Aging Increases Susceptibility to Ovarian Cancer Metastasis in Murine Allograft Models and Alters Immune Composition of Peritoneal Adipose Tissue

Abstract

Ovarian cancer, the most deadly gynecological malignancy in U.S. women, metastasizes uniquely, spreading through the peritoneal cavity and often generating widespread metastatic sites before diagnosis. The vast majority of ovarian cancer cases occur in women over 40 and the median age at diagnosis is 63. Additionally, elderly women receive poorer prognoses when diagnosed with ovarian cancer. Despite age being a significant risk factor for the development of this cancer, there are little published data which address the impact of aging on ovarian cancer metastasis. Here we report that the aged host is more susceptible to metastatic success using two murine syngeneic allograft models of ovarian cancer metastasis. This age-related increase in metastatic tumor burden corresponds with an increase in tumor infiltrating lymphocytes (TILs) in tumor-bearing mice and alteration of B cell-related pathways in gonadal adipose tissue. Based on this work, further studies elucidating the status of B cell TILs in mouse models of metastasis and human tumors in the context of aging are warranted.

Introduction

Ovarian cancer (OvCa) is the leading cause of death due to gynecological malignancy in women in the United States. Often diagnosed with metastases, patients with ovarian cancer receive poor prognoses [1]. The vast majority of OvCas are epithelial in origin with predominantly serous (70%–85%) and endometrioid (10%) histotypes [2,3]. OvCa metastasizes uniquely when tumor cells or multicellular aggregates detach from the primary tumor and disseminate throughout the peritoneal cavity, forming metastatic sites on the peritoneum [1]. Additionally, hematogenous metastasis with metastatic homing to the ovary has been seen in model systems [4,5].

Aging is a significant risk factor and prognostic factor for women with OvCa, wherein the median age at diagnosis is 63 [6]. Furthermore, elderly patients have poorer prognoses, with unfavorable progression-free survival (PFS) and overall survival associated with increased age [7–11]. While treatment disparities in the elderly...
may contribute to this difference, the aged host may also be more susceptible to disease progression. There is a paucity of studies addressing the impact of aging on OvCa metastasis. The fact that OvCa is so often diagnosed at metastatic stages of the disease confounds the ability of researchers to draw conclusions about the relationship between metastasis and age from patient data. Thus, to evaluate this relationship, research models of metastasis are required. In vitro models using human omental mesothelial cells (HOMCs) have demonstrated that HOMCs from elderly patients are in a pro-inflammatory state [12]. Another study showed an increase in adhesion of OvCa cells to HOMCs in a state of induced senescence in vitro [13]. While a single ovarian cancer metastasis study has utilized middle-aged mice [14], studies designed to evaluate the impact of age on metastasis in vivo have not been reported.

The purpose of this study was to test the hypothesis that age impacts the metastatic outcome of OvCa. Here we utilize distinct syngeneic murine models of OvCa metastasis to demonstrate that age increases the susceptibility of the host to metastasis. Our data suggest that the immune composition of aged peritoneal adipose tissue, specifically B cells, may contribute to this age-related disparity in metastasis.

### Materials and Methods

#### Murine Aging Models

Two age groups of C57Bl/6 female mice (Jackson Laboratories) were used for this study. Retired breeders were purchased around 8 months of age and allowed to age until they reached 20 months, at which point they are devoid of ovarian follicles [15]. Young mice were bred once beginning at 8 weeks and were used between 4–6 months of age. Mouse age time points were chosen based on research on mouse-human lifespan equivalencies [16]. In this murine model of aging, mice 3–6 months (young) and 20–23 months (aged) correspond to humans 20–30 and 60–67 years of age, respectively (Figure 1A). The same methods were used for experiments with FVB/NJ mice (Jackson Laboratories), which have a comparable lifespan to C57Bl/6 mice [17]. All animal procedures were carried out according to the regulations of the Institutional Animal Care and Use Committee at The University of Notre Dame.

#### Murine Allograft Models

Two allograft models were utilized in this study. Parental ID8 cells, a C57Bl/6 syngeneic mouse ovarian surface epithelial (MOSE) cell
line [18] were tagged with red fluorescent protein (RFP) and maintained as previously described [19]. ID8 cells with a CRISPR/Cas9-generated Tp53 gene deletion (designated ID8-Tp53<sup>−/−</sup>) were generously provided by Dr. McNeish, Glasgow, UK [20] and were RFP-tagged. Cells derived from the oviductal epithelium of FVB/NJ mice and genetically engineered to lack PTEN expression and express oncogenic KRAS [21] (FVB/N-PTEN<sup>shRNA</sup>/KRAS<sup>G12V</sup>) were generously provided by Dr. Burdette, Chicago, USA, and were maintained and RFP-tagged as previously described [19,21].

C57Bl/6 young and aged mice (n = 14) were intraperitoneally (IP) injected on the right side of the peritoneal cavity with ID8-RFP cells (3.7x10<sup>6</sup>) or ID8-Tp53<sup>−/−</sup>-RFP cells (8.7x10<sup>6</sup>) to model dissemination and colonization events of ovarian cancer metastasis. Mice were imaged under isoflurane anesthesia using the Bruker Xtreme In Vivo Imaging system and were observed for signs of lethargy or ascites accumulation. Mice were sacrificed by CO<sub>2</sub> anesthesia followed by cervical dislocation. If present, ascites was harvested and volume noted. The ventral skin was pulled away and the peritoneal cavity was exposed with incisions down the center and the sides of the ventral parietal peritoneum. The abdominal organs were scanned <i>in situ</i> [19,22], then the organs were removed and imaged <i>ex vivo</i>. Note that because tumor cells were injected on the right side, the right and left fat pads were analyzed separately, such that lesions on the left side (designated FatL) represent metastatic seeding whereas the right fat pad (FatR) underwent injection. Images underwent spectral unmixing as previously described [22]. Using ImageJ, tumor burden was analyzed by calculating the tumor area and the intensity of the RFP signal (Raw Integrated Density). In the case of abdominal images, the measurements were corrected using the adjusted body weight of the mouse (weight<sup>2/3</sup>). The adjusted weight of the organs (weight<sup>2/3</sup>) was used to control for animal-to-animal differences in organ size. Statistical analysis used Student’s T-test. Studies using FVB/N-PTEN<sup>shRNA</sup>/KRAS<sup>G12V</sup> cells were conducted in parallel fashion following IP injection of cells (3.4x10<sup>6</sup>) into young and aged FVB/NJ mice (n = 14). At ~4.5 weeks, mice were sacrificed and abdominal and organ tumor burden was assessed as described above.

**In Vivo Adhesion Assay**

To evaluate early events in <i>in vivo</i> adhesion [23], young and aged C57Bl/6 mice (n = 10) were injected IP with 4.3x10<sup>6</sup> ID8-RFP cells, sacrificed the following day, and excised peritoneal fat depots were rinsed with PBS and imaged as described [19,22]. RFP signal was quantified using ImageJ. Statistical analysis was carried out using Student’s T-test. The FVB/N-PTEN<sup>shRNA</sup>/KRAS<sup>G12V</sup> <i>in vivo</i>.

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**Figure 2.** Evaluation of ID8 tumor burden <i>in situ</i> of young (Y) and aged (A) C57Bl/6 mice. A. Longitudinal imaging of tumor progression in C57Bl/6 aging cohorts. Mice were injected IP with 3.7x10<sup>6</sup> ID8 mouse ovarian cancer cells tagged with RFP and were imaged under anesthesia at 4.5, 5.5, 6.5 and 7.5 weeks post injection to monitor tumor progression. Individual mice were followed over the course of 4 weeks. B. Abdominal ID8 tumor burden <i>in situ</i>. Mice were sacrificed at 8 weeks post IP injection and each abdominal cavity was exposed and imaged. C, D. Quantification of abdominal tumor burden. Abdominal tumor area (C) and intensity (D), respectively, were quantified by dividing either the tumor area or the tumor intensity by the scale-adjusted body weight of each mouse. N = 14. P-values for tumor area and tumor intensity are .02 and .03, respectively. Double asterisk indicates P < .05.
adhesion study was carried out in a parallel fashion (3.2 x10^6 PTENshRNA/KRASG12V-RFP cells were injected into young and aged FVB mice, n = 10 and 9 respectively).

**Histological Analysis**

Organs were fixed in 10% formalin and paraffin embedded. Tissue sections were stained with H&E according to the specifications of Leica’s H&E protocol using SelecTech Define MX-aq, SelecTech Blue Buffer 8, SelecTech Hematoxylin 560 MX, SelecTech Alcoholic Eosin 515. Immunohistochemical analysis of CD45R (protein tyrosine phosphatase receptor type C; lymphocyte common antigen) was performed as described[19] using rat monoclonal anti-CD45R (clone RA3-6B2, 1:800 dilution). Anti-rat IgG and peroxidase detection system reagents (DAB chromogen) were purchased from Abcam, Vector Laboratories, and BioGenex, respectively. Slides were scanned into the eSlide Manager database (version 12.3.2.5030) with the Aperio ScanScope CS (Leica, Biosystems, Inc.). Using Aperio macros, the number of cells stained with DAB chromogen and the total number of cells in tumor sections were quantified. Statistical analysis was carried out using a Student’s T-test.

**RNA Isolation, RNAseq and Bioinformatics**

Gonadal fat was harvested from young and aged mice (n = 4), placed into QIAzol Lysis Reagent (QIAGEN) according to the manufacturer’s specifications. RNA from gonadal adipose tissue was converted to cDNA using a QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer’s protocol. The Notre Dame Genomics and Bioinformatics Core Facility carried out the RNAseq and bioinformatics analyses. All samples had an RNA Integrity Number (RIN) value greater than or equal to 9. Libraries were constructed using the TruSeq RNA Sample Prep Kit v2 set A and Guide 15,026,495 Rev. F (Illumina, Inc., San Diego, CA). To calculate final library concentration, the quantity and average fragment length was measured using the Agilent Bioanalyzer DNA 7500 Assay (Agilent Technologies, Santa Clara, CA) and Qubit DNA High Sensitivity Assay (Life Technologies Corp.). Libraries were normalized to 20 nM in Buffer EB (Qiagen, Santa Clarita, CA). Equal molar amounts were combined for 100-bp paired-end sequencing on two lanes of HiSeq2500. An average of 46 million raw reads were obtained per sample. Raw sequences were trimmed with Trimmomatic[24] v0.32 and assessed for quality with FastQC[25] v0.11.2. Trimmed sequences were aligned to the Mus_musculus.GRCm38 Ensembl version of the mouse genome, using Mus_musculus.GRCm38.81 annotations,[26] with TopHat2 [27], v2.0.11, Linux_x86_64, using Bowtie 2 [28], v2.2.2. Corresponding alignments were sorted with SAMtools [29], v0.1.19. Read counts were generated with HTSeq-count [30], v0.6.1, and were merged with a python script [31]. Subsequent statistics were completed in R [32], v3.1.0, implementing the EdgeR library [33–35]. Gene names and GO terms were identified using the Ensemble version of BioMart [36]. Metacore Pathway Analysis and Ingenuity Pathway Analysis (IPA) were used to view pathways enriched in differentially expressed genes using a cutoff of P = .05.

**Quantitative Real-Time PCR**

RNA from gonadal adipose tissue was converted to cDNA using a QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer’s specifications. qRT-PCR reactions were set up using iTAQ Universal SYBER Green Supermix (BIO-RAD) according to the manufacturer’s specifications. A StepOnePlus Real-Time PCR Thermal Cycler System (Applied Biosystems, StepOne Software V2.2.2) was used for qPCR. Primers were custom ordered from Integrated DNA Technologies (Coralville, Iowa). Primers are listed in...
Supplemental Table 1. RSP13 and HPRT were used as endogenous controls to calculate ΔCt values. PCR thermal cycling conditions were as follows: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. Biological replicates of n = 4 and three technical replicates were utilized for each experiment. The nonparametric Mann–Whitney U Test was selected to determine if ΔCt values of the young and aged samples were significantly different from each other (significance cutoff $P = .05$, analysis carried out in R [32]).

Figure 4. RNASeq analysis of gonadal adipose tissue. A. Representation of differentially regulated pathways in aged gonadal adipose. RNA harvested from gonadal adipose was subjected to RNASeq analysis using the HiSeq2500. Raw sequences were processed and evaluated using Metacore Pathway Analysis. The top 20 pathways from Metacore Pathway Analysis are shown. B, C. IHC analysis of tumor infiltrating lymphocytes in C57Bl/6 ID8 tumor tissue. CD45R+ lymphocytes in (B) mesentery and (C) omental tumor sections were identified by staining with an anti-CD45R antibody (1:800 dilution) followed by a peroxidase-conjugated anti-rat-IgG and peroxidase detection using DAB as described in Methods. Representative images of young and aged tumor tissues are shown. Analysis was carried out with the Aperio Image Analysis Tools package. N = 13, 14 for young (Y), aged (A), respectively. Scale bar = 100 μm. Triple asterisk indicates $P < .01$, double asterisk indicates $P < .05$. 

### Table 1

| Pathway Description                                      | -log(P-value) |
|-----------------------------------------------------------|---------------|
| B cell signaling in hematological malignancies            |               |
| Immune response_B cell antigen receptor (BCR) pathway     |               |
| SLE genetic marker-specific pathways in B cells           |               |
| Immune response_PIP3 signaling in B lymphocytes           |               |
| Role of B cells in SLE                                     |               |
| Immune response_CCL2 signaling                            |               |
| Immune response_CCR3 signaling in eosinophils             |               |
| T follicular helper cell dysfunction in SLE                |               |
| Renal tubulointerstitial injury in Lupus Nephritis         |               |
| Dysregulation of germinal center response in SLE           |               |
| Inhibition of neutrophil migration by proresolving lipid mediators in... |               |
| Immune response_Platelet activating factor/PTAFR pathway signaling |               |
| Immune response_CD40 signaling                            |               |
| Immune response_NF-AT in immune response                  |               |
| Oxidative stress_Activation of NADPH oxidase              |               |
| Cell adhesion_Integrin inside-out signaling in T cells     |               |
| Cell adhesion_Integrin inside-out signaling in neutrophils |               |
| Vascular endothelial cell damage in SLE                    |               |
| Oxidative stress_Role of IL-8 signaling pathway in respiratory burst |               |
| Immune response_C5a signaling                             |               |

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| Dysregulation of germinal center response in SLE           |               |
| Inhibition of neutrophil migration by proresolving lipid mediators in... |               |
| Immune response_Platelet activating factor/PTAFR pathway signaling |               |
| Immune response_CD40 signaling                            |               |
| Immune response_NF-AT in immune response                  |               |
| Oxidative stress_Activation of NADPH oxidase              |               |
| Cell adhesion_Integrin inside-out signaling in T cells     |               |
| Cell adhesion_Integrin inside-out signaling in neutrophils |               |
| Vascular endothelial cell damage in SLE                    |               |
| Oxidative stress_Role of IL-8 signaling pathway in respiratory burst |               |
| Immune response_C5a signaling                             |               |
Epidemiologic data indicate that older women are at greater risk for developing ovarian cancer and receive worse prognoses than younger women [6,8]. As the impact of age on ovarian cancer metastasis has not been evaluated in vivo, two syngeneic allograft models of aging and metastasis were selected; the C57Bl/6 ID8 and the FVB/N-PTENshRNA/KRAS G12V models [18,21].

To explore how age impacts initial adhesion in vivo, young and aged C57Bl/6 mice (Figure 1A) were injected IP with ID8-RFP cells (4.3x10^6) and sacrificed the following day. Peritoneal adipose tissues, the earliest sites of adhesion in this mouse model of metastasis, were imaged ex vivo and analyzed for tumor cell adhesion. Increased tumor cell adhesion was observed to aged adipose tissues relative to the corresponding tissues in young mice (Figure 1B-D).

To investigate if the aged host is more susceptible to metastatic success over an extended period of time during which tumor cells survive and proliferate, young and aged C57Bl/6 mice were injected IP with ID8-RFP cells (3.7x10^6) and were imaged longitudinally beginning at 4.5 weeks to monitor tumor progression. Live imaging demonstrated that aged mice developed greater metastatic burden (Figure 2A). Abdominal tumor area and abdominal tumor intensity, parallel methods of analysis, consistently demonstrated aged mice had significantly greater overall tumor burden than their young counterparts (Figure 2B-D). Dissection and analysis ex vivo revealed significant age-related differences in tumor burden in the gonadal adipose tissue, with a notable difference also in the mesentery (Figure 3A-B). No difference in metastasis to the omentum was detected, suggesting that over the 8-week incubation, the initial difference in adhesion to the aged omentum was compensated for by matched survival or growth of tumor cells in the young and aged omental fat band. Ascites accumulation was not significantly different. The same trend was observed using virgin young and aged mice, confirming that the parity status of the retired breeders did not confound the results (Supplemental Fig. 1A-D).

RNAseq analysis of gonadal adipose tissue was used to explore factors potentially contributing to metastatic success in aged vs young animals [37]. Over 1500 genes were significantly differentially expressed (P < .05) to varying degrees, ranging from log2(fold change) -4.86 to 4.35 in the aged animals. Metacore Pathway Analysis and Ingenuity

### Table 1

| Gene | RSP13 Average Fold Change | RSP13 P-Value | HPRT Average Fold Change | HPRT P-Value |
|------|---------------------------|---------------|--------------------------|--------------|
| Bank1 | 8.57                      | .057          | 9.18                     | .029         |
| Blnk  | 15.78                     | .057          | 16.90                    | .029         |
| Cad1l | 9.59                      | .029          | 10.27                    | .029         |
| CD22  | 18.25                     | .029          | 19.54                    | .029         |
| CD72  | 15.45                     | .029          | 16.54                    | .029         |
| CD79a | 29.68                     | .029          | 31.78                    | .029         |
| CD79b | 26.55                     | .029          | 28.43                    | .029         |
| Ikarf2| 11.52                     | .029          | 12.34                    | .029         |
| Pex5  | 15.78                     | .029          | 16.90                    | .029         |
| Prkcb | 6.20                      | .057          | 6.64                     | .029         |

Figure 5. Evaluation of ID8-Trp53−/−-RFP tumor burden of young (Y) and aged (A) C57Bl/6 mice. A, B. Quantification of abdominal ID8-Trp53−/−-RFP tumor burden in situ. Mice were injected IP with 8.7x10^6 ID8-Trp53−/− mouse ovarian cancer cells tagged with RFP. Mice were sacrificed at 5.5 weeks post IP injection and each abdominal cavity was exposed and imaged. Abdominal tumor area (A) and intensity (B), respectively, were quantified by dividing either the tumor area or the tumor intensity by the scale-adjusted body weight of each mouse. N = 14. P-values for tumor area and tumor intensity are 0.005 and 0.006, respectively. C, D. Quantification of organ specific tumor burden. The organ area fraction (C) and intensity (D) were calculated by dividing the tumor area or intensity by the adjusted organ weight. N = 14. Triple asterisk indicates P < .01, single asterisk indicates P < .05.
Pathway Analysis (IPA) were used to view pathways enriched in differentially expressed genes and these analyses revealed that several upregulated pathways involving lymphocytes, and most notably B cells, were prominent (Figure 4A; Supplemental Table 2). As B cells are altered in aging and have prognostic significance in OvCa [38,39], B cell pathway-related genes were chosen for validation by qRT-PCR. Of 11 genes evaluated, 10 were significantly differentially regulated in aged gonadal adipose tissue (Table 1). Concomitant with the increase in B cell markers, CD45R+ lymphocytes were significantly upregulated in tumors from aged mice relative to young mice (Figure 4B-C).

During the course of this study, new sequencing data showed that parental ID8 cells contain wild type Trp53, whereas mutations in this gene are highly prevalent in human OvCa [20]. An ID8 derivative was therefore generated using CRISPR/Cas9 to delete Trp53, designated ID8-Trp53−/− [20]. Using ID8-Trp53−/−-RFP cells, our results confirm those of Walton and coworkers and show much more rapid IP growth (8 weeks vs 5.5 weeks for parental vs mutant cells). Furthermore, a significant increase in both overall and organ-specific tumor burden was found in aged mice relative to young mice in this model (Figure 5A-D).

To determine if the age-related effect observed with ID8 cells in C57BL/6 hosts extends to another allograft model, cells derived from the mouse oviductal epithelium were used [21]. These cells mimic fallopian tube-derived OvCa and form aggressive high-grade carcinomas [21]. Young and aged FVB/N mice were injected with FVB/N-PTENshRNA/KRASG12V cells (3.2x10⁶) and sacrificed the following day, followed by imaging of peritoneal adipose tissue ex vivo. Surprisingly, analysis revealed the young FVB/N mice were more susceptible to tumor cell adhesion at this time point (Figure 6A-C). However when mice were sacrificed and imaged in situ and ex vivo at 5.5 weeks, aged mice had twice as much abdominal tumor as young mice (Figure 7A-C), with significantly greater tumor burden in multiple tissues including the omentum, mesentery and gonadal adipose tissues (Figure 8A-B).

**Figure 6.** Short-term in vivo assay of PTENshRNA/KRASG12V cell adhesion to peritoneal adipose tissues. A. Representative tumor cell adhesion images. Young (Y) and Aged (A) mice were injected IP with 3.2x10⁶ PTENshRNA/KRASG12V RFP-tagged mouse ovarian cancer cells and sacrificed the following day. Major peritoneal adipose depots were rinsed with 1xPBS imaged using the Bruker In Vivo Xtreme imaging system ex vivo. B, C. Quantification of organ-specific tumor cell adhesion. Tumor Cell Adhesion Area fraction (B) and Intensity Fraction (C) and were quantified using ImageJ as described in Materials and Methods. N = 10, 9 for young and aged, respectively. For Omentum Area Fraction P = .02; Omentum Intensity Fraction P = .001. Triple asterisk indicates P < .01, double asterisk indicates significant P < .05.

**Discussion**

Age is a well-established risk factor for developing OvCa [6]. Furthermore, poorer prognoses are observed in aged individuals, with older women twice as likely to die from OvCa as younger women [8]. A recent epidemiologic study of patients with serous OvCa reported a median overall survival of 37.4 months and 47.6 months for women ages 65 and older and for women under the age of 65, respectively [10]. While a number of factors may be responsible for decreased survival in older women, such as age-related disparities in patient care [11] or coexisting medical conditions, altered cancer aggressiveness in the aged host may also contribute to this phenomenon [8]. Cancer aggressiveness could include properties of the tumor cells themselves and/or the receptivity of the aged host. Studies addressing the impact of the aged peritoneal cavity on OvCa metastasis may offer insights into mechanisms underlying epidemiologic data, particularly the...
observed decrease in survival of aged patients, and may expose new therapeutic targets for treatment of aged women with OvCa [6].

It has been proposed that age-related senescence of human omental mesothelial cells (HOMCs) may create a more receptive environment for metastasis as OvCa cells adhere more readily to induced-senescent mesothelial cells in vitro [13,40]. Furthermore, HOMCs are in an inflammatory state in the elderly [12]. A single study using a syngeneic murine model of OvCa metastasis reported that age did not significantly alter the immune profile of the murine omental fat band and was ascribed to be a negligible factor in an in vivo study addressing the role of parity on OvCa metastasis [14]. This study used middle-aged mice that were 11–12 months old; however there have been no reports regarding the impact of age on OvCa metastasis in vivo using truly aged mice (>20 months). Syngeneic murine allograft models with immunocompetent mice provide several advantages in aging and metastasis studies. Such mouse models allow for an intact immune system with which to study the impact of immune cells on cancer progression. Immunocompetent mice also age better than immunocompromised mice without succumbing to other diseases. Working with aged animals can be both expensive and challenging [16], but the current data suggest age is an important factor when investigating OvCa metastasis, warranting further investigation.

Interestingly, various events in metastatic dissemination appear to be differentially effected by age. Short-term in vivo adhesion studies with the ID8 C57Bl/6 model revealed that ID8 cells adhered more readily to aged omentum and mesentery compared to young and, at 8 weeks post injection, aged mice had larger overall tumor burden. In contrast, in short-term in vivo adhesion studies with the FVB/NJ model, PTENshRNA/KRASG12V cells adhered with greater efficacy to the young omentum. Similarly, however, in an extended tumor study, the aged animals also had substantially greater tumor burden. It is interesting to speculate that differential adhesion observed at early time points may reflect distinct profiles of mesothelial senescence in the two murine hosts. Alternatively, differential expression of adhesive ligands and receptors on C57Bl/6 versus FVB/NJ host animals and on tumor cells derived from the ovarian surface epithelium versus oviductal epithelium may also influence early adhesive events in vivo. These may include expression of molecules such as P-cadherin, mesothelin/Muc16, CD44/hyaluronic acid, and fibronectin/integrins. Nevertheless, while the two distinct models of age and metastasis varied in the age-related impact on early adhesive events, both consistently demonstrated that the aged host is ultimately more susceptible to IP metastatic colonization.

Our data suggest that age-related changes in the immune cell composition in peritoneal adipose tissue may contribute to metastatic efficiency in aged animals. An unbiased RNAseq approach revealed enrichment of a number of pathways related to B cells in aged C57Bl/6 mouse gonadal adipose tissue and a panel of B cell markers and regulators were validated as significantly upregulated in aged mice relative to young animals. A greater number of TILs were also found in aged ID8 tumors. B cell TILs are associated with prognosis, but reports vary on whether they correspond to positive or negative prognostic significance. Survival in patients with both CD20+ B cell and CD8+ T cell TILs was greater than in patients with CD8+ only TILs [41].

**Figure 7.** Evaluation of abdominal PTENshRNA/KRASG12V tumor burden in situ of young (Y) and aged (A) FVB/NJ mice. A. Representative abdominal tumor burden images. Mice were injected IP with 3.4 x 10⁶ PTENshRNA/KRASG12V-RFP cells. Mice were sacrificed at 4.5 weeks post IP injection and each abdominal cavity was exposed and imaged. B, C. Quantification of abdominal tumor burden. Tumor area (B) and intensity (C), respectively, were quantified by dividing either the tumor area or the tumor intensity by the scale-adjusted body weight of each mouse. N = 14. P-values for tumor area and tumor intensity are .0006 and .0007, respectively. Triple asterisk indicates significant P < .01.
A separate study also found that both the presence of CD20+ in the epithelial portion of the tumors and the combination of CD20+ CD8+ were associated with better survival in debulked patients with high-grade serous OvCa [42]. Furthermore, analysis using The Cancer Genome Atlas data revealed that certain B cell receptor expression signatures were predictive of improved survival in immuno-reactive and mesenchymal OvCas [43]. Interestingly, mice with a depleted CD20+ B cell population developed greater tumor burden when injected with ID8 cells [44]. B cells have also been reported as a negative prognostic factor by multiple groups [45,46]. A greater number of CD19+ B cells was correlated with poor survival [46], while more recent data show that high expression of CD19+ B cells in omental ovarian tumors is associated with decreased survival [45]. This same group demonstrated CD19+ B cells promote angiogenesis via STAT3, thereby contributing to disease progression [47]. As CD19 was upregulated in the aged gonadal adipose RNAseq dataset, it is interesting to speculate that the enhanced metastatic tumor burden seen in aged animals in this study may be due to increased angiogenesis.

Among the immunological changes that accompany aging, age-related changes in B cell development and function in humans and in mice have been described [38]. Several groups have recently reported the presence of an age-associated B cell population in aged mice [48,49]. While beyond the scope of this study, further investigation into which subpopulation of B cells are present in the metastatic niche in aged murine models is warranted. Given our findings on the impact of age on metastasis and the literature on age-related changes to B cell populations, future studies which take age into account when investigating the prognostic significance of B cell TILs are needed. Additionally, researchers considering the possibility of modulating B cell response as a therapeutic strategy must consider that age-related differences may impact the contribution of B cells to disease progression.

In summary, here we demonstrate that the aged host is more susceptible to OvCa metastasis in two distinct murine models. Our data suggest a potential contribution of B cells to this age-related disparity in metastatic success. These murine models harbor potential for evaluating immunotherapy interventions in the context of the aged host. Investigating the impact of age on metastasis is timely research, as there is an aging global population and the number of older women diagnosed with OvCa is expected to rise [8]. In addition to continued research in murine models, future research is needed to elucidate the status of B cells in human tumors from patients of varying ages. Such studies are required to predict whether targeting B cells with immunomodulatory therapies is an effective approach in older patients.

**Conflict of Interest**

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. The manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not

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**Figure 8.** Evaluation of organ-specific PTENshRNA/KRASG12V tumor burden of young (Y) and aged (A) FVB/NJ mice. A. Representative tumor burden images. Individual organs were dissected from the mouse peritoneal cavity and imaged ex vivo. B. Quantification of organ-specific tumor burden. The Organ Area Fraction was quantified by dividing the tumor area by the adjusted organ weight. N = 14. For Organ Stom P = .001; Omen P = .00002; Ovary P = .000002; PerR P = .019; Mes P = .018; FatL P = .0006. Triple asterisk indicates P < .01 and double asterisk indicates P < .05.
listed. The Corresponding Author (Dr. MS Stack; sstack@nd.edu) is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

Acknowledgements
This work was supported by Grants RO1CA109545 (MSS) from the National Institutes of Health/National Cancer Institute; from the Leo and Anne Albert Charitable Trust (MSS); National Science Foundation Graduate Research Fellowship Program grant DGE-1313583 (EAL); National Institutes of Health/ National Cancer Institute KO1 CA218305 (TSH). We thank Freimann Life Sciences Center at the University of Notre Dame, especially Emily Cronberger, for their care of the mice and contributions to the breeding strategy.

Appendix A. Supplementary Data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.03.007.

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