc-NF-κB signaling also increased expression of the proliferation marker PCNA and the pro-proliferative factor M-CSF, and induced expression of the M2 marker CD206.

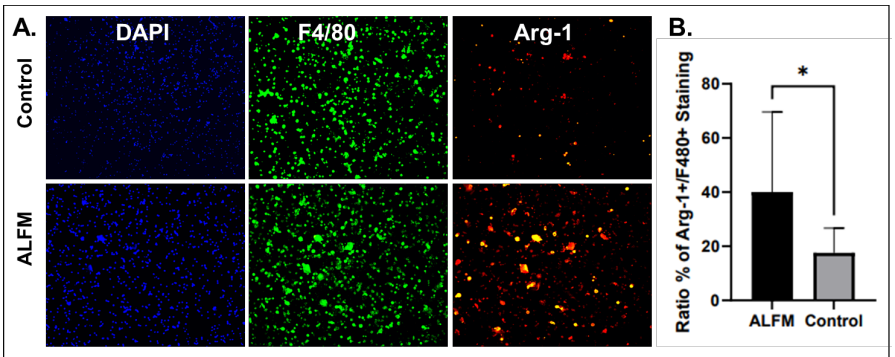


Figure 3. M2-polarization of peritoneal macrophages in tumor-bearing ALFM mice. Control and ALFM mice were injected with TBR5 cells (n=3). Mice were treated with 1g/L dox 3 days prior to tumor injection and for a further 7 days before collection of the peritoneal fluid by lavage and cytospin onto slides. **A)** Co-IF staining for the pan-macrophage F4/80 (green) and the M2 macrophage marker arginase-1 (Arg-1) in cytospin slides. DAPI-stained nuclei are in blue. **B)** Percentage of F4/80 positive macrophages that were also arginase-1 positive were counted in 5 independent fields per slide. Values are mean+SD and show the ratio of cells co-staining for F4/80 and arg-1. * p<0.05, Student's test.

Second, we injected control and ALFM mice with mouse TBR5 ovarian cancer cells, a well-established syngeneic model in the Yull laboratory (1, 2). Dox treatment was started 3 days prior to tumor injection and continued until mouse sacrifice 7 days later. This time course allowed us to assess early changes in the behavior of tumor-associated macrophages (TAMs) in the peritoneal fluid in the presence of implanting tumor cells. As shown in **Fig 3A&B**, the ratio of M2 polarized macrophages was significantly increased in cytospin sections of macrophages harvested from dox-treated ALFM mice compared to littermate control mice. Thus, we have evidence from independent models of ALFM macrophages that increasing nc-NF-κB signaling has the potential to promote the M2 pro-tumor phenotype and thus support tumorigenesis.

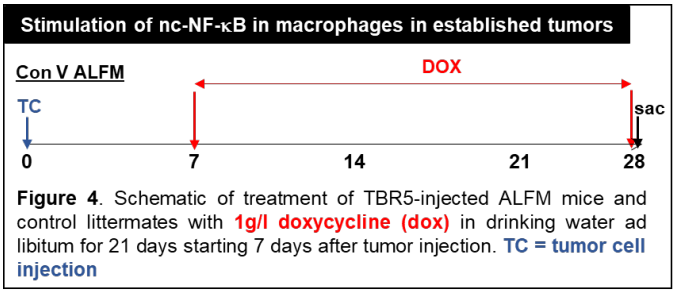


Figure 4. Schematic of treatment of TBR5-injected ALFM mice and control littermates with **1g/l doxycycline (dox)** in drinking water ad libitum for 21 days starting 7 days after tumor injection. **TC = tumor cell injection**

Our proposed studies in the TBR5 model in ALFM model were designed to directly study impacts of increased nc-NF-κB signaling on tumorigenesis. We have now generated sufficient experimental mice, cohorts of 5 ALFM double transgenics and 5 littermate controls, to perform an initial study over a time course of 28 days. Having performed our preliminary studies validating the ALFM model, we designed an initial experiment using cohorts of 5 ALFM double transgenics and 5 littermate controls. The dox treatment schedule over 3 weeks, starting 1 week after intraperitoneal injection of TBR5 cells, is shown in the experimental timeline in **Fig 4**. Thus, effects of increasing nc-NF-κB activity in macrophages in the progression of established tumors will be examined. The longer period of dox stimulation will allow us to determine effects on tumor growth and formation of ascites fluid, two major indices of tumor burden in our studies (1, 2). This treatment parallels our earlier published study using transgenics that increase canonical NF-κB activity in macrophages during defined stages of tumorigenesis (1). This experiment is ongoing, and results will be presented in next year's report. One notable observation is that the dox is well-tolerated by both control and ALFM mice.

Major Task 2: Conduct experiments in cultured human and mouse ovarian cancer cells *in vitro* treated with the SN52 peptide or SN52mut control

The subtasks proposed in the approved Statement of Work are:

Subtask 1: Measure expression of p100/p52 in an expanded panel of human cell lines by western blot, including OVCAR-3 and OVCAR-4. (Months 0-6).

Subtask 2: Compare effects of SN52 and SN52mut on assays of cell growth (SRB assay, clonogenic assays, PCNA expression by western blot and high-content IF measuring adherent cell number and cell cycle indices in DAPI-stained cells in our panel of 3 human (SKOV-3, OVCAR-3, OVCAR-4) and 2 mouse (ID8-luc, TBR5) ovarian cancer cell lines. (Months 0-6).

Subtask 3: Compare effects of SN52 and SN52mut on nc-NF-κB signaling in our cell line panel by western blots analysis of p52 and RelB in nuclear fractions. (Months 0-6).

Outcomes during 2022-23:

S1 (Yull and Wilson): By western blot, we have shown expression of p100 and p52 in a panel of human (SKOV-3) and two mouse ovarian cancer cell lines directly relevant to the mouse studies, ID8-luc and TBR5 (see funded grant Fig 1A). We proposed to also measure their expression in human OVCAR-3 and OVCAR-4 cells because they are considered representative of high-grade serous histology, the most common and deadliest of the ovarian cancer subtypes in patients (3, 4). As shown in **Fig 5**, p52 is expressed in both cell lines, suggestive of active non-canonical signaling.

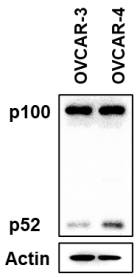


Figure 5. Expression of p100 and p52 in two human ovarian cancer cell lines representative of high-grade serous histology. Western blot analysis of p100 and p52 expression in OVCAR-3 and OVCAR-4 cells.

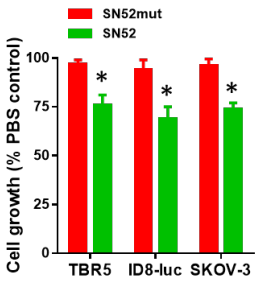


Figure 6. Effects of 24h treatment with 40 μg/ml SN52 or mutant control peptide (SN52mut) on growth of mouse ovarian cancer cell lines TBR5, ID8-luc and human ovarian cancer cell line SKOV-3, expressed relative to PBS treated cells. Cell growth was measured by sulforhodamine B assay; *p<0.05 compared to SN52mut, Student's t test.

S2 (Yull and Wilson): Using the SN52 and SN52mut peptides used to generate our preliminary data for the funded application (see funded grant Fig 1B), we tested the effects of inhibiting nc-NF-κB signaling in two independent growth assays. First, we used a well characterized method in our group, the sulforhodamine B (SRB) colorimetric assay (4, 7). This assay measures cell growth and viability via staining of cellular protein in the monolayer. Similar to previous observations in ID8-luc and SKOV-3 cells presented as Preliminary data for the funded application, SN52 significantly reduced growth compared to the SN52mut control **(Fig 6)**. A second growth assay used was based on immunofluorescent cell cycle analysis of DAPI-stained nuclei using the MetaXpress imaging and analysis platform (8). Using our mouse ovarian cancer cells, we showed that SN52 significantly reduced the number of adherent cells after 24 hours' treatment compared to SN52mut in both cell lines (**Fig 7A&B**), consistent with our SRB data. Cell cycle analyses are shown in **Fig 7C-E**. They revealed that while SN52 significantly reduced the percentage of cells undergoing DNA replication (S phase) in both cell lines, there was a difference in the pattern of cell cycle arrest. ID8-luc cells showed a prominent G_2/M arrest, while TBR5 cells were significantly arrested only in the G_0/G_1 phase. A literature search revealed a potential mechanism for this difference between the cells. Notably, ID8-luc cells are p53 wild-type while TBR5 cells are p53-mutated. While a well-known gene target of p52 is cyclin D1, which controls the transition of cells from G_0/G_1 to S phase, p52 induction of the cyclin dependent kinase inhibitor p21, a major regulator of the G_2/M transition, is p53-dependent (9).

S3 (Yull and Wilson): Preliminary experiments using two readily available NF-κB reporter cell lines generated in the Yull laboratory, ID8-NGL and BMDM-NGL cells derived from FVB mice (5, 6) confirmed that the SN52 peptide significantly reduced NF-κB signaling compared to the mutant control **(Fig 8)**. The reporter does not distinguish between the binding of canonical or non-canonical

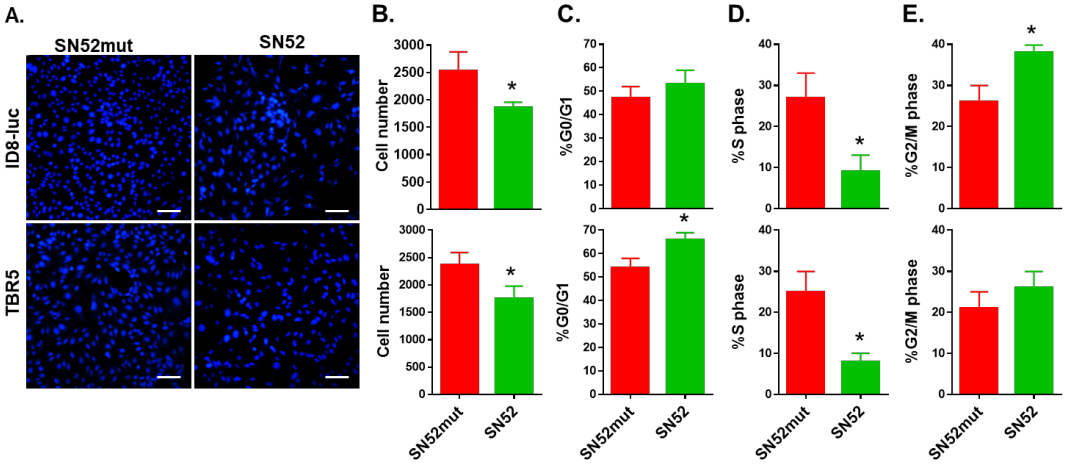


Figure 7. Cell cycle arrest following inhibition of non-canonical NF-κB signaling. Cultured ID8-luc and TBR5 cells were treated for 24h with 40 μg/ml SN52 or mutant control peptide (SN52mut). **A)** Cells were then fixed and stained blue by DAPI. High-content IF analysis off DAPI-stained nuclei was used to measure **(B-E)** adherent cell number and the percentage of cells in G_0/G_1, S and G_2/M phases. Images were acquired and analyzed using the MetaXpress platform. Values are mean+SEM of 5 fields/well of 8 replicate wells per treatment; *p<0.05 compared to SN52mut, Student's T test. Scale bars represent 10μm.

components, but the observation suggests that nc-NF-κB signaling is a significant contributor to baseline NF-κB activity in these cells. To show a specific reduction nc-NF-κB signaling by the SN52 peptide, we performed subcellular fractionation to measure p52 levels in nuclear and cytosolic fractions. As shown in **Fig 9**, we confirmed that there were reduced levels of nuclear p52 in TBR5 cells treated with SN52 compared to the SN52mut control peptide.

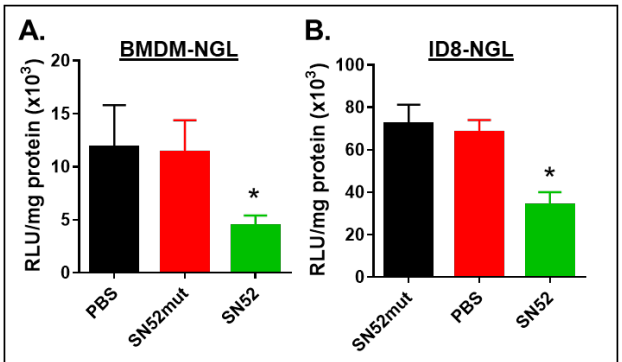


Figure 8. The SN52 inhibitory peptide reduces NF-κB reporter activity in macrophages and ovarian cancer cells. A) Immortalized BMDM-NGL cells derived from FVB NF-κB (NGL) reporter mice and **B)** ID8 cells stably transfected with the NGL reporter plasmid were treated for 6h with 40 μg/ml SN52 or mutant control peptide (SN52mut) or a PBS control. Values are mean+SEM of relative luciferase units (RLU) expressed relative to corresponding cellular protein (Bradford assay.); *p<0.05 compared to SN52mut or PBS, Student's T test..

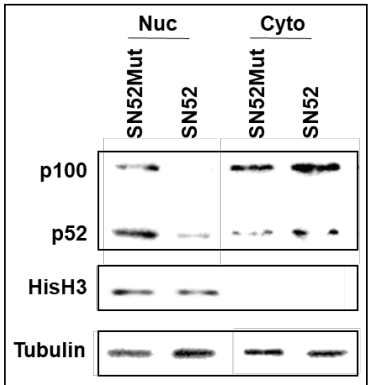


Figure 9. Non-canonical peptide inhibitor reduces nuclear p52 localization in mouse ovarian cancer cells. Effects of 6h treatment with 40 μg/ml SN52 or mutant control peptide in TBR5 cells. Western blot analysis of expression of p100/p52, the nuclear marker histone H3, and tubulin in nuclear and cytosolic fractions.

Major Task 3: Determine therapeutic potential of SN52 peptide in mouse models of ovarian cancer.

The subtasks proposed in the approved Statement of Work are:

Subtask 1: Treat WT C57BL/6 mice injected IP with ID8-luc cells or WT FVB mice injected with TBR5 cells with SN52, SN52mut or 1:1 (v/v) water-saline vehicle injected IP. (Months 1-12).

Subtask 2: Treat Nude mice injected IP with an established ovarian PDX model with SN52 or SN52mut injected IP. (Months 12-18).

Outcomes during 2022-23:

S1 (Yull and Wilson): We have bred sufficient cohorts of wild-type FVB and C57BL/6 mice to begin these experiments. We will purchase a new stock of the SN52 and SN52mut peptides from Elim Pharmaceuticals for the *in vivo* experiments (unmodified, 26 amino acids, 95% purity). This will ensure consistency in manufacturer in the peptides from the studies outlined in Major Task 2 and between *in vivo* experiments.

S2 (Yull and Wilson): Nude mice for this experiment will be purchased once we have established optimal treatment conditions for the peptide and have IRB and OHRO approval for these studies.

Major Task 4: Use mannosylated nanoparticles (Mn-NPs) with p100/p52 siRNA payload to inhibit nc-NF-κB in macrophages.

The subtasks proposed in the approved Statement of Work are:

Subtask 1: Compare effects of Mn-NP-p100/p52 and Mn-NP-Scr (scrambled control) on nc-NF-κB signaling in models of cultured macrophages, including BMDMs and TAMs isolated from mouse ascites fluid (Months 6-12).

Subtask 2: Treat WT C57BL/6 mice injected with ID8-luc cells with PBS, Mn-NPs containing p100/p52 siRNA or Scr siRNA for 3 consecutive days for delivery studies and assessment of target protein expression knockdown and nc-NF-κB activity. (Months 6-12).

Subtask 3: Treat WT C57BL/6 mice injected with ID8-luc cells or WT FVB mice injected with TBR5 cells with Mn-NPs containing p100/p52 siRNA or Scr siRNA. (Months 12-24).

Outcomes during 2022-23:

S1 (Yull and Wilson): We have identified and purchased siRNA sequences targeting p100/p52 or a scrambled control from Thermo Scientific that were previously used by the Yull/Giorgio groups in nanoparticle formulations (10). These siRNAs will be incorporated into mannose-decorated micelles with assistance from Ms. Sydney Henriques, a graduate student in the laboratory of Dr. Todd Giorgio (study Co-I).in order to validate their efficacy *in vitro* in preparation for *in vivo* studies.

S2&3 (Yull and Wilson): These *in vivo* experiments have not been started because, as explained above, we have not generated nor tested the particles required for the studies.

Opportunities for training and professional development: During the reporting period, we provided an employment opportunity for a recent college graduate, M. Francisca Adeniran, as a Research Assistant 1. We also provided the opportunity for a high school senior, Robert Cheng, to gain laboratory research experience as part of the Vanderbilt Research Experience for High School Students (REHSS) program in the summer of 2022.

Dissemination of results: Dr. Yull presented a poster at the 2023 American Association for Cancer Research (AACR) Meeting in Orlando, FL: "Investigating the role of non-canonical NF-kappaB signaling in tumor cells and macrophages in ovarian cancer".

Plans for the next reporting period. By the end of Year 2, we anticipate presenting our data at the 2024 AACR meeting and submitted a manuscript to a high-quality cancer research Journal based on data obtained primarily in Major Tasks 1&2.

Major Task 1. We have performed initial important validation showing that: 1) we can distinguish between double transgenic ALFM and control mice by DNA-based PCR genotyping; 2) dox in drinking water is well tolerated by ALFM and control mice; 3) we can induce the p52-FLAG transgene in primary BMDMs harvested from ALFM mice selectively by dox treatment and 4) that upregulated nc-NF-κB signaling in macrophages is associated with increased proliferation and increased expression of M2 macrophage markers. We aim to perform all proposed *in vivo* studies in dox-treated ALFM and control mice in Year 2.

Major Task 2. We have validated that the SN52 peptide selectively inhibits nc-NF-κB signaling in cultured ovarian cancer cells. The majority of the proposed experiments have been completed at least once. Most require additional replicate experiments. Experiments using a third model of cancer cell growth, the clonogenic assay, will be performed. The major observation of a clear pro-proliferative role for nc-NF-κB signaling in ovarian cancer cells is consistent with our hypothesis.

Major Task 3. We will obtain sufficient peptides to perform multiple *in vivo* experiments. We will obtain IRB and OHRO approval for human ovarian PDX studies and purchase Nude mice. We aim to perform all proposed studies in Year 2.

Major Task 4. We will generate the Mn-NP reagents containing p100/p52 or scrambled siRNA and validate selective knockdown of p52 expression and reduced nc-NF-κB in cultured macrophage models before their use *in vivo*. We aim to perform all proposed studies in Year 2.

4. Impact

Impact on the development of the principal discipline(s) of the project

Identification of new therapeutic strategies in ovarian cancer, particularly in advanced chemoresistant disease, is a key clinical challenge for patients and their doctors to improve survival and quality of life. The primary goal of this Project is to define a new role(s) for nc-NF-κB signaling in malignant tumor epithelial cells and in a key cell type in the tumor microenvironment, macrophages, for promotion of the progression of ovarian cancer progression and as a novel target for therapy. Two approaches that allow the specific inhibition of nc-NF-κB in one or both of these cell populations will be tested in mouse models of ovarian cancer to provide important supportive evidence of their potential to treat ovarian cancer patients in future clinical trials. Importantly, both of our strategies involve classes of drugs that are already in use in the clinic, namely small therapeutic peptides and nanoparticle-based delivery of a therapeutic payload. Since both strategies have the potential for rapid translation into the clinic, successful completion of the studies may have high impact for patients. Initial results during the first year of the Project are consistent with our Hypothesis that nc-NF-κB promotes growth of ovarian cancer cells and macrophages and also promotes an M2-like pro-tumor phenotype in macrophages. Studies to determine effects of increasing nc-NF-κB in macrophages in specific time windows in tumor-bearing mice are underway. We are ready to begin testing our therapeutic inhibitory peptide in mice and to generate and characterize our therapeutic nanoparticles in cultured cells before using them in mice.

Impact on other disciplines?

Nothing to Report

Impact on technology transfer?

Nothing to Report

Impact on society beyond science and technology?

Nothing to Report

5. Changes/Problems

Changes in approach and reasons for change

Nothing to Report

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

Francisca Adeniran was recruited as a Research Assistant as a new college graduate. She joined the group on June 20th 2022. Francisca completed a broad range of introductory training learning techniques such as PCR and immunofluorescence. She also completed the necessary training in order to be able to manage and work with our mouse models. Unfortunately, she discovered that she had a fear of handling mice that neither of us had anticipated. This likely contributed to her decision to hand in her notice and leave the group on October 8th 2022. At this stage we commenced efforts to recruit a replacement. However, the pool of available personnel was limited and we experienced some difficulty in finding a good fit. However, we have recently recruited Dr. Kirsten Hoek. She has extensive experience as an immunologist, in the analysis of mouse models and has previously served as a lab manager such that she proficient in most lab techniques. While her salary is greater than the research assistant originally proposed on the budget, we feel that she will enable greater progress in the second year of this funding. Robert Cheng, the high school student who joined the lab as part of the Research Experience for High School students for several weeks last summer has requested to rejoin the lab as a Research Intern for several weeks this summer. We propose to partially support his efforts using this funding source.

Changes that had a significant impact on expenditures

As noted in the section above we experienced challenges relating to hiring and training of appropriate staff. The approval for commencing the mouse work portion of the studies was not obtained until the beginning of 2023, thus mouse related costs started at this point.

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

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. Products

Publications, conference papers, and presentations

Wilson AJ, Parker D, Cheng R, Crispens M, Giorgio TD, **Yull FE.** Investigating the role of non-canonical NF-kappaB signaling in tumor cells and macrophages in ovarian cancer. AACR Annual Research Meeting. April 14-18, 2023. Orlando, FL. Poster presentation.

Cheng R, Parker D, Adeniran F, Bennett N, Henriques S, Wilson A, **Yull F**. Investigating the role of non-canonical NF-kB transduction pathway on macrophage phenotype and ovarian tumor progression. Research Experience for High School Students Symposium July 8, 2022. Poster presentation.

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. Participants & Other Collaborating Organizations

Name: *Fiona Yull*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): *0000-0003-1636-7942*
Nearest person month worked: *1*
Contribution to Project: Dr. Yull is the Principal Investigator of this pilot. Dr. Yull is an Associate Professor of the Departments of Pharmacology and Obstetrics and Gynecology. She has obtained the necessary IACUC and ACURO approvals to commence the studies involving mouse models. She has been involved in recruiting personnel, experiment planning, interpreting and troubleshooting. She is responsible for presentation of data derived from this work.

Name: *Todd Giorgio*
Project Role: *Co-Investigator*
Researcher Identifier (e.g. ORCID ID): *0000-0002-9557-1425*
Nearest person month worked: *1*
Contribution to Project: Dr. Giorgio is Professor of Biomedical Engineering and Chemical Engineering. Dr Giorgio has extensive experience with the design of nanoparticles and nanotargeting approaches. The nanoparticle formulations that are capable of delivering siRNA specifically to tumor associated macrophages, to be used in these studies, were generated as part of an ongoing collaboration between Dr. Giorgio and Dr. Yull by three sequentially co-mentored graduate students. Dr. Giorgio has attended weekly group lab meetings to provide intellectual input. His current graduate student, Sydney Henriques is helping with the generation of the mannosylated nanoparticles that we require for these studies and will assist with training Dr. Kristen Hoek in techniques required for this aspect of the studies.

Name: *Francisca Adeniran*
Project Role: *Research Assistant*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *5*
Contribution to Project: Francisca was recruited as a Research Assistant as a new college graduate. We were hoping that she would be able to join us close to the beginning of the grant funding but there was a delay because she is a Nigerian citizen and we needed to wait for her Optional Practical Training (OPT) to be approved. Therefore, she joined the group on June 20th 2022. Francisca completed a broad range of introductory training learning techniques such as PCR and immunofluorescence. She also completed the necessary training in order to be able to manage and work with our mouse models. Unfortunately, she discovered that she had a fear of handling mice that neither of us had anticipated. This likely contributed to her decision to hand in her notice and leave the group on October 8th 2022.

Name: *Kristen Hoek*
Project Role: *Staff Scientist*
Researcher Identifier (e.g. ORCID ID): *0000-0001-9917-1480*
Nearest person month worked: *1*
Contribution to Project: Dr. Hoek is an experienced biomedical researcher with over 25 years of experience. She has extensive experience as an immunologist with a previous focus on B cell and T cell biology. She is a relatively recent recruit to our group but she will contribute her expertise to the characterization of changes in immune populations with a major focus on flow cytometry. Dr. Hoek will also assist with management of mouse colonies and be responsible for the maintenance and preparation of cell lines for *in vivo* studies. She will assist with collection of mice at sacrifice and perform downstream analyses of harvested mouse tissue by flow cytometry and other techniques, and also with experimental design, collation of results, interpretation of data and preparation of manuscripts. Dr. Hoek has been developing and validating antibodies and panels for the flow cytometric analysis of samples from in vitro and in vivo study components.

Name: *Andrew Wilson*
Project Role: *Co-Investigator*
Researcher Identifier (e.g. ORCID ID): *0000-0002-8656-7723*
Nearest person month worked: *1*
Contribution to Project: Dr. Wilson is a Research Assistant Professor of Obstetrics and Gynecology. His laboratory research program is particularly interested in the role of inflammatory pathways, particularly NF-κB signaling, in ovarian cancer progression, and in identifying and testing novel therapeutic strategies targeting tumors and the tumor microenvironment. He has collaborated with Dr. Yull (PI) since 2010 and has worked very closely with her and her research staff since then. He has extensive experience with Dr. Yull's transgenic mouse models. He assisted with training and supervision of Francisca Adeniran (RA). He is working on gaining the appropriate IRB approval for the human subject studies at VUMC. He attends weekly lab meeting and contributes to overall experimental design, interpretation and presentation of data.

Name: Robert Cheng
Project Role: *High School Student – Research experience for High School Students*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *2*
Contribution to Project: Robert was supported by the Research experience for High School Students Program to join the lab for approximately 6 weeks. He gained an understanding of the project and performed some cell-based studies.

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

No change to the requested level of support on this budget.

Completed
1R01CA214043-01A1 (Yull, Giorgio, Khabele) 03/10/2017-02/28/2023 (NCE)
"Macrophage-based ovarian cancer immunotherapy"
This study will define the impact of increasing NF-kB signaling specifically in macrophages on the tumor microenvironment and ovarian tumor progression and will show that targeted modulation of NF-kB in macrophages can be harnessed as a novel therapy.

New funding
W81XWH2211086 (Yull – PI, Rhoades Partnering PI) DOD BCRP 09/15/2022 – 09/14/2025
Support: 3.6 calendar months (Yull) 2.4 calendar months (Wilson) 0.6 calendar months (Giorgio)
"Modulation of lung and bone microenvironments to treat breast metastases"
The goal of these studies is to eliminate the mortality associated with metastatic breast cancer by determining methods to alter the functions of tumor-associated macrophages (TAMs) to achieve tumor cytotoxic effects. Eliminate the mortality associated with stage IV metastatic breast cancer by increasing canonical NF-kB in tumor associated macrophages (TAMs) to achieve tumor cytotoxic effects. Inducible transgenics, liposomal MTP-PE or nanoparticle delivery of IkBa siRNA will be used in models of existing lung and bone metastases to demonstrate efficacy.

What other organizations were involved as partners?

Nothing to report.

8. Special Reporting Requirements

None.

9. Appendices

Appendix 1.
References

Appendix 2.
Poster presentation at the 2023 American Association for Cancer Research Meeting, Orlando, FL.

Appendix 1.

1. Hoover AA, Hufnagel DH, Harris W, Bullock K, Glass EB, Liu E, Barham W, Crispens MA, Khabele D, Giorgio TD, Wilson AJ, Yull FE. Increased canonical NF-kappaB signaling specifically in macrophages is sufficient to limit tumor progression in syngeneic murine models of ovarian cancer. BMC cancer. 2020;20(1):970. doi: 10.1186/s12885-020-07450-8. PubMed PMID: 33028251; PubMed Central PMCID: PMC7542116.
2. Glass EB, Hoover AA, Bullock KK, Madden MZ, Reinfeld BI, Harris W, Parker D, Hufnagel DH, Crispens MA, Khabele D, Rathmell WK, Rathmell JC, Wilson AJ, Giorgio TD, Yull FE. Stimulating TAM-mediated anti-tumor immunity with mannose-decorated nanoparticles in ovarian cancer. BMC cancer. 2022;22(1):497. doi: 10.1186/s12885-022-09612-2. PubMed PMID: 35513776; PubMed Central PMCID: PMC9074180.
3. Domcke S, Sinha R, Levine DA, Sander C, Schultz N. Evaluating cell lines as tumour models by comparison of genomic profiles. Nature communications. 2013;4:2126. doi: 10.1038/ncomms3126. PubMed PMID: 23839242; PubMed Central PMCID: PMC3715866.
4. Wilson AJ, Sarfo-Kantanka K, Barrack T, Steck A, Saskowski J, Crispens MA, Khabele D. Panobinostat sensitizes cyclin E high, homologous recombination-proficient ovarian cancer to olaparib. Gynecologic oncology. 2016;143(1):143-51. doi: 10.1016/j.ygyno.2016.07.088. PubMed PMID: 27444036; PubMed Central PMCID: PMC5031537.
5. 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. Journal of ovarian research. 2013;6(1):63. doi: 10.1186/1757-2215-6-63. PubMed PMID: 24020521; PubMed Central PMCID: PMC3846584.
6. Glass EB, Masjedi S, Dudzinski SO, Wilson AJ, Duvall CL, Yull FE, Giorgio TD. Optimizing Mannose "Click" Conjugation to Polymeric Nanoparticles for Targeted siRNA Delivery to Human and Murine Macrophages. ACS omega. 2019;4(16):16756-67. doi: 10.1021/acsomega.9b01465. PubMed PMID: 31646220; PubMed Central PMCID: PMC6796989.
7. Wilson AJ, Saskowski J, Barham W, Yull F, Khabele D. Thymoquinone enhances cisplatin-response through direct tumor effects in a syngeneic mouse model of ovarian cancer. Journal of ovarian research. 2015;8:46. doi: 10.1186/s13048-015-0177-8. PubMed PMID: 26215403; PubMed Central PMCID: PMC4517540.
8. Wilson AJ, Stubbs M, Liu P, Ruggeri B, Khabele D. The BET inhibitor INCB054329 reduces homologous recombination efficiency and augments PARP inhibitor activity in ovarian cancer. Gynecologic oncology. 2018;149(3):575-84. doi: 10.1016/j.ygyno.2018.03.049. PubMed PMID: 29567272; PubMed Central PMCID: PMC5986599.
9. Schumm K, Rocha S, Caamano J, Perkins ND. Regulation of p53 tumour suppressor target gene expression by the p52 NF-kappaB subunit. The EMBO journal. 2006;25(20):4820-32. doi: 10.1038/sj.emboj.7601343. PubMed PMID: 16990795; PubMed Central PMCID: PMC1618099.
10. Ortega RA, Barham W, Sharman K, Tikhomirov O, Giorgio TD, Yull FE. Manipulating the NF-kappaB pathway in macrophages using mannosylated, siRNA-delivering nanoparticles can induce immunostimulatory and tumor cytotoxic functions. International journal of nanomedicine. 2016;11:2163-77. doi: 10.2147/IJN.S93483. PubMed PMID: 27274241; PubMed Central PMCID: PMC4876941.

Appendix 2.

#4973

Investigating the role of non-canonical NF-kappaB signaling in tumor cells and macrophages in ovarian cancer

Andrew J Wilson[1], Dominique Parker[2], Robert Cheng[2], Todd D. Giorgio[3], Marta Crispens[1], & Fiona Yull[1,2]

Dept. OB/GYN, Vanderbilt University Medical Center, [2] Dept. Pharmacology & [3] Dept. Biomedical Engineering , Vanderbilt University, Nashville, TN

Introduction

- Ovarian cancer has a 5-year survival rate of 48.5% and ranks fifth in causes of cancer-related deaths in women.
- To improve clinical outcomes, a better understanding of disease progression and new treatment strategies are urgently required.
- Targeting the behavior of a key cell type in the tumor microenvironment (TME), tumor-associated macrophages (TAMs), is emerging as a viable therapeutic option.

A key goal of macrophage-directed therapy is to shift the phenotype of pro-tumor TAMs towards M1-like anti-tumor functions

- Our group seeks to understand and exploit pathways that regulate macrophage phenotype and behavior, including **NF-kappaB (NF-κB)** .
- Canonical NF-κB signaling has been widely studied as a therapeutic target in cancer; while reducing activity in tumors is beneficial, we have shown that **increasing** activity specifically in macrophages induces inflammatory, anti-tumor (M1-like) functions and reduces ovarian tumor burden in mice.[1,2]
- The second major NF-κB pathway, **non-canonical (nc-NF-κB)** signaling, is relatively understudied in cancer.
- We recently showed that higher **nc-NF-κB** signaling in ovarian tumors is associated with worse patient outcomes.[3]
- In macrophages, **nc-NF-κB** signaling is thought to play a role in wound healing, characteristics of which resembles M2-like TAM behavior (pro-tumor). immune suppression

Aims

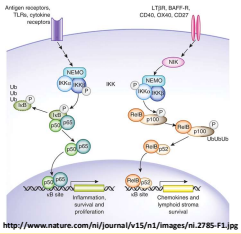
To determine the effects of modulating nc-NF-κB signaling in ovarian cancer cells and macrophages, and to determine whether the pathway represents a novel therapeutic target

Methods

- We assembled a panel of human and mouse ovarian and mammary cells, and mouse bone marrow-derived macrophages (BMDMs)
- Cell growth was measured in 2-D sulforhodamine B assays
- Activity of **nc-NF-κB** pathway measured by western blot analysis of cytoplasmic p100 and nuclear p52 levels.
- Activity of **nc-NF-κB** pathway inhibited by the SN52 peptide (CelTek, TN)
- Macrophage phenotype measured by western blot (M2 marker CD206)

Two distinct NF-κB signaling pathways

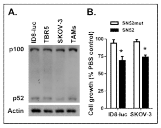
Canonical **Non-canonical**



http://www.nature.com/ni/journal/v15/n1/images/ni.2785-F1.jpg

nc-NF-κB in cancer cells and macrophages

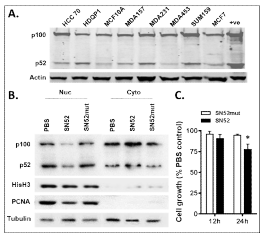
Figure 1. Ovarian cancer cell lines



A) Expression of p100/p52 in a panel of mouse and human ovarian cancer cells, and in ovarian TAMs isolated from ascites fluid of ID8-luc-injected C57BL/6 mice

B) Effects of 24h treatment with 40 μg/ml SN52 or mutant control peptide (SN52mut) on cell growth (*p<0.05, Student's T test

Figure 2. Breast cancer cell lines

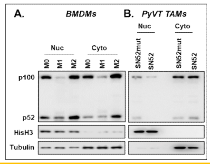


A) Expression of p100/p52 in a panel of human breast cancer cells,

B) Effects of 12h treatment with 40 μg/ml SN52 or mutant control peptide (SN52mut) on nuclear p52 levels and on the proliferation marker PCNA

C) Effects of 12-24h treatment with 40 μg/ml SN52 or mutant control peptide (SN52mut) on MDA231cell growth (*p<0.05, Student's T test

Figure 3. Macrophages (BMDMs and mammary TAMs)



A) Effects of M1 polarization (LPS, IFNγ-treated) and M2 polarization (IL-4-treated) on nuclear p52 levels compared to unpolarized (M0) in an immortalized mouse BMDM cell line 24h,

B) Effects of 4h treatment with 40 μg/ml SN52 or mutant control peptide (SN52mut) on nuclear p52 levels in mouse mammary TAMs

Manipulation of nc-NF-κB signaling in transgenic ALFM mice

Dox-inducible expression of a p52-FLAG transgene is targeted to macrophages in double transgenic ALFM mice

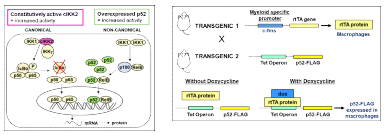
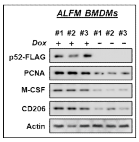


Figure 4. Validation experiments in BMDMs isolated from ALFM mice



Effects of dox (48h stimulation) or no dox control on BMDMs harvested from 3 confirmed double ALFM transgenics on transgene expression, and markers of proliferation (PCNA) and M2 polarization (CD206).

Summary

- p52 is expressed in human and mouse ovarian and breast cancer cells, and in mouse ovarian and mammary TAMs, indicating active **nc-NF-κB** signaling (**Figs.1A, 2A, 3A**).
- Inhibition of p52 nuclear import by SN52 reduces cell growth in ovarian and mammary cancer cell lines (**Figs. 1B, 2C**).
- In immortalized BMDMs, M2-polarized cells showed elevated nuclear p52 levels (**Fig 3A**). In primary ALFM BMDMs, there was a selective increase in expression of the p52-FLAG transgene, proliferation and M2 polarization in dox-treated cells compared to no dox controls (**Fig 4**).
- We show that **nc-NF-κB signaling** promotes tumor growth and M2 macrophage polarization, suggesting it is a novel therapeutic target.

Experiments in 1) ALFM or transgenic controls, and 2) testing the therapeutic effects of nc-NF-κB inhibition, in an established syngeneic ovarian cancer model (IP injection of TBR5 cells) are underway

References

1. Hoover *et al*, BMC Cancer. 2020;20(1):970; PMID: 33028251
2. Glass *et al*, BMC Cancer. 2022;22(1):497; PMID: 35513776
3. Hufnagel *et al*, Biomarker Research. 2020;8:45; PMID: 32974032

Funding

CDMRP OCRP Pilot Award
W81XWH-22-1-0308 (Yull PI)

Also supported by a generous gift from Mr. Chris Hill through Anglo-American Charity Ltd.