PIM KINASE CONTROL IN INFLAMMASOME SIGNALING IN MACROPHAGE SURVIVAL

by

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Abstract:

The tumor microenvironment consists of a variety of different cell types that contribute to helping maintain tumor cell survival in a harsh and ever-changing environment. The goal of this study was to determine how PIM kinases impact cellular ROS production and survival in tumor associated macrophages, and how the NLRP3 inflammasome is affected by PIM activity and changes in cellular ROS. We found that PIM inhibition significantly increases intracellular ROS in macrophages in a concentration- and time-dependent basis. Through western blotting and RT-PCR, we utilized protocols to activate the inflammasome and show how different treatment techniques, such as the inhibition of ROS, can modulate expression and activation of caspase - 1. We primarily demonstrate a direct relationship between the inhibition of PIM kinase and increased cellular ROS. The inhibition of PIM increases ROS and kills macrophages through a caspase – 1 dependent manner. This data helps to improve our current understanding of PIM kinase signaling in macrophages and can be used as a steppingstone to direct future studies investigating the potential role of PIM kinase inhibitors as a novel therapeutic approach to combat pro-tumorigenic effects of the immune system to improve cancer treatment.
Introduction:

The tumor microenvironment (TME) is comprised of proliferating tumor cells, stromal cells, blood vessels, infiltrating inflammatory cells and a wide variety of immune cells. Together, these cells enhance tumor survival and tumor proliferative growth. While various immune effector cells are recruited to the tumor site, some of their anti-tumor functions are downregulated, primarily due to signals derived from the tumor itself [1]. In addition, these immune cells tend to promote tumor growth through activation of signal transduction pathways, such as the NF-κB pathway, which promotes tumor survival and drives abortive activation of immune cells. An understanding of the complex relationships that different immune cell types have within the TME and how they promote tumor growth provides an opportunity to target different nuances of the TME and develop new therapeutic strategies to treat solid tumors.

The innate immune system consists of a relatively non-specific approach to pattern recognition allowing for immediate response to intruders based on certain, unique molecules. Activation of pattern recognition receptors by pathogen-associated molecular patterns (PAMPs), derived from invading pathogens, and danger-associated molecular patterns (DAMPS), induced because of endogenous stress, triggers downstream signaling cascades that lead to production of type 1 Interferons (Interferon-α and Interferon-β) and proinflammatory cytokines [2,3]. These proinflammatory cytokines generate an inflammatory response in the microenvironment, promoting angiogenesis, tissue remodeling and tumor cell proliferation. My research is focused on understanding the relationship between PIM kinase and inflammatory cytokines and how regulation of the inflammasome prevents the activation of many of these cytokines. Inflammasomes are multimeric protein complexes that assemble in the cytosol after sensing PAMPS or DAMPS [4]. Upon recognition of certain stimuli, the relevant pattern recognition
receptor (PRR), Toll-Like Receptors can oligomerize to form a Caspase–1–Activating Scaffold. When active, caspase-1 cleaves the proinflammatory IL-1 family of cytokines into their bioactive form, IL-1β, causing pyroptosis, which is a type of inflammatory cell death. This secretion of proinflammatory IL-1β, through pyroptosis, can occur regardless of stimulus, in monocytes and macrophages, and maintains a direct relationship with caspase-1. As a result, caspase-1 inhibitors have been shown to decrease secretion of the mature IL-1β cytokine [5]. Other studies investigating IL-1β show that during chronic inflammation, factors such as cellular ROS that led to the formation of the inflammasome can also support tumor development, but in cases of too much cellular ROS, both the immune system and tumor cells will die [6]. While the M1 macrophage causes tissue damage through the killing of pathogens, the M2 macrophages clean up and try to repair the damage through the release of cytokines, such as TGF-β and IL-10, that have anti-inflammatory effects and deal with wound repair [7,8]. Under the right conditions, M2 phenotype macrophages create an immunosuppressive microenvironment by producing an array of cytokines, chemokines, and growth factors, while also triggering the binding of inhibitory immune surface proteins on T cells, such as PD1-PDL1 interaction, allowing for cancer initiation and angiogenesis [9].

When LPS, or other agonistic stimuli are recognized by their respective Toll like receptor (TLR), the NF-κB transcription factor is translocated into the nucleus, upregulating production of the NLRP3 protein, the precursor for the NLRP3 inflammasome, as well as pro-IL-1β, an inactive cytokine that is crucial for host cell responsible to injury and infection [10]. As this is happening, mitoROS production is increased in the cell due to pattern recognition sensing of pathogens. Mitochondrial ROS has a switch-like interaction with the NF-κB signaling pathway; transcription of NF-κB-dependent genes influences the levels of ROS, while NF-κB activity is also regulated
by ROS, potentially making ROS a rate-limiting factors in NLRP3 activation [11]. At the same time, the NLRP3 adaptor protein, adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), is being linearly ubiquitinated and phosphorylated, which is required for inflammasome assembly to occur. The ASC protein bridges interactions between TLRs and pro-caspase-1, recruiting pro-caspase-1 to the inflammasome. As the NLRP3 inflammasome complex localizes to the mitochondria, other stimuli, such as mitochondrial ROS, DNA, and potassium efflux, and ATP work to activate the NLRP3 inflammasome. From what is understood, the NBD domain of NLRP3 oligomerizes the N-Terminal Pyrin Domain of NLRP3 allowing for the nucleation of CARD domains. When pro-caspase 1 interacts with the CARD domains, through CARD-CARD interactions, the proximity of pro-caspase 1 proteins induces an autoproteolytic maturation of this pro-caspase 1 into an active caspase 1 [12,13]. Once activated, caspase 1 cleaves the pro-domain off the inactive IL-1β cytokine, allowing its activation and secretion from the cell [14].

Proviral Integration Site for Moloney Murine Leukemia Virus (PIM) is a family of serine-threonine kinases that have been implicated in driving cell growth, survival, and proliferation thorough interaction with numerous tumorigenic pathways [15]. Notably, studies by our lab and others have shown that PIM upregulation causes resistance to conventional chemotherapy and other targeted therapeutics [16]. PIM1 is overexpressed in prostate tumors possessing inflammatory features and markers of stemness [17]. Recent work from our lab has demonstrated that PIM is a critical regulator of oxidative stress and ROS production in tumor cells. First, in a hypoxic environment, where there is reduced oxygenation within the TME, there tends to be excess generation of reactive oxygen species (ROS) [18]. To combat this, PIM1/2 are stabilized in tumor cells to reduce ROS to physiological levels and maintain survival. Mechanistically, PIM1 can
promote the stabilization and nuclear localization of the NRF2 transcription factor, which binds to Antioxidant Response Element (ARE) sequences in the genome that initiate transcription of many cytoprotective genes that reduce ROS [19,20]. Alternatively, previous studies performed in my lab demonstrated that PIM inhibitors cause fragmentation to the mitochondria leading to an increase in ROS release. Inhibition or loss of PIM1 increases Dynamin-related protein 1 (Drp1) levels and localization to the mitochondria, increasing mitochondrial fission [17]. Drp1 is a cytoplasmic protein that, when recruited, colocalizes to the mitochondria, forming around the mitochondria constricting and separating the mitochondrial membrane. Increased levels of Drp1 have been shown to upregulate cytochrome – c release, caspase activation and downstream factors that lead to cellular apoptosis [17,21]. Thus PIM kinases help to promote stability of the mitochondria by preventing this fission and downstream apoptotic activation [22]. Together, these data indicate that PIM1 overexpression will reduce ROS, while inhibiting PIM causes an increase in ROS. Thus, expression of PIM is a major kinase responsible for protecting against oxidative damage. Based on these findings, we generated the hypothesis that PIM could play a key role in negatively regulating the inflammasome and mediating inflammation-driven cell death.

Due to the relationship between active caspase-1 and the IL-1β cytokine, there is credence to the idea that identifying ways to increase the expression of caspase-1 components within the cell will effectively kill tumor associated macrophages (TAMs) via pyroptosis, but also promote inflammation and in cases, tumorigenesis. There is a lack of understanding of the stimuli that lead to the formation of the NLRP3 inflammasome in solid tumors. Recently, hypoxia has been proposed as a major driver of mitochondrial based stimuli that help form the inflammasome [23]. More specifically, it was shown through in vivo studies in a diabetic mouse model system, that hypoxia can activate the NLRP3 inflammasome in LPS-primed mice, resulting in initiation of β-
cell inflammatory response and cell death [24]. Therefore, we hypothesize that upregulation of PIM proteins (as is seen in hypoxia) will decrease inflammasome-mediated pyroptosis, and PIM inhibition will dramatically increase ROS and push inflammasome-production past thresholds needed for cell survival, leading to apoptosis.

Tumor associated macrophages are key cells that exist within a TME that contribute to immunosuppression in solid tumors. By producing a variety of stimulating factors, growth factors, cytokines, and chemokines, TAMs help to signal a series of inhibitory immune checkpoints in T cells [25]. Thus, TAMs promote tumor growth, survival and metastatic tendencies. In prostate cancer, it is becoming clearer that TAMs are key players related to tumor aggressiveness. Specifically, high macrophage infiltration in the prostate TME was significantly associated with phenotypic traits of metastasis, such as extracapsular extension [26]. In relation to prostate cancer and TAMs, we are interested in understanding the potential relationship between the inflammasome, the IL-1β cytokine, and the modulation of cell survival by PIM; specifically how overexpression and inhibition of PIM could affect inflammation and survival in TAMs. Our hypothesis is that PIM decreases inflammation by reducing ROS levels, failing to activate caspase-1-mediated cleavage of proIL-1β. This phenomenon can be reversed using PIM inhibitors, leading to increased secretion of active IL-1β and further inflammation, ultimately reducing protumorigenic TAMs and increasing tumor cell death.
Methods and Materials:

Reagents and Animals:
DMEM, Opti-MEM, Streptomycin, Penicillin, and Fetal Bovine Serum (FBS) were obtained from Thermo/Fisher/Gibco (Carlsbad, CA, USA). Primary antibodies against PIM1 (Rabbit) (ID: 3247S), PIM2 (Rabbit) (ID: 4730S), and PIM3 (Rabbit) (ID: 4165S) were all purchased from Cell Signaling Technologies (Danvers, MA, USA). The NLRP3 specific pro and cleaved Caspase-1p20 Antibody (Mouse) (ID: AD-20B-0042) was purchased from AdipoGen (San Diego, CA, USA). LPS-EB Ultrapure was obtained from InvivoGen (Catalog #: tlrl-3pelps) and was dissolved in LAL Water, also purchased from InvivoGen (Catalog #: h2nlal-1.5) at a concentration of 1 mg/mL and stored at -20°C. The sheep-α-mouse IgG(H+L) HRP Conjugate Secondary Antibody was obtained from Genesee Scientific (ID: 84-848) (El Cajon, CA, USA), while the Anti-Rabbit IgG HRP-linked Antibody was obtained from Cell Signaling Technologies (ID: 7074S) (Danvers, MA, USA). Stock solutions of 100mM ATP Concentration were obtained from Fisher Scientific (ID: FERR0441) (Waltham, MA, USA), and were aliquoted into eppendorf tubes at 25µL volume and stored at -80°C. The PIM447 inhibitor was purchased from Selleck Chemical LLC, via Fisher Scientific, diluted to a concentration of 3mM, aliquoted, and stored at -20°C. Both the Mouse IL-1 beta / IL-1F2 DuoSet ELISA Kit (Catalog #: DY401-05) and DuoSet Ancillary Reagent Kit 2 (Catalog #: DY008) were obtained from R&D Systems (Minneapolis, MN, USA) and stored in conditions between 2-8°C once opened. For ROS Fluorescence, the 2’,7’-Dichlorofluorescin diacetate, 2,7-DCFDA, was purchased from Sigma-Aldrich (ID: D6883) (St. Louis, MO, USA), diluted to a concentration of 20mM, aliquoted in light sensitive eppendorf tubes, and stored at -20°C. C57BL/6 mice carcasses were obtained from the University of Arizona Experimental Mouse Shared Resource (EMSR).
Cell Culture Line:
Mouse macrophage cell line J774A.1 was obtained from ATCC. The cells were maintained in complete DMEM medium (containing 10% FBS, 100 ug/ml of penicillin, and 100ug/mL of Streptomycin) and cultured at 37°C in a humidified incubator with 5% CO₂. These cells were subcultured every 2-3 days through scraping and the removal / addition of media to the T75 flask they are maintained in.

Bone Marrow Derived Macrophages Cell Culture:
Bone Marrow Derived Macrophages were harvested from C57BL/6 mice carcasses obtained from the University of Arizona Experimental Mouse Shared Resource and were cultured based on protocol provided by Dr. Justin Wilson of the University of Arizona Cancer Center. With possession of the mice, the femurs were harvested and cleaned, and prepared by storage in PBS. Both tips of each femur were cut allowing for a 26G needles to be inserted into the bone, where we flush the bone marrow with 2.5 ml of DMEM media into a 15 ml centrifuge tube, repeating the process on the other side of the femur. Once each bone is flushed, large particles will settle to the bottom of the tube, and the rest of the media is collected in new centrifuge tubes, where they are spun down at 450xg for 5 min at 4°C. After repeating this wash process, add ~2x10⁶ cells to a 10cm Non-Cell Culture Treated Petri Dish and add 9 mls of Bone Marrow Macrophage Media. Incubate for 6 to 7 days, adding an addition 10mLs of bone marrow mac media on day 4 to prevent acidification of media. Bone Marrow Macrophage media is comprised of DMEM, heat inactivated FBS, CMG14-12 media (containing RPMI, heat inactivated FBS, 100x Penicillin / Streptomycin, Sodium Pyruvate, and non-essential amino acids), and 100x Penicillin / Streptomycin.
**Inflammasome Activation:**

J774A.1 cell were plated in 6 well dishes at approximately 1.5x10^6 cells per well and allowed to adhere overnight. Cells were treated over a course of 5 hours, being placed on a plate rocker for 3-5 minutes to provide equal dispersion of treatment throughout the entire well. Cells were treated with 1ug/mL of LPS for 4 hours and 30 minutes. Half an hour prior to completion of this treatment, cells were treated with PIM447 (PIM Kinase Inhibitor) at concentrations specified by treatment conditions. Upon completion of LPS treatment, cells were treated with 50 um of Nigericin for 1 hour to promote cleavage of Pro-Caspase 1 and resultant cleavage of Pro-IL-1β. Supernatant of treated cells was collected and stored in -80°C temperature for ELISA, while cell lysates were collected and stored in ice for western blot analysis. When not in use, lysates are stored at -20°C.

**Western Blot Analysis:**

Whole cell lysates were collected through 100-120 ul of RIPA Lysis Buffer + Protease Inhibitor, and protein levels were measured through Bradford Testing. Total proteins were separated by SDS-PAGE and then transferred onto Polyvinylidene Difluoride Membranes (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was blocked and incubated with primary antibody overnight, followed by incubation sheep-α-mouse IgG(H+L) HRP Conjugate Secondary Antibody or Anti-Rabbit IgG HRP-linked Antibody, depending on the primary antibody. Bands on the membrane were revealed by either SuperSignal West Femto Chemiluminescent Substrate (ID: P134095) (Thermo Fisher Scientific, Waltham, MA, USA), or Clarity™ Western ECL Substrate (Catalog #: 170-5061) (Bio-Rad, Hercules, CA, USA) and images were recorded on ProSignal™ Blotting Film (Catalog #: 30-810L) (Prometheus Protein Biology Products; Genesee Scientific, El Cajon, CA, USA) and Syngene G: BOX Chemi (Frederick, MA, USA) for high resolution fluorescence.
**ELISA:**
An enzyme linked immunosorbent assay (ELISA) was performed to quantify the pro-inflammatory mediators (IL-1β) after the exposure of the J774A.1 cells to LPS, Nigericin, and PIM447 treatments. Using the DuoSet reagent ELISA 1L-1B kit, dilute the capture antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 ul per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes, with a volume of 400 ul. Each well in the plates are blocked with 300 ul of reagent diluent and the wells are incubated for 1 hour. After repetitive wash steps, add 100 ul of sample or standards in Reagent Diluent, or an appropriate diluent per well, and incubate the microplate for 2 hours. Once plate has been washed, add 100 ul of Detection Antibody, diluted in a reagent diluent to each well, and incubate for 2 hours. Add 100 ul of a working dilution of Streptavidin-HRP to each well. Incubate this plate for 20 minutes at room temperature in a dark environment. After the 20-minute incubation period, add 100 ul of Substrate Solution, a color reagent, to each room, incubating for 20 minutes. Like with Streptavidin HRP, avoid incubating in direct light. After the 20-minute incubation period, add 50 ul of Stop Solution to each well, gently tap the plate to ensure thorough mixture. Plate is read almost instantly at a wavelength of 450nm.

**Cell Viability Assay:**
Between $5 \times 10^4$ and $1 \times 10^5$ J774A.1 macrophages were plated uniformly in a 96 well plate and cells could adhere overnight. These cells were treated with a 2-fold dilution set from concentrations starting as high as 320 um of PIM447 to 1 um of PIM447 for a time course of either 72 or 96 hours. Data was analyzed through usage of an XTT Cell Viability Kit, following online
protocols, with time measurements taken 1 hour and 18 hours. Absorbance was read at a wavelength of 450nm. Immediately before use, add 100 ul of the XTT activator to ~5 ml of XTT reagent to make XTT working solution. Prepared XTT Working Solution should be added to cells within several minutes.

**RT-PCR Assay:**
With identification of mRNA concentration, you add specific concentrations of a qScript Super Mix that contains required primers and enzymes to allow for amplification of cDNA from mRNA. Once converted from mRNA to cDNA, design a master mix for a 10 ul SSOAdvanced Universal Sybr-Green Super Mix, 1 ul of a diluted forward and reverse primer, and nuclease free water. With the master mix prepared, add 2 ul of cDNA to each well and 18 ul of master mix, place film on top of plate and lightly vortex. Spin the microplate for 1 minute at 1000rpm and run and analyze the RT-PCR program.

**Statistical Analysis:**
Differences across groups were determined by unpaired two-tailed Student’s t test. One-way analysis of variance was used to analyze differences between more than two groups across one time point. P values were adjusted using Bonferroni’s multiple comparison test. The data are presented as the mean SD or mean SEM as indicated, and a p value. The IC50 value for viability assay was determined in GraphPad PRISM, analyzing the data for Absolute IC50, with X being the concentration.
Results:

Relationship between PIM and Mitochondrial ROS in Macrophages.

To identify the relationship between PIM and the NLRP3 inflammasome within macrophages, we asked the following questions: How do PIM inhibitors affect macrophages and how does PIM activity influence the production of components required for NLRP3 inflammasome formation? Within the TME, the hypoxic environment will upregulate both expression of the PIM Kinases, as well as ROS. These two cellular components counter each other allowing the tumor cell to survive (Model 1). My lab previously tested the logistics of this model in a variety of immortalized and primary prostate and lung cancer cells, ultimately confirming that PIM inhibitors generate excess ROS that can lead to tumor cell death.
**Model 1.** This model was designed in BioRender to show the current known pathway linking PIM and ROS within the tumor microenvironment. In a hypoxic tumor microenvironment, both the PIM Kinase family and ROS are upregulated, and PIM serves to keeping ROS levels in check to maintain cell survival by upregulating Nrf2 target genes inhibit cellular ROS and promote cell survival [10].

The tumor microenvironment does not consist solely of tumor cells; other cell types, including TAMs are key for promoting signaling pathways that allow for tumor cell survival. Based on our understanding of how PIM regulates oxidative stress in cancer cells, we initially hypothesized that PIM Inhibition in macrophages should increase mitochondrial ROS [17]. To test this, H2DCF Staining was used to selectively measure superoxide levels in a J774A.1 cell line, an immortalized mouse macrophage cell line. J774A.1 cells were treated PIM447 (a pan-PIM kinase inhibitor), H2O2 (a positive control known to induce cellular ROS) for 1 and 24 h. H2DCFDA is a cell-permeable probe that diffuses into cells and, when deacetylated, forms H2DCF. In the presence of cellular ROS, this H2DCF is highly fluorescent and allows for quantitative measurements of cellular ROS levels [26]. Through live cell imaging of H2DCF, we observed marked visual increases in fluorescence of the J774A.1 cells at both the 1 hour and 24-hour treatment intervals. Quantitatively, using ImageJ software, we demonstrate that PIM447 treatment significantly increases in the fluorescence in J774A.1 cells, and this increases with time (Figure 1a). To confirm these results, J774A.1 cells were treated with a consecutive two-fold dilution of PIM447, starting at 1 um to 10 um. After two hours of treatment, we saw little increase in CTCF fold change, with increases primarily being at the higher concentration (10 uM). When intensity was measured after 24 hours, we saw significant increases in CTCF fold change, even at lower concentrations of PIM447 (Figure 1b). These data confirm the first part of our hypothesis that PIM inhibition increases cellular ROS in macrophages.
Figure 1. PIM Inhibition leads to total cellular ROS production in macrophages. **a.** Cellular ROS was detected by H$_2$DCF staining in J774A.1 Macrophages when treated with the same concentration (3 um) of PIM447 inhibitor at different time points. 500 um concentration of H$_2$O$_2$ was used as a positive control due to its known ability to induce cellular ROS. Fold Change in ROS levels was measured by ImageJ count of the fluoresced cells in part a. *p < 0.05, **p < 0.01, ***p < 0.001 vs. DMSO. **b.** Cellular ROS was detected by H$_2$DCF staining in J774A.1 Macrophages when treated with a range of concentrations of PIM447 inhibitor at two different time points. Fold Change in ROS levels was measured by ImageJ count of the fluoresced cells in part b.

PIM Inhibition aids NLRP3 Inflammasome Activation

With confirmation that PIM Inhibition within macrophages increases cellular ROS production, we sought to identify how this PIM Inhibition effects both pro-caspase 1 production and eventual inflammasome activation within macrophages. Based on what is known regarding the inflammasome, when an external stimuli, such as LPS interacts with TLRs located on the cytoplasmic membrane of macrophages, the NF-kB signaling pathway is activated to produce NLRP3 and pro-IL-1β, which is then deubiquitinated (Model 3). Based on this knowledge, we designed a stimulation protocol that uses both LPS and Nigericin. Nigericin is a potent compound, we applied, that disrupts the mitochondrial membrane allowing for stimulation and release of mtROS and mtDNA, as well as causes K$^+$ efflux, all of which are secondary and important activators to form the NLRP3 inflammasome.

J774A.1 cells were chosen for these experiments because they constitutively express ASC components required to activate the NLRP3 Inflammasome, making them an appropriate model to recapitulate the effect on human or mouse derived macrophages. As we previously identified that 24-hour treatment with a PIM inhibitor significantly increased cellular ROS, we pretreated our cells with PIM447 for 24-hour at different concentrations, ranging from 0.5 um to 40 um. Following the 24-hour treatment with PIM447, cells were treated with a 1 ug/mL of LPS and, after 4 hours, we added 50 um of Nigericin for 1 hour. As expected, we noticed cleaved caspase–1 in wells treated with both LPS and Nigericin. Strikingly, treatment with PIM447 increased
expression of cleaved caspase-1 when combined with LPS and nigericin. Caspase-1 cleavage continued to increase in response to increasing concentrations of PIM inhibitor and reached a maximum at concentrations above 10 μm (Figure 2a). Using ImageJ, we quantified the levels of cleaved caspase-1, confirming that the PIM inhibitor significantly increased cleaved caspase-1 levels (Figure 2b).

Model 3. This model shows the relationship between LPS interaction with TLR4 and how that leads to pro-caspase 1 accumulation within the cytosol, potential IL-1β activation, and NLRP3 Inflammasome activation. When enough components of pro-caspase 1 interact, the CARD-CARD domains within the ASC components will cause autoproteolytic maturation of pro-caspase 1 into activate caspase 1. Active Caspase will cleave the pro-domain off IL-1β allowing for secretion of active IL-1β.
A. 

Figure 2. PIM Inhibition induces increased Cleaved Caspase expression. a. Western Blot analysis for the detection of procaspase – 1 in the cell lysate and the culture medium of J774A.1. Cells are pretreated with different concentrations of PIM447, followed by treatment of 1μg/mL of LPS for 4 hours. After LPS treatment, cells are treated with 50μM of Nigericin for 1 hour prior to cell harvesting for Western Blot and ELISA. The cells in a multiple 6-well plate were lysed with 100μL of RIPA Buffer to allow for maximum concentration of protein. Blots were developed via film with a one second exposure and finalized with SynGene Technology for clean bands. b. Fold change in Cleaved Caspase Expression was quantified in

B.
Caspase Inhibition Promotes J774A.1 Cell Survival

Next, we sought to identify the IC50 value of PIM447 in J774A.1 cells. Within the NLRP3 inflammasome complex, cleaved caspase-1 components work together to induce inflammatory cellular stressors that cause programmed cellular death. Thus, we hypothesized that the PIM inhibitor is killing macrophages by activating Caspases. To test this, cells were plated at 3x10^4 per well in a 96 well plate and treated over a course of 72 hours or 96 hours. We treated cells with a combination of PIM447 concentrations ranging from 0.05 um and to 320 um alone and 5 um of QVD-OPH, a non-specific Caspase Inhibitor that inhibits activity of caspase 1,3,4 and others. 50uL of XTT was added and cells were incubated with XTT for 18 hours. Data was collected at 450 nm wavelength reader and normalized in Excel. PIM447 alone treated cells had an IC50 value of approximately 8.08 um and 5.84 um at 72 and 96 h, respectively (Figure 3a and b). At both time points tested, treatment with the Caspase inhibitor dramatically reduced PIM447 mediated cell death, raising the IC50 to approximately 100 um at both timepoints (Figure 3a and b).
Figure 3. PIM Inhibition leads to decreased Cell Survival. a. Analysis of Cell Viability was performed through XTT Viability Assay. Cells were plated at 30000 per well in a 96 well plate and treated over a course of 72 hours. 50uL of XTT was added and cells were incubated with XTT for 18 hours. Data was collected at 450nm wavelength reader and normalized in Excel. Graph was created through using GraphPad prism. b. Analysis of Cell Viability was performed through XTT Viability Assay. Cells were plated at 30000 per well in a 96 well plate and treated over a course of 96 hours. 50uL of XTT was added and cells were incubated with XTT for 18 hours. Data was collected at 450nm wavelength reader and normalized in Excel. Graph was created through using GraphPad prism.
Next, we asked whether PIM447-induced cell death required NLRP3. To this end, we used OLT1177, a specific NLRP3 inhibitor that prevents NLRP3-ASC and NLRP3-caspase 1 interaction, inhibiting NLRP3 Inflammasome Oligomerization [28]. A recent study in animal models showed that OLT1177 blocks activation of the NLRP3 inflammasome. The study also showed a direct binding between NLRP3 and OLT1177 blocked ATPase activity. In different immune cells, the study also showed that OLT1177 reduced cleaved caspase-1 activity and downstream IL-1β secretion [29]. Treatment with multiple concentrations of OLT1177 alone showed no effect on cell viability (Figure 4a). Notably, OLT1177 was able to rescue macrophage cell death when combined with PIM447, indicating that PIM inhibition kills cells through the NLRP3 and caspase-1 pathway (Figure 4b).
Figure 4. NLRP3 Inhibition rescues TAM cell viability. a. Analysis of Cell Viability was performed through XTT Viability Assay. Cells were plated at 30000 per well in a 96 well plate and treated over a course of 72 hours. 50μL of XTT was added and cells were incubated with XTT for 18 hours. Data was collected at 450nm wavelength reader and normalized in Excel. Graph was created through using GraphPad prism. b. Multiple concentrations of OLT1177 were used to test the effectiveness of OLT1177 with a range of PIM447 concentrations. Analysis of Cell Viability was performed through XTT Viability Assay. Cells were plated at 30000 per well in a 96 well plate and treated over a course of 96 hours. 50μL of XTT was added and cells were incubated with XTT for 18 hours. Data was collected at 450nm wavelength reader and normalized in Excel. Graph was created through using GraphPad prism.

ROS alone does not stimulate cleaved caspase-1 production.

Cellular ROS has been shown to promote the expression of cellular components, such as cleaved Caspase-1, when in combination with LPS and Nigericin. To further examine the relationship between PIM, ROS, and the Inflammasome, we asked whether ROS alone was sufficient to increase caspase-1 cleavage and how that can affect downstream production and stimulation of the IL-1β Cytokine.

To understand this relationship, we plated out the same cell lines and treatments described above and harvested mRNA for RT-PCR. Through amplification of this cDNA, we looked at the expression of specific genes related to the NLRP3 Inflammasome and downstream signals (Table 1). Analysis of this data showed trends verifying the results of our western blotting data, but we also gleaned an understanding of how our treatments truly modify the cells. Previous data from other labs had shown that inhibition of PIM blocks downstream effectors of the NFkB signaling pathway, and this was confirmed by our PCR data, showing no significant changes to NFkB in cells treated with either H2O2 or PIM447 alone without any stimulating factor. Along with this, we saw significant increases in IL-1β expression in cells treated with LPS, Nigericin and PIM447 as compared to LPS and Nigericin alone (Figure 6). This confirms our hypothesis that PIM Inhibition will lead to downstream increases in production of IL-1β. Interestingly, induction of
cellular ROS alone using H₂O₂ affected key genomic components in the NLRP3 inflammasome, primarily increase in IL-1β expression to a similar extent as LPS and Nigericin treatment.

<table>
<thead>
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<tr>
<td>NFkB</td>
<td>GAAATGCCACCTTTTGACAGTG</td>
<td>CATGCCTGAAATATGATCAC</td>
</tr>
</tbody>
</table>

Table 1. Primers used for RT-PCR. All the primers were researched from previous work done involving the NLRP3 Inflammasome, and all the primers are designed specifically for mouse related samples.
Figure 5. **PIM Inhibition, alongside LPS and Nigericin increase IL-1β expression.** Quantitative transcriptional expression of key components of the NLRP3 Inflammasome based on how they change in relation to specific treatment regimen. Some protocol of treatment as was used in Western Blot. Total treatments represent LPS, Nigericin, and PIM447. Data was analyzed through Bio-Rad Prime PCR and Excel and graphed in Excel. *p < 0.05, **p < 0.01, ***p < 0.001

### Inhibition of ROS decreases cleaved caspase-1 expression

With our data showing that cellular ROS allows for the recruitment and production of factors that can release IL-1β in tumor associated macrophages, we investigated how inhibition of ROS affected IL-1β and caspase-1 activity. We tested the hypothesis that the increase in ROS in response to PIM inhibition is required for PIM447-mediated cell death in TAMs. To study this, we incorporated an ROS Scavenger, N-Acetyl Cysteine (NAC), that reduces the accumulation of cellular ROS. We compared the viability of cells treated with different concentrations of PIM447 in the presence or absence of 2 um NAC. Treatment with NAC significantly reduced PIM447-induced cell death. The viability of the NAC treated cells did tend to decrease after the 10 um concentration, but at a significantly slower rate compared to PIM447 alone (Figure 6). While the
viability is maintained at these higher concentrations, it is not maintained as well when compared with caspase Inhibitors, suggesting that the PIM inhibitor is still causing caspase-mediated cell death that is independent of ROS.

Figure 6. NAC maintains cellular viability at higher concentrations. Analysis of Cell Viability was performed through XTT Viability Assay. Cells were plated at 30000 per well in a 96 well plate and treated over a course of 72 hours. 50uL of XTT was added and cells were incubated with XTT for 18 hours. Data was collected at 450nm wavelength reader and normalized in Excel. Graph was created through using GraphPad prism.

A.
B.

Figure 7. NAC reduces the expression of cleaved caspase 1. a. a. Western Blot analysis for the detection of procaspase – 1 in the cell lysate and the culture medium of J774A.1. Cells are pretreated with different concentrations of PIM447,1uM of OLT1177 and 2mM of NAC, followed by treatment of 1ug/mL of LPS for 4 hours. After LPS treatment, cells are treated with 50uM of Nigericin, or 500uM of H₂O₂ for 1 hour prior to cell harvesting for Western Blot and ELISA. b. Fold change in Cleaved Caspase Expression was quantified in ImageJ, with resulting expression values being normalized based on LPS + Nigericin as baseline at 1. *p < 0.05, **p < 0.01, ***p < 0.001

To determine how NAC treatment affected cleaved caspase-1 production in cell, we treated cells in duplicate under the following conditions: one group with our standard LPS, Nigericin and PIM447 treatments and the other with the same treatments, but the addition of NAC. Lysates were collected for gel electrophoresis and western blotting. There was decreased caspase 1 cleavage in the cells that were treated with NAC, suggesting that ROS does contribute to the activation of the inflammasome. There was increased visual expression of PIM1 in the lines that were treated with our PIM Inhibitor, which is a known phenomenon, and this effect was further increased in the presence of NAC (Figure 7a). Through quantification via ImageJ, we confirmed a significant decrease in cleaved caspase-1 expression in the presence of NAC (Figure 7b). It is interesting to note, however, that there was the least amount of decrease in the cells that were treated with the NLRP3 Inhibitor.
Discussion:

The tumor microenvironment does not consist solely of tumor cells, and other cells play a major role in modulating signals that allow cell survival and growth. One significant cell type within the microenvironment is the macrophage, particularly those switched from the M1 phenotype to the M2 phenotype [7]. These cells provide growth signals and overall survival signals that help allow tumor cells to survive and thrive. Therefore, identifying techniques to either kill these macrophages or switch them back to an M1 phenotype, is a promising approach to improve cancer therapies [8]. Here, we focus on PIM inhibition as a strategy to limit TAMs within the tumor microenvironment.

PIM Kinases are commonly overexpressed in tumor cells. However, they can also be upregulated in normal and immune cells through external factors, such as hypoxia, and aberrant signaling through pathways such as JAK/STAT regulate the transcriptional activation of PIM genes [32]. Regardless of the mechanism, PIM Kinase overexpression in tumor cells has been linked to drug resistance and cell survival. My studies sought to expand our knowledge of the role PIM kinases play in immune cells and how targeting PIM could be useful in combatting their pro-tumorigenic effect. Previous research performed by the Warfel Lab and others demonstrated that inhibition of the PIM Kinase increases cellular ROS. Taking that knowledge, we tested the role of PIM in regulating cellular ROS in macrophages. Using an immortalized mouse macrophage line, J774A.1, we show that PIM inhibition significantly increases ROS. Treatment with PIM447, a pan-PIM Kinase inhibitor, increased cellular ROS in a dose- and time-dependent manner (Figure 1). Next, we sought to understand the relationship between PIM Kinase inhibition and the NLRP3 Inflammasome. The NLRP3 inflammasome is a unique, and specific type of inflammasome that regulates the activation of Caspase-1 and induces inflammation in response to a variety of
microbial and host derived stimuli [12,13]. As a microbial stimuli, we used LPS due to its’ interactive nature with TLR4 found in the macrophage membrane. LPS is an endotoxic component found on the surface of gram-negative bacteria, which when interacts with TLR4, it activates signaling pathways that results in the deubiquitinating of cellular NLRP3 components. Treatment of J774A.1 cells with our pan-PIM Kinase inhibitor, significantly elevated the levels of cleaved caspase–1 compared to the standard activation regimen of LPS and Nigericin alone. This data suggests that while Nigericin stimulates and disrupts the mitochondrial membrane, the inhibition of PIM kinases adds an additional internal stress, in the form of increased cellular ROS, which increases the activation of the NLRP3 inflammasome and resultant production of cleaved caspase 1.

Because caspase-1 activation is the initiating step in inflammatory apoptosis and upstream of IL-1β activation, we focused on elucidating whether this caspase was essential for PIM inhibitors to kill macrophages. Running a series of XTT Viability assays, we treated cells with a combination of PIM447 and a nonspecific caspase inhibitor, and we noticed a maintenance of cell survival until excessively high concentrations of PIM447. PIM447, when alone, had an IC50 value between 8 um and 12 um, while PIM447 in combination with our caspase inhibitor had an IC50 value of above 100 um. This result demonstrates that activation of caspases is required for PIM inhibitor-mediated cell death. This also confirmed the importance of caspases regarding cell death when increasing cellular ROS. Modifying the protocol, we tested whether inhibiting the activation of caspase-1 was sufficient to block cell death by PIM447 [33]. To this end, we combined PIM447 with a specific caspase 1 inhibitor. Thus, caspase-1 inhibition alone can maintain cell survival at concentrations of PIM447 past its’ IC50 value.
Next, we designed experiments to study the relationship between PIM, ROS and cleaved caspase-1 activity. First, we treated J774A.1 cells with H$_2$O$_2$ to increase ROS. H$_2$O$_2$ did not significantly alter the expression of cleaved caspase-1 at the protein level, although we observed increased relative expression of caspase-1 at the transcriptional level. Additionally, we noticed increased expression of IL-1β. Thus, cellular ROS might activate IL-1β through another pathway, regardless of the NLRP3 Inflammasome. Based on these findings, we propose that cellular ROS, while it may not affect cleaved caspase-1 production, does elevate the levels of IL-1β. This could mean that cellular ROS production, by disrupting the mitochondrial lining, could release enough stimulating factors to bypass the LPS and Nigericin stimulation and produce increased levels of IL-1β.

To determine whether cell death during PIM inhibition requires ROS, we treated cells with PIM447 and NAC, a compound that scavenges cellular ROS. The addition of NAC significantly reduced cell death, further solidifying the relationship between PIM447 mediated cell death and ROS in macrophages. While we did still observe cell death at higher concentrations of PIM447, it was significantly blunted by NAC. Notably, reducing ROS levels lowered the amount of cleaved caspase-1, justifying what we visualized through the viability assay. By having less cleaved caspase-1, the cells undergo cell death at a slower rate due to it taking longer to produce enough cleaved caspase to surpass cytoprotective components within cells and the surrounding environment.

Based on our understanding of TAMs, it would be pertinent to study how PIM inhibition and inflammasome activation affects inhibitory stimulating pathways turned off by these protumor macrophages. Another interesting area of research would be understanding how PIM could affect the efficacy of PDL-1 inhibitors, and how treatment with PIM447 could alter the recruitment of
new immune cells and alter tumor progression. Thus, treatment with PIM447 could selectively eliminate these macrophages, and in combination with PDL-1, PIM447-mediated inflammasome activity and secretion of IL-1β could recruit more M1 phenotype macrophages. It is possible that this combination could blunt the inhibitory effects of the PDL-1 receptor. Further studies need to be performed \textit{in vivo} using syngeneic cancer models to determine how this approach would change tumor growth and aggression. Therefore, studies on the combined effects of PDL-1 and PIM inhibitors would be beneficial as a way of designing a new therapeutic technique where the body’s own immune system is able to bypass checkpoints that inhibit macrophage activity and successfully recruit new CD8 T cells to the tumor environment [31]. This, in theory, would allow the immune system to target and recruit new immune cells to a tumor environment and allow for increased recognition and immune defense against cancer cells.
Bibliography:


