# Endothelial Cell's Inflammasomes and Breast Cancer

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## General Abstract

The environment of tumors is critical in cancer formation, progression, and spreading. Inflammation and especially the multi-protein cytoplasmic complexes called inflammasomes are key players in the inflammatory microenvironment . Here, we determined whether inflammasomes were present in vessel cells (i.e., endothelial cells) and the effects of their activation. The consequences for breast cancer are discussed.

## Technical Abstract

**Background:**
- Despite progress in understanding cancer, incidence and associated mortality of breast cancer remain significant.
- Recently, the tumor microenvironment, especially inflammation, has been recognized as a critical mediator of tumor progression and metastasis.
- Besides specific immunity, innate components including the cytoplasmic multi-protein complexes inflammasomes have been detected in immune and other cell types within the tumor environment.

**Approach:**
- Using known inflammasome activators such as LPS and ATP, we determined the presence of inflammasomes in 2H11endothelial cells via co-localization of three cytoplasmic proteins: NLRP3, ASC1, and pro-caspase 1.
- After confirming this cytoplasmic presence through confocal microscopy and semi-quantitative dot-blots, the effects of inflammasome activation on IL-1ß, IL-18, and VEGF secretion were assessed.
- Furthermore, the effects of the tumor microenvironment were tested using conditioned media from 4T1 mammary tumor cells.

**Results:**
- Results indicate that following ATP and 4T1 conditioned media + ATP treatments, 2H11 cells secreted significantly more IL-18. In contrast, 2H11 cell secretions of IL-1ß were increased following exposure to LPS+ATP, and LPS treatment alone. VEGF secretions were only significantly increased following LPS+ATP treatment.

**Conclusion:**
- These data confirm the presence and activity of inflammasomes in 2H11 endothelial cells and suggest that endothelial cells may contribute to the tumor's inflammatory microenvironment in breast cancer.

## Introduction and Significance

- Breast cancer is the most common diagnosed cancer in women within the United States with 30% of diagnosed breast cancers becoming metastatic [1]. **(Fig 1)**
- In addition to tumor cells, the breast tumor microenvironment includes various cell types and proteins, including extracellular matrix proteins, that participate in tumor progression [2]. **(Fig 2)**
- In particular, increasing density of vessels has been associated with poor prognosis [2].
- While interplays between the local environment and tumor cells are complex, the role of inflammasomes in promoting inflammation through the secretion of IL-1ß and IL-18 has emerged in multiple cancers [3,4].
- As shown in **Fig 3.**, inflammasomes are protein complexes of NLRP3, ASC1 and pro-caspase1 leading to the generation of activated caspase1, which in turn promotes the secretion of the pro-inflammatory cytokines IL-1ß and IL-18. Reduced inflammasome activity correlates with reduced inflammation, in turn, leading to reduced tumor progression.
- Here, the presence of the proteins forming inflammasomes and their activation in vessel cells (2H11) were investigated *in vitro*. 2H11 cells were assessed following inflammasome activation using known activators and also in the presence of media from 4T1 murine breast tumor cells.


**Fig 1.** Incidence of Breast Cancer among women

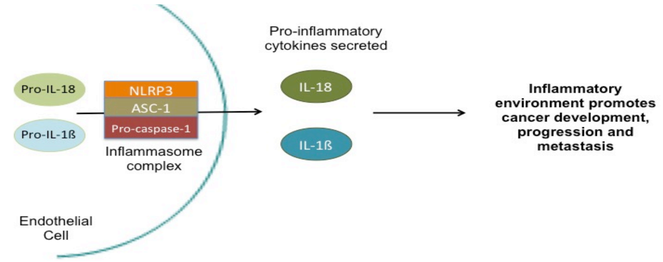



**Fig 3.** Schematic of inflammasome activation leading to IL-1ß and IL-18 secretions in endothelial cells

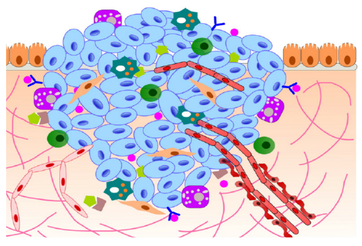


**Fig 2.** The local tumor microenvironment consists of many different cell types. [5]

## Hypothesis

**Vessel cells express inflammatory proteins and participate to the inflammation in breast tumors.**

## Results

### Expression and co-localization of three key proteins confirms inflammasome complex formation in endothelial cells.

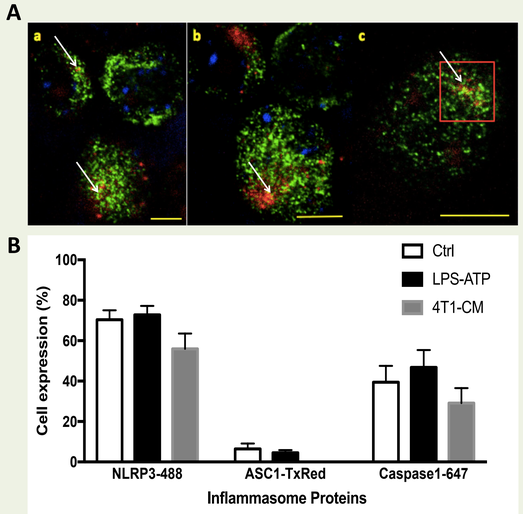
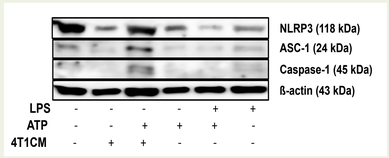



**Fig 4. Inflammasome protein expression in 2H11 cells. (A)** Representative confocal microphotographs depicting 2H11 cells stained with inflammasome proteins [NLRP3 (Green), ASC1 (Red) and nuclei (Blue). Scale bars (5 µm) are displayed. Arrows point to NLRP3-ASC1 co-localization. **(B)** 2H11 cells positive (%) for NLRP3, ASC1 and Caspase1 as determined by flow-cytometry.**p<0.01 and ***p<0.001.

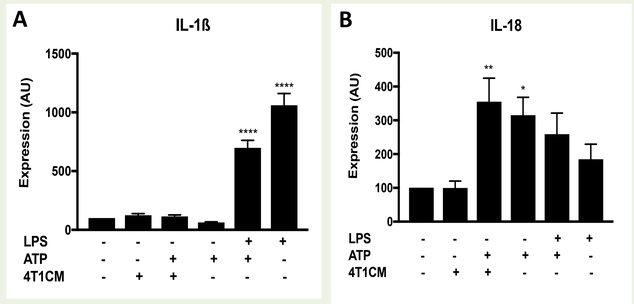
### Breast cancer environment activates 2H11 cell inflammasomes.




Briefly, following incubation in media alone or with LPS, ATP or 4T1 conditioned media (4T1CM), cell lysates were assessed by Western blots for expression of inflammasome proteins NLRP3, ASC1 and Caspase 1. ß-actin serves as loading control

**Fig 5. In the breast cancer environment (4T1CM+ATP), inflammasome complex proteins are expressed.**

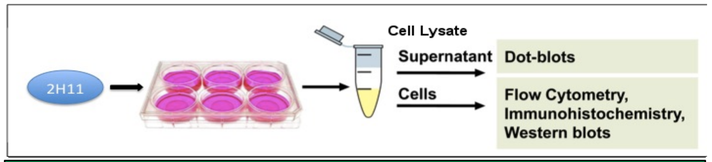
### Breast cancer cells activated 2H11 cell inflammasomes leading to the secretion of inflammatory signals.




Following incubation in media alone or with LPS, ATP or 4T1 conditioned media (4T1CM), IL-1ß and IL-18 relative expression was determined by semi-quantitative dot blots of cell supernatant. *p<0.05, **p<0.01 and ***p<0.001.

**Fig 6. Pro-inflammatory cytokines IL-1ß and IL-18 are released from endothelial cells cultured in inflammatory environments.**

## Experimental Design




## Materials and Methods

**Cell and culture conditions:** The murine 2H11 cell line was used to mimic endothelial cells. Aggressive stage of human breast cancer was represented by murine mammary 4T1 cells. Cells were grown and cultured in DMEM supplemented with antibiotic, antifungal, and 10% of FBS (Atlanta biologic, Atlanta, GA). For experiments, 2H11 cells were incubated for 6hrs with FBS-free media alone (negative control), or with FBS-free media supplemented with LPS 5ug/ml + ATP 5mM (positive control) or 4T1 conditioned media. Cells were then harvested and analyzed by flow-cytometry and confocal microscopy. Additionally, both cell lysates and supernatants were collected for Western blots and cytokine measurements.

**Flow-cytometry & Confocal microscopy:** Post-treatment, cells were fixed in formalin and stored at 4°C prior to permeabilization with saponin and immunostain with antibodies to NLRP3 (R&D system, Minneapolis, MN); ASC1 (Santa Cruz Biotechnology, Santa Cruz CA) and Caspase1 (Santa Cruz) as described previously [2]. The presence of NLRP3+, ASC1+ cells was determined by flow-cytometry using a FACS-Calibur (BD BioSciences, CA). Additionally, cells were mounted and visualized using confocal microscopy. 3D image stacks were collected at 0.3-µm z increments on a DeltaVision workstation (GE) based on an inverted microscope (IX-70; Olympus) using a 60×1.4NA oil immersion lens. Images were captured at 24°C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics) and deconvolved using the iterative-constrained algorithm and the measured point spread function.

**Western blots & Dot blots:** Cell lysates were analyzed by western blots as described previously [2]. Briefly after protein quantification using BCA assays, heating and reducing the samples in loading buffer, samples were separated by electrophoresis and transferred to nitrocellulose membrane. After blocking, incubation with primary antibodies against NLRP3, ASC1, Caspase1, IL-1ß, IL-18, a specific secondary HRP conjugated antibody was used along with a chemiluminescent substrate to detect the presence of proteins. ß-actin expression served as loading control. For dot-blots, samples were loaded (65µl) onto a nitrocellulose membrane, blocked and immunodetected as described for Western blots. For quantification of the signal, Image J and Protein Array Analyser were used. Protein expression are expressed as % of control conditions.

**Statistical analysis:** Data are presented as mean ± SEM. Differences between groups were analyzed using ANOVAs and post-hoc tests with an *a priori* significance level of p<0.05.

## Results/Conclusion

- In 2H11 endothelial cells, inflammasome complexes are
  - present and
  - activated.
- Within the breast cancer microenvironment, endothelial cells
  - release pro-inflammatory cytokines (IL-1ß and IL-18) and
  - participate in tumor inflammation.

➢ **Data confirm that Vessel cells express inflammatory proteins and contribute to the inflammation in breast tumors.**

## Relevance

As inflammation is key to the clinical progression of breast cancer, an intervention modulating vessel cell pro-inflammation activity may be helpful in preventing breast cancer progression.

## Acknowledgements/References

The author acknowledge the active participation of Madison Anderson, UNC Charlotte Honors in Biological Sciences graduate and the expertise and assistance of Richard Chi, PhD (UNC Charlotte) with confocal imaging. This work was in part funded by the Center for Biomedical Engineering and Science and the University of North Carolina at Charlotte.

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