# UNIVERSITY OF CALIFORNIA, MERCED

## Purinergic Receptors Responsible for ATP-dependent ROS Production and Activation of Inflammasomes in Gingival Epithelial Cells.

In Quantitative and System's Biology

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Master of Science

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The Master's of Science Thesis by Marcus Ho is approved and is acceptable in quality and form for publication on microfilm and electronically.

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# DEDICATION:

To my mother and sister, who have always been there to hear my complaints and make sure I am eating enough. You both have provided me with limitless and unquestioning love, and can cheer me up when I am at my worst.

To the memory of my father, who taught me the importance of family, health, and hard work.

I would also like to thank the lab members of the Ojcius lab (2010-2012) as well as the undergraduate students working there. Without their guidance and support throughout this year, I would never have been able to maintain my sanity, and this thesis may not have existed.

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# Abstract:

$P2X_4$ and $P2X_7$ are purinergic receptors prevalent in human immune and epithelial cells. Some of their functions in the cell include mediating cell death and the inflammatory response. The multiprotein inflammasome complex in the innate immune system helps process and release cytokines such as interleukins 1β and 8. Pannexin-1 is a membrane channel suggested to play a role in inflammasome activation by interacting with purinergic receptors. This thesis evaluates whether extracellular ATP activates the inflammasome in a $P2X_7$, $P2X_4$ or pannexin-1-dependent manner. The characterization of this mechanism is to be elucidated in gingival epithelial cells (GECs), which are part of the oral cavity's first line of defense in the innate immune system. GECs express $P2X_4$ and $P2X_7$ receptors as well as pannexin-1, and are sensitive to ATP-induced apoptosis. However, the mechanisms by which the inflammasomes are activated are uncertain. In this study, we compared reactive oxygen species (ROS) production levels between GECs treated with ATP and those without ATP. ROS production has been shown to promote the assembly of inflammasomes, leading to caspase-1 activation. We also did treatments with various receptor and channel inhibitors, and generated GEC $P2X_4$, $P2X_7$ and pannexin-1 knockdown cell lines to compare the amount of ROS and caspase-1 activation due to ATP stimulation. Our results show that depletion of $P2X_4$, $P2X_7$ and pannexin-1 by either specific antagonists or shRNA diminished the ability of ATP to induce ROS production and to a lesser extent, caspase-1 activation in GECs. $P2X_4$, $P2X_7$ and pannexin-1 are therefore likely to be important mediators of the inflammatory response in GECs. Lastly, we sought to find protein-protein interactions between the three proteins, and how they may affect inflammasome activation. So far, this study shows that $P2X_4$ and $P2X_7$ are able to interact, and are enhanced upon ATP stimulation.

# Introduction:

## Gingival epithelial cells:

GECs are among the first host cells encountered by colonizing bacteria in the oral cavity. Epithelial cells are crucial to the innate immune response not only by acting as a physical barrier but also to sense and respond to pathogen associated molecular patterns (PAMPS). GECs utilize pattern-recognition receptors (PRRs) that sense bacteria and result in the secretion of inflammatory cytokines to initiate an immune response in the host. Numerous studies have shown that GECs secrete pro-inflammatory cytokines and chemokines in response to periodontal bacteria such as *Porphyromonas gingivalis* [1]. The cells can also modulate other signaling pathways and phenotypic properties common in epithelial cells including inhibition of interleukin-8 secretion, modulation of intracellular calcium concentrations, and activation of integrin receptor-associated signaling pathways [2]. However, there are many signaling pathways and characteristics that are not well understood in GECs, which could provide insight to potential treatment options for our understanding of periodontal health. Periodontitis is the inflammation of the tissues that surround and support the teeth. Chronic inflammation of these tissues can lead to bleeding, pus formation, and many other detrimental oral health issues such as gradual bone resorption and tooth decay. Consequently, studying the mechanism behind the molecular signaling of GECs as an important component of the immune system in can help us further understand how a quick and effective response is developed towards infection by oral pathogens.

## Inflammasome Activation:

The production and secretion of inflammatory cytokines are tightly controlled, requiring the activation of an intracellular inflammasome multiprotein complex. The inflammasome is fully operational in regulating cytokine release once it encounters both an exogenous pathogen associated molecular pattern (PAMP) as well as an endogenous danger-associated molecular pattern (DAMP). Both of these signals together potentiate the immune response because they

not only warn the GECs of a foreign organism in the vicinity, but they give indications that the neighboring cells are stressed or undergoing cell death.

GECs have been shown to express different types of PRRs such as toll-like receptors (TLRs), nod-like receptors [1]. The ligation of PAMPs to a PRR from an invading organism is sufficient to trigger transcription and synthesis of many pro-inflammatory cytokines such as pro-IL-1β, IL-8, or IFNγ [3, 4]. Once the DAMP is also sensed by the cell, the assembly of the inflammasome will take place in the cytoplasm, resulting in processing and secretion of the mature cytokines. These danger signals could be released from neighboring cells that are dying, infected, or stressed. Examples of danger signals include extracellular ATP, monosodium urate crystals, fibronectin, or rapid potassium ion efflux [5, 6].

NLRP3:

Bioinformatics studies have shown that there are 22 NLR genes in the human genome [7]. While epithelial cells have TLRs to sense extracellular and endosomal PAMPs, one advantage of cells using NLRs is to have pathogen sensors that are in intracellular compartments.

One of the well established inflammasomes that exist in GECs is Nalp3, also known as NLRP3 or Cryopyrin [8]. This protein complex is composed of a Nod-like receptor, and ASC (apoptosis-associated speck-like protein containing a caspase activation recruitment domain) adaptor. The NLR has an N-terminal pyrin domain, a nucleotide-binding domain, and a leucine-rich repeat (LRR) motif [9]. The C-terminal LRR domain is responsible for recognizing different pathogens by protein-protein interactions, and is highly conserved within the NLR family. Upon activation of the inflammasome, by the LRR region, the molecule will undergo a conformational change that triggers oligomerization through the nucleotide-binding oligomerization domain (NOD). Furthermore, the NLR will expose the N-terminal effector domains to recruit caspase activation recruitment domains (CARD).

Cysteinyl aspartate specific proteinases (Caspases) are a type of protease with essential roles in apoptosis, necrosis, and inflammation [10]. The discovery of caspases came from developmental studies of C. *elegans* as important for apoptosis. The cell death gene ced-3 was

found to have a similar mammalian homolog for interleukin-1 beta-converting enzyme (ICE, now known as caspase-1) [11]. These evolutionary conserved proteases are synthesized as inactive zymogens (pro-caspase), which are then regulated at the post-transcriptional level resulting in a rapid cascade of signaling events. The CARD domain is one of the prodomains of caspase responsible for its activation [12]. Once the caspase proteases are activated, they will regulate the activation of a variety of other inflammatory molecules such as pro-IL-1β or pro-IL-18 into biologically active and mature IL-1β and IL-18.

Previous research has shown that ATP stimulates the assembly of the NLRP3 inflammasome in GECs. In the GECs that had NLRP3 knockdown, ATP was unable to stimulate IL-1β secretion from GECs infected with Porphyromonas *gingivalis*; while GECs that normally expressed NLRP3 protein showed a significantly greater amount of IL-1β secretion [1]. However, the downstream signaling events after ATP ligation are yet to be discovered.

IL-1 Beta:

The cytokine IL-1β is a very important molecule involved in signaling. The transcription of pro-IL-1β can be generated from many different pathways, namely NF-kB and type I interferon responses in inflammasome pathways. Signaling through these two pathways due to microbial insult will generate immature pro-IL-1β to prepare for an immune response. Once a DAMP is sensed by the cell in addition to the pathogen component, then the cell will produce active caspases to cleave pro-IL-1β to generate large amounts of mature IL-1β to be released [1]. Once produced, IL-1β follows an unconventional method of secretion out of the cell, in which it does not normally traffic through the Golgi complex before exocytosis. It is not yet known how exactly the cytokine is secreted following cleavage of its precursor, but has been suggested that it can occur via active transport by a multi-drug resistance transporter and/or following cell death [57]. In this way, the role of inflammasome activation most likely involves cross talk between other pathways such as NF-kB or the type I interferon response. Following secretion, IL-1β can then act as a danger signal for neighboring cells, activating receptors that lead to induction and release of several other pro-inflammatory cytokines such as IL-6, IL-8, tumor necrosis factor (TNFα), or interferon (IFNɣ) [57].

## Reactive Oxygen Species:

There is a possibility of reactive oxygen species being produced after ATP treatment of gingival epithelial cells that serve to stimulate caspase-1 activation and processing of downstream cytokines. The production of ROS such as hydrogen peroxide has long been considered a detrimental consequence of oxidative phosphorylation. Due to activity of the electron transport chain, some of the mitochondrial oxygen consumed is incompletely reduced, interacting with electrons to form superoxide anions, which may then dismutate into $H_2O_2$ which will diffuse out of the mitochondria [13-16]. Furthermore, ROS has been associated with diseases as diverse as aging, cancer, and neurodegenerative disease [17]. $H_2O_2$ has been shown to be a physiological mediator in cell signaling pathways involved in differentiation, proliferation, migration, and cytokine secretion [18, 19]. Lastly, an ROS response in psychologically stressed mice led to caspase-1 activation, secretion of IL-1β, and up-regulation of IL-18 precursor proteins [8]. It seems as though redox regulated gene transcription and metabolic activity recently play a larger role in signaling than previously thought. Proteins, particularly those with thiol or heme transition metal centers can bind to DNA as transcription factors once they are oxidized by ROS. Given the ability of ROS to act as a secondary messenger to activate caspase-1 and downstream interleukin cytokines, we investigated the role of ROS production and how it may have a correlation to inflammation activation in GECs.

## Purinergic Receptors:

The ionotropic P2X receptors are a family of ligand-gated ion channels whose ligands are purines and pyrimidines. Extracellular ATP has been shown to bind to the $P2X_7$ and $P2X_4$ receptors, which have been known to be highly expressed in macrophages and some epithelial cells [1]. Depending on cell type, the binding of ATP to $P2X_7$ can lead to apoptosis, shedding of cell-adhesion molecules, cell proliferation, or the killing of intracellular pathogens [20-22]. The binding of ATP to a P2X receptor results in a conformational change that opens the ion channel, causing a depolarization of the cell membrane, and subsequent $K^+$ efflux. $P2X_7$ is also shown to be an inducer of nonselective macropores that are permeable to large ( ≤ 800 Daltons)

inorganic and organic molecules. Thus, activation of this receptor by extracellular ATP rapidly induces a complete collapse of ionic gradients that switches the cytosol from a high $K^+$, low $Na^+$, low $Cl^-$ ionic milieu to a low $K^+$, high $Na^+$, and high $Cl^-$ environment [23].

The ligation of the $P2X_7$ and $P2X_4$ receptors with ATP has also been shown to cause the formation of reactive oxygen species consequently leading to caspase-1 activation in macrophages [24]. These inflammasome mechanisms have been characterized in monocytes, macrophages, as well as HeLa cells, but never in GECs.

In GECs, it has been shown that pre-incubation with the $P2X_7$ irreversible antagonist, oxidized (ox)ATP before treatment with ATP leads to significant inhibition of ATP-induced apoptosis. Conversely, using the $P2X_7$ receptor agonist 2',3'-O-(4-benzoyl-benzoyl) ATP (bzATP), to stimulate the receptor does induce apoptosis, suggesting that the ATP-induced apoptosis mechanism is $P2X_7$-dependent [25].

So far, $P2X_7$ is the most established of the purinergic receptors in having a physiological role in the immune system. $P2X_4$ however has been shown to be expressed in most immune cells, including mast cells, B and T lymphocytes, monocytes, macrophages, microglia, and osteoclasts [26]. The role of $P2X_4$ is less well understood; however it is known to have a higher binding affinity to ATP, is predominantly intracellular, and has been shown to be up-regulated following peripheral nerve injury [27]. Furthermore, molecular and inhibitor studies suggested a main role for $P2X_4$ receptor in nucleotide-induced apoptosis in human mesangial cells, indicating a relevant role for purinergic signaling in regulating death rate in these cells [28]. Because $P2X_4$ has been shown to mediate ATP-induced apoptosis, further examination of this receptor in GECs is necessary. Immunofluorescence microscopy of protein expression revealed that $P2X_4$ is detected at very low levels in GECs [25], therefore we cannot exclude the possibility that $P2X_4$ may contribute to ATP induced caspase-1 and inflammasome activation.

### Cross-Talk Between $P2X_4$ and $P2X_7$:

Of the seven human P2X receptor family subunit types ($P2X_1$- $P2X_7$), all but $P2X_6$ are able to assemble as homotrimers. Of these homotrimers, it has been shown that different combinations of these subunits can form heterotrimers with each other. These reports are

based on studies of co-localization, co-immunoprecipitation, structural evidence such as chemical cross-linking, single-molecule imaging, in situ proximity ligation, co-isolation on affinity beads, and atomic force microscopy [29-31]. The field of electrophysiology has heightened our understanding of the functional role of extracellular ATP acting as a signal transducer by binding to purinergic receptors. Kinetic characteristics such as the rate of current decay, reversal potential values, and concentration response curves to determine ATP sensitivity of ATP-activated currents in cell membranes vary between the members of the family of receptors, [32].

Interestingly, much work has been done on the interaction between $P2X_4$ and $P2X_7$ receptors, showing that they are frequently co-expressed in immune and epithelial cells [33]. Point mutations of $P2X_4$ have demonstrated a functional interaction between the two receptors; one mutant exerted a dominant negative effect on $P2X_7$ receptor currents, whereas another mutation conferred $P2X_4$-like properties on the whole cell currents [34]. These results suggest the interaction between the two receptors has a physiological relevance to produce specific downstream events. Since different P2X subtypes are frequently co-expressed in the same cell, the potential for mixing and matching of subunits can diversify responses that are mediated by this class of receptors. Protein-protein interactions between these two receptors are worth exploring in GECs, because both of these receptors may have a role in the activation of the inflammasome or ROS production.

## Pannexin-1:

Stimulation of the $P2X_7$ receptor with ATP induces a rapid opening of the potassium-selective channel, followed by the gradual opening of a larger pore [35]. One of the pores is mediated by the hemichannel composed of pannexin-1, which also contributes to the formation of phagosomes [36]. The transmembrane protein is located at the cell surface and in the endoplasmic reticulum organelles, and has been found to be involved in caspase-1 activation by heat-killed bacteria and several PAMPs such as lipoplolysaccharide (LPS). The exact mechanism behind this is not yet established, but these results suggest that pannexin-1

and $P2X_7$ work in conjunction to mediate TLR-independent transport of bacteria-derived molecules through endosomal compartments to activate NLRP3 in mouse macrophages [37].

Another study in primary neurons and astrocytes shows that high extracellular potassium is able to open pannexin-1 channels leading to caspase-1 activation. Probenecid, a pannexin-1 channel blocker was shown to inhibit activation of caspase-1 in astrocytes stimulated with KCl [38]. Furthermore, nigericin, a polyether ionophore which disrupts membrane potential and transports monovalent cations such as $K^+$ across membranes is a potent activator of caspase-1 in multiple cell types [39]. It works similarly with ATP in that it decreases intracellular levels of potassium in a pannexin-1 signaling manner to activate the inflammasome [40].

Whether pannexin-1 is involved in a potassium efflux, reactive oxygen species, or PAMP internalizing dependent manner, or even a combination of these types of pathways, it must be further characterized in its role in the innate immunity. Therefore, the possible function of pannexin-1 being a TLR-independent way of signaling for caspase-1 activation must be further studied to give a comprehensive understanding of inflammasomes.

## Autoinflammatory and Inflammasome Related Diseases:

Autoinflammatory diseases represent a heterogeneous group of pathologies characterized by spontaneous periodic inflammation and fever in the absence of infectious or autoimmune causes [41]. Investigation of the diverse biological activities of IL-1β has significantly increased our understanding of the pathogenesis of several diseases, including type I diabetes, gout, and many autoinflammatory disorders, including the NLRP3-associated disorders known as Cryopyrin-Associated Periodic Syndromes (CAPS) [42-44]. Blocking IL-1β with IL-1 receptor antagonists in autoinflammatory diseases often results in a decrease in the activity of the inflammasome, suggesting that IL-1β is driving its own synthesis, as well as the activation of the inflammasome [42]. A correlation has been observed between IL-1β single-nucleotide polymorphisms and periodontal disease. Significantly higher levels of IL-1β have also been detected in saliva from patients with periodontitis, compared to healthy controls, suggesting a role for this cytokine in development of disease [45-46].

Interestingly, other components of the inflammasome have also been shown to cause illness. For example, polymorphisms in $P2X_7$ have been associated with Crohn's disease, cancer, and susceptibility to tuberculosis [47-49]. Researchers have developed potent and specific $P2X_7$ receptor antagonists with human specificity such as AZD9056. This molecule, in its Phase II clinical trial produces clinically relevant improvements by reducing swollen and tender joints of patients with rheumatoid arthritis [50]. In relation to microbial infection, the study of inflammasome components is critical to understanding how certain pathogens are able to evade or manipulate immune responses. It has been reported that *Porphyromonas gingivalis* produces the molecule nucleoside diphosphate kinase (NDK), which once released into the oral cavity, scavenges for extracellular ATP, preventing its signaling effects on purinergic receptors. In this way, P. gingivalis can evade apoptosis of GECs, allowing it to live intracellularly for an extended period of time [25].

The more details emerge on inflammasomes and their activation, the more evidence also points to a direct contribution of inflammasome defects, such as hereditary mutations in inflammasome activators leading to exaggerated immune responses. There must be other mutations in molecules involved in the evolutionarily conserved inflammasome complex that are yet to be studied. The work done towards this thesis hopes to bring insight on these complexes and their relation to periodontal health defects (Refer to diagram 1 below). What purinergic receptors are responsible for a robust and swift immune response to keep an organism healthy? How can cells sense danger by communicating with their neighbors and how do they prepare accordingly? Lastly, how does reactive oxygen species play a role in inflammasome activation?

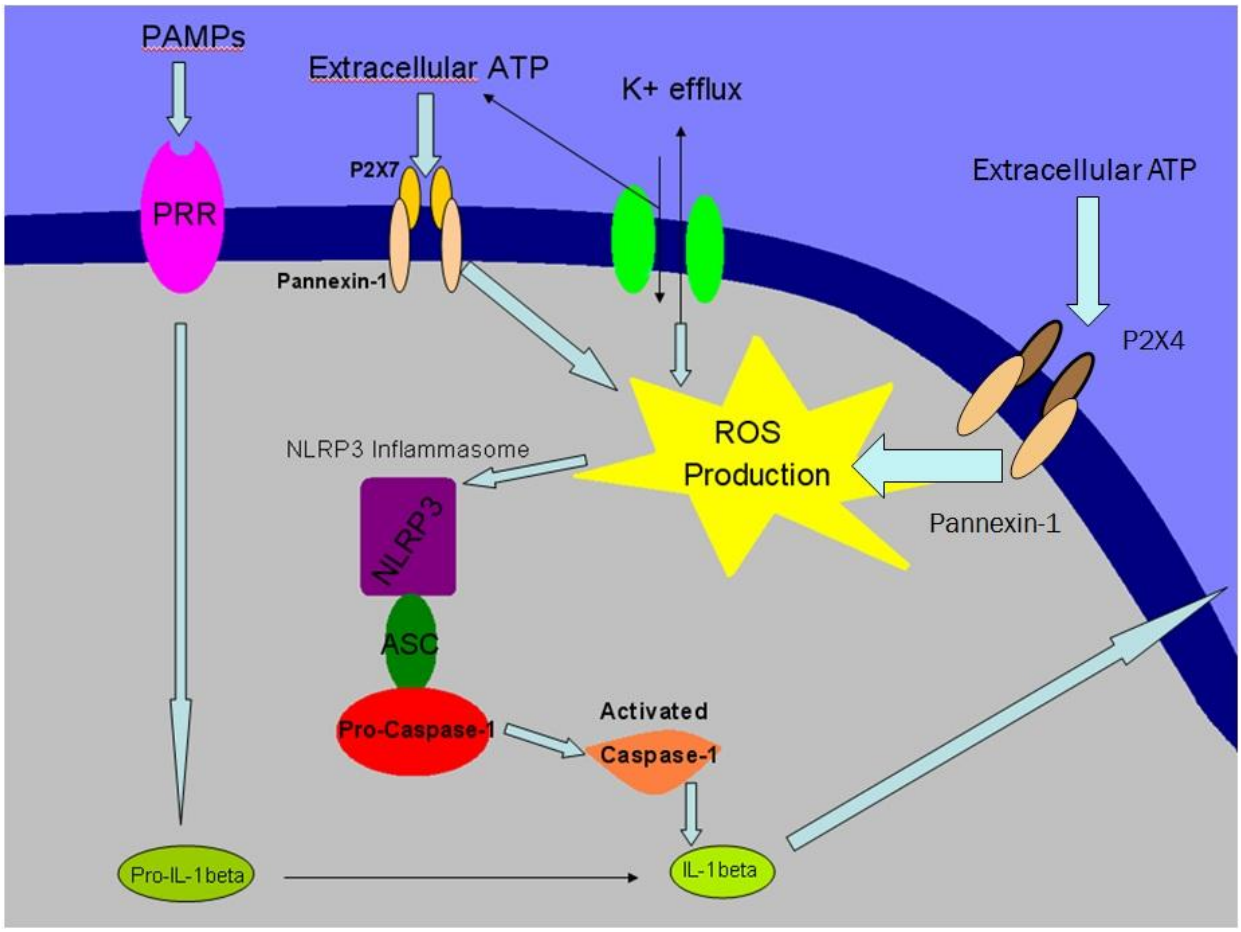



Diagram 1: Proposed Inflammasome mechanism in GECs

# Methods and Materials:

## Cell Culture

Gingival epithelial cells (GECs) were obtained from the Ozlem laboratory (Gainesville, FL), and cultured in a humidified incubator at 37°C with 5% $CO_2$. GECs were cultured in tissue culture flasks containing defined keratinocyte-SFM (1X) media (KM) (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, bovine pituitary extract (BPE), and embryonic growth factor (EGF). Knocked-down GECs were grown under same media conditions and then grown with media containing 5 µg/mL puromycin. To eliminate possibility of mycoplasma contamination of cells that would influence signaling pathways, polymerase chain reaction was performed on the supernatants using targeted primers for eight of the most common mycoplasma species. Passage of cells to new flasks was done by using TrypLE Express 1x (Invitrogen)

## Generation of KD cell lines

Short-hairpin (sh)-lentivrial particles (Sigma Aldrich) for Pannexin-1, $P2X_7$, $P2X_4$, and shCtrl were added to GECs cultured at about $2 \times 10^4$ cells/mL at a multiplicity of infection of 10. At increasing confluence, cells were moved to successive larger flasks. Cells were cultured for 2 passages until they were grown with media containing 5 µg/mL puromycin to select for stable knockdown cells lines. Cells were not to be cultured past twenty passages for risk of loss of phenotype.

## Cell Treatment

GECs were grown to 70-90% confluence in tissue culture plates (Corning Inc). Media was changed 6 hours prior to treatment with phenol red free Optimem media (Invitrogen) without FBS, and incubated at 37°C in 5% $CO_2$ in-between treatments, and during dye loading. Treatments with reagents were performed at the indicated times and concentrations.

## Chemical Reagents

Adenosine 5'-triphosphate disodium salt hydrate (ATP), Probenecid, oxidized ATP, and nigericin were all obtained from Sigma (St. Louis, MO). Treatment with inhibitors or other reagents was performed at the indicated times and concentrations. 5-(3-Bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one (5-BDBD) and Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS) were obtained from Tocris Bioscience, London, UK.

## RNA Extraction

Total RNA was extracted from the GECs using the Qiagen RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The total RNA was quantified by measuring the optical density with the NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE) and converted into cDNA by standard reverse transcription with Taqman® reverse transcriptase kit (Applied Biosystems, Foster City, CA).

## cDNA Synthesis, PCR Amplification

After reverse-transcription, cDNA was amplified with respective primers using the Qiagen Fast Cycling PCR kit (Qiagen, Valencia, CA) following the manufacturer's instructions. All primers used are listed in table 1.

Table 1: Sequences of Primers used for polymerase chain reactions

| | |
|---|---|
| GAPDH Forward | 5'- CGACCACTTTGTCAAGCTCA -3' |
| GAPDH Reverse | 5'- AGGGGAGATTCAGTGTGGTG -3' |
| $P2X_4$ Forward | 5'- GATACCAGCTCAGGAGGAAAAC -3' |
| $P2X_4$ Reverse | 5'- GCATCATAAATGCACGACTTGAG -3' |
| $P2X_7$ Forward | 5'- TGATAAAAGTCTTCGGGATCCGT -3' |
| $P2X_7$ Reverse | 5'- TGGACAAATCTGTGAAGTCCATC -3' |
| Pannexin-1 Forward | 5'- GGTGAGACAAGACCCAGAGC -3' |
| Pannexin-1 Reverse | 5'- GGCATCGGACCTTAACACCTA -3' |
| $P2Y_1$ Forward | 5'- CTTGGTGCTGATTCTGGGCTG -3' |

| | |
|---|---|
| $P2Y_1$ Reverse | 5’- GCTCGGGAGAGTCTCCTTCTG -3’ |
| $P2Y_2$ Forward | 5’ - CCGCTCGCTGGACCTCAGCTG -3’ |
| $P2Y_2$ Reverse | 5’ - CTCACTGCTGCCCAACACATC -3’ |
| $P2Y_4$ Forward | 5’ - CGCTGCCCACCCTCATCTAC -3’ |
| $P2Y_4$ Reverse | 5’ - CCGAGTGGTGTCATGGCACAG -3’ |
| $P2Y_5$ Forward | 5’ - TGTTCAGTCTACCCACTCTCAG -3’ |
| $P2Y_5$ Reverse | 5’ - CTTACTGCTGCCACTACTGAGC -3’ |
| $P2Y_6$ Forward | 5’ - AACCTTGCTCTGGCTGACCTG -3’ |
| $P2Y_6$ Reverse | 5’ - GCAGGCACTGGGTTGTCACG -3’ |
| $P2Y_{11}$ Forward | 5’- GAGGCCTGCATCAAGTGTCTG -3’ |
| $P2Y_{11}$ Reverse | 5’- ACGTTGAGCACCCGCATGATG -3’ |
| $P2Y_{12}$ Forward | 5’ - ACCGGTCATACGTAAGAACGAG -3’ |
| $P2Y_{12}$ Reverse | 5’ - GCAGAATTGGGGCACTTCAGC -3’ |
| $P2Y_{13}$ Forward | 5’- GGAAGCAACACCATCGTCTGTG -3’ |
| $P2Y_{13}$ Reverse | 5’- GACTGTGAGTATATGGAACTCTG -3’ |

The Fast Cycling PCR protocol for all the primers was a 5 min enzyme activation step at 95°C, then 45 cycles of 96°C for 5 seconds, 55°C for 5 seconds, and 68°C for 20 seconds. At the end, there was a 10 min extension step at 72°C. In order to check whether the GECs expressed the purinergic receptor genes, after the PCR amplification for those specific human genes was carried out, the products were separated by electrophoresis on a 2% agarose gel. The gel was run at 100 Volts for 45 minutes and visualized by ethidium bromide staining on a gel doc system (Bio-Rad).

## Quantitative Polymerase Chain Reaction

qPCR of DNA samples was prepared using primer concentrations of 10uM and SYBR green Brilliant III (Agilent). Stratagene's MXPro3000P was used for quantitative PCR and had the thermal profile of 3 minute enzyme activation step at 95°C, then 50 cycles of 95°C for 5 seconds (denaturing), and 55°C for 20 seconds (annealing). At the end, there was a melting cycle specific to SYBR green kit with the profile of 1 minute heating step at 95°C followed by a 55°C extension step for 30 seconds ending with the final 30 seconds of 95°C. Analysis of gene expression levels were normalized to GAPDH housekeeping gene. All primers were checked for homologous dissociation melting curves and each replicate on the triplicates were checked for $C_T$ value deviations no more than 1 cycle from each other.

## Fluorescence Microscopy

Cells were grown to 70% confluence on a 12-well plate (Corning Inc.) and prior to chemical treatment. Following indicated treatments, supernatant was aspirated from treatment plates and each well was washed twice with pre-warmed 37°C Hanks Balanced salt solution (HBSS) (Invitrogen) prior to loading the ROS indicator dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate,acetylester (CMH2DCFDA; DCF for short), reconstituted per the manufacturer's suggestion. The treated cells were incubated with 2.5 µM DCF for 15 minutes, and the plate was covered with foil to avoid light exposure. Some cell images were taken with a blue color, in which 5 minutes prior to the end of the DCF incubation, Hoechst stain (from FLICATM Caspase-1 Detection kit at 200µg/mL, ImmunoChemistry Technologies) was loaded 2.5 µL/500 µL. After incubation at 37°C, cells were washed gently three times with HBSS in the dark to wash out background staining. Then each well was loaded with 1 mL of phenol red-free RPMI media (Invitrogen) before microscope imaging. Images were captured on a widefield fluorescence microscope (Leica, Deerfield, IL). Brightfield images were viewed at 15.7 – 41.6 ms exposure, while total cytoplasmic ROS production (DCF staining) was captured between 218.5 – 704 ms exposure

## Quantification of Fluorescent Intensity

The production of ROS with respect to the number of cells was quantified by measuring the intensity of the fluorescence emitted from the acquired images with NIH ImageJ analysis software. Using the freehand selection tool, each individual cell was selected in the image field, and then the RGB was measured based on pixel intensity. In order to determine the relative fluorescence intensity, the ratio of the mean intensities of green (DCF) to the number of cells in the field of view (that were circled for measurement) were performed in triplicate, and averaged. Some pictures were taken with Hoechst dye, in which the ratio of the mean intensities of green (DCF) to blue (Hoechst) were taken in each field, then that ratio was averaged with consideration to the number of cells total over the fields. The results were then expressed either as mean fluorescence, or the fold change in intensity compared to the control GECs with no treatment.

### Western Blotting

Samples were lysed using RIPA Lysis Buffer (Millipore) and collected using a disposable cell lifter (Fisher). Protein concentrations were evaluated by the Bradford assay (Bio-Rad) and standardized using Bovine serum albumin (Sigma). Lysate samples were then loaded into a 12% SDS polyacrylamide gel, and then transferred to a polyvinylidene difluoride membrane (Millipore) by using a semi-dry apparatus (Bio-Rad) at 19 volts for 42 minutes. Gels were then stained with Coomassie blue (Bio-Rad) to verify transfer efficiency. The blots were blocked for 1 hour in 5% (w/v) nonfat dried milk in TTBS. The membrane was incubated overnight at 4°C with rabbit anti human caspase-1 antibody (Millipore) at 1:1000 dilution and then washed 3 x 10 minutes with TTBS. The blots were then incubated with HRP conjugated goat anti-rabbit IgG antibody (Millipore) at 1:1000 dilution for one hour at room temperature. Membranes were then washed three times for 10 minutes each followed by a fourth wash for 3 minutes before incubation with Luminata Forte Western HRP substrate (Millipore) for 5 minutes. Proteins were then visualized using a gel doc system by ECL (Bio-Rad). Supernatants of cells for analysis were precipitated with trichloroacetic acid (TCA, Sigma) and sodium deoxycholate (Sigma). All samples from lysate and supernatant were treated with phenylmethanesulfonylfluoride (PMSF, Sigma) and protease inhibitor cocktail (Sigma)

### Trypan Blue Viability Test

Cells were plated on a 6-well plate at 80% confluency. 3mM ATP was then added for the indicated time points, and cells were trypsinized, spun down and counted on a hematocytometer. Cell viability was expressed as a percentage of live cells to total cells (live and blue colored dead cells)

### Exogenous Expression of $P2X_4$

Generation of fusion protein plasmid was done by using the pcDNA3.1/nV5-DEST Gateway Vector kit (Invitrogen). Transformation of plasmid was done in DH5α Competent Cells (Invitrogen). Successful reaction done by LR clonase recombination technique in plasmid DNA was then extracted using Maxiprep kit (Qiagen, Valencia, CA) confirmed by sequencing (UC Berkeley DNA Sequencing Facility) using a forward primer in the $P2X_4$ sequence and the V5 reverse primer. Once plasmid DNA was cloned, 4 ug of pcDNA3.1- $P2X_4$ -V5 as well as the empty vector provided in the Vector kit was transfected into GECs. Transfection was done using lipofectamine2000 (Invitrogen) on a 12-well plate at 80% confluency. Selection of stable cell lines was done using Geneticin (G418) (Gibco) since pcDNA3.1 plasmid contains a neomycin resistance gene. The concentration of G418 used for selection was 1.1 mg/mL while the concentration used for maintenance was 700 ug/mL. Verification of expression of recombinant fusion protein was done by Western blot of GEC cell extracts using anti-V5 antibody (Invitrogen).

### Co-Immunoprecipitation

Co-immunoprecipitation of stably expressed $P2X_4$ was done using the Dynabeads Co-Immunoprecipitation kit (Invitrogen). $P2X_4$ polyclonal rabbit antibody was obtained by Alomone labs. Co-immunoprecipitation was done following manufacturers' instruction manual. GECs were harvested in two T150 flasks grown to about $3.6 \times 10^7$ cells to obtain a pellet weight of 50 mg. One deviation from the protocol was addition of *n*-Dodecyl β-D-maltoside

(DDM) (Sigma) to the lysis buffer to aid in precipitating surface receptor proteins. Polyclonal rabbit $P2X_7$ antibodies were obtained from Santa Cruz Biolabs and Alomone Labs.

# Results:

GECs express common purinergic receptors:

We first checked to see whether GECs express common purinergic receptors. mRNA was extracted from wild type GECs and was converted to cDNA by reverse transcription. Then, PCR amplification was used with primers specific for the human $P2X_2$-$P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_{4\text{-}6}$ $P2Y_{11\text{-}13}$, $P2Y_{18}$ and Pannexin-1 genes. The cDNA was then run on an agarose gel with GAPDH as the housekeeping control. We found that GECs definitely express $P2X_7$ and pannexin-1. $P2X_4$ was also detectable, although at a much lesser extent. The metabotropic purinergic P2Y receptors were also expressed such as $P2Y_1$, $P2Y_2$, $P2Y_{4\text{-}6}$, $P2Y_{11\text{-}13}$ and $P2Y_{18}$. $P2X_2$, $P2X_3$, $P2X_5$ and $P2Y_4$ did not amplify. Confirmation that these

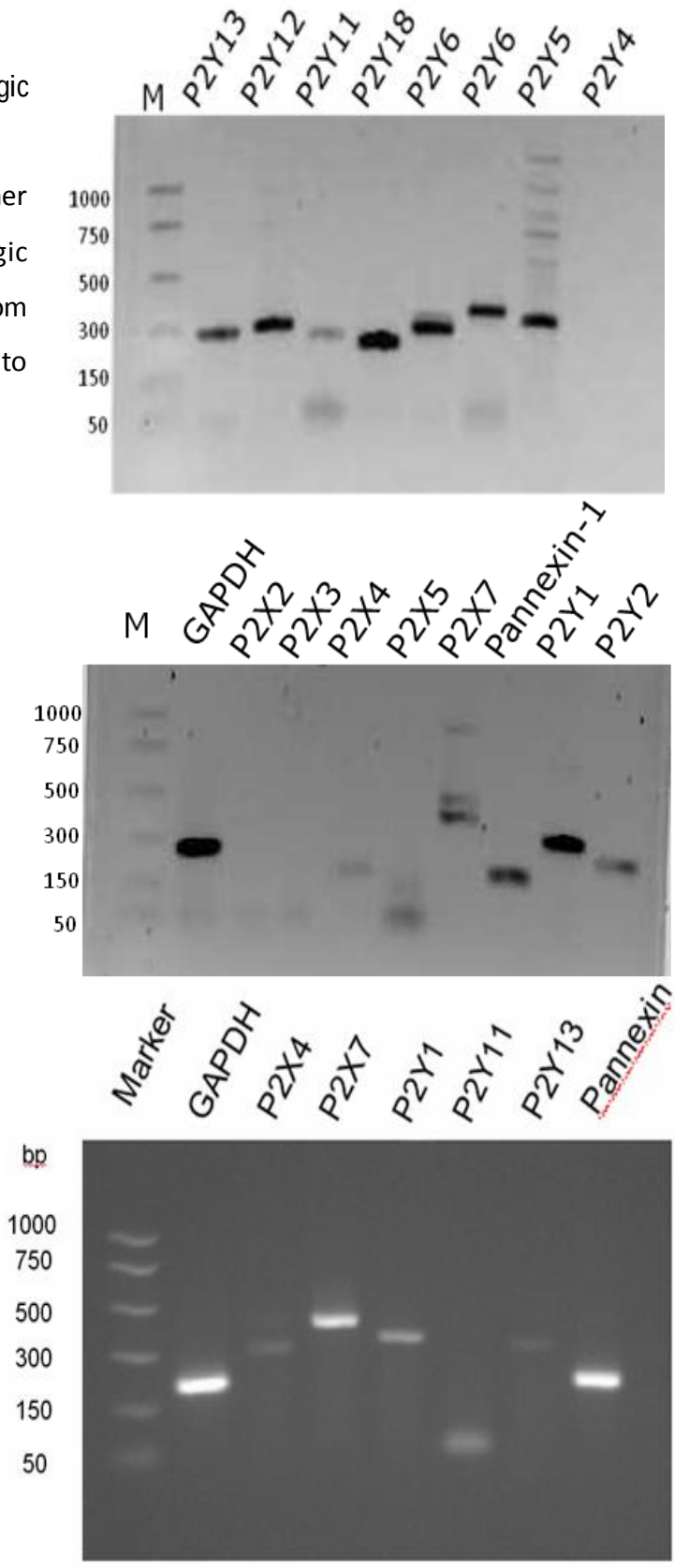



Fig. 1 PCR amplification was carried out with primers specific for $P2X_2$-$P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_{4\text{-}6}$ $P2Y_{11\text{-}13}$, $P2Y_{18}$ and Pannexin-1 genes as described in the Methods and Materials.

receptors are present gives us reason to start experiments on them to explore their functions in GECs.

## Optimizing ATP treatment time

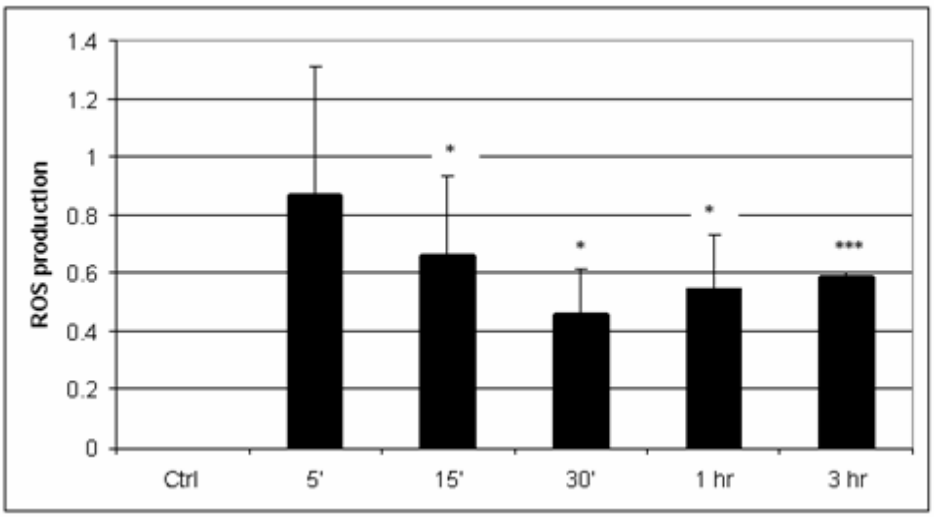



Fig. 2 ROS mean fluorescence was measured from ATP treated GECs at different time points. Experiments were done in triplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to untreated cells.

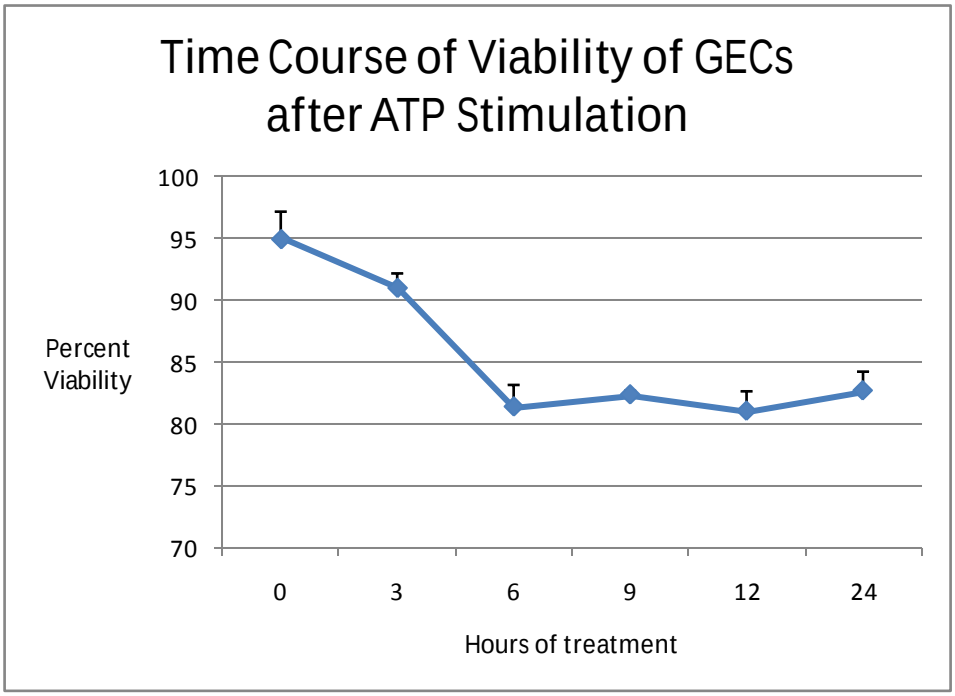



Fig. 3 Trypan blue viability test of ATP treatment in GECs. 3 mM ATP treatment was applied for 0, 3, 6, 9, 12 and 24 hours. After the indicated time points, cells were detached by Trypsin and counted on a hemocytometer. Experiment was done twice.

Optimization of ATP treatment time was done to show fluctuations of ROS production. In previous studies, ATP treatment was done for 3 hours [26], but we wanted to see if a shorter time would suffice for measuring significant ROS production. So we treated cells with ATP for 5 min, 15 min, 30 min, 1 h, and 3 hr. We found that there was a statistically significant

increase in ROS production for all conditions when compared to the control, except for the 5 min condition (Fig 2). ATP treatment at 5 min resulted in a greater amount of ROS production followed by a 15 min treatment, 3 h, 1 h, and finally 30 min. While the 3 h condition was the most statistically significant, and while the 15 min and 30 min conditions were also reasonably good times to use, since our positive control treatment was set at 1 h incubation, we chose to do the same for ATP. 1 hour stimulation provided for quick experiments with a strong fluorescent signal while keeping a cell viability of over 90%.

GECs stimulated with 3mM ATP for 3, 6, 9, 12 and 24 hours showed some of the cells dying after 6 hour incubation. Cell viability for the above time points were 94.9%, 90.95, 81.3, 82.3, 80.9, and 82.6 respectively. The rate of cell death then slowed at time points post 6 hour treatment (Fig. 3). This result gives us a time frame in which we can do experiments without losing too many cells that would affect downstream applications. Measuring gene expression

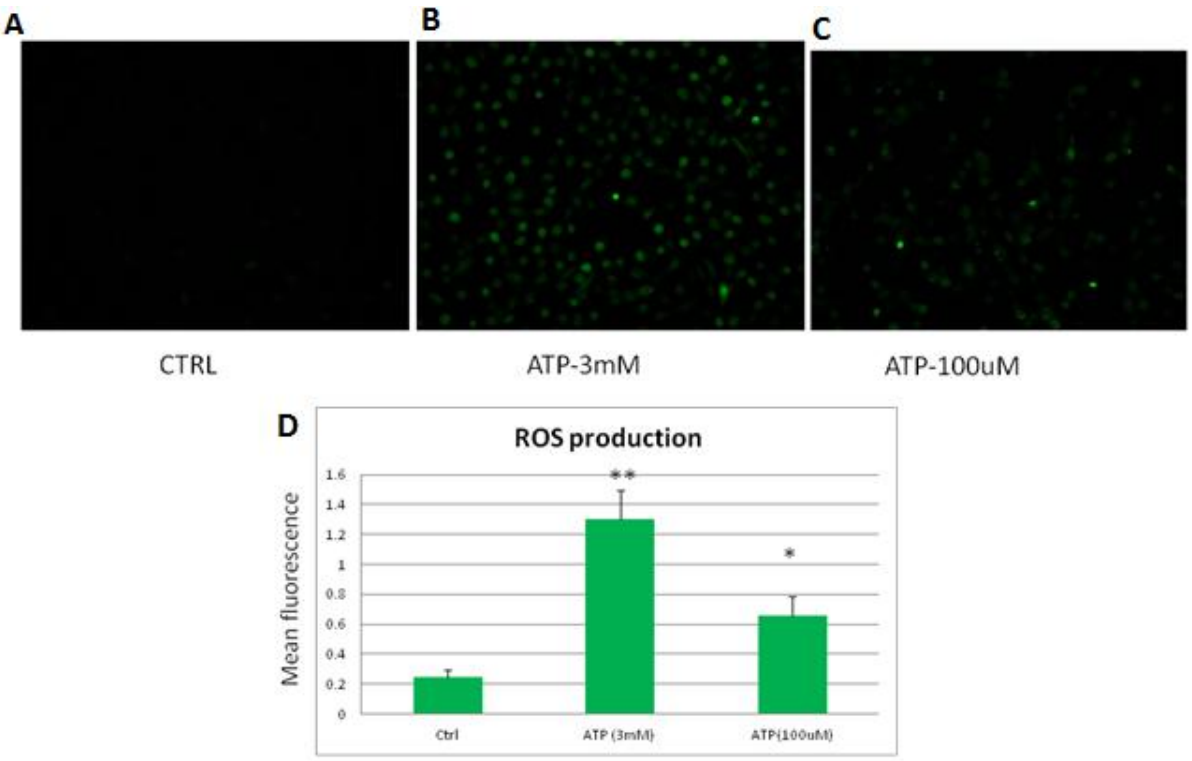



Fig. 4 (A – C) Fluorescent microscopy pictures of GECs stimulated with ATP. Images were taken of wild type cells (A), cells stimulated with 3 mM ATP (B), and 100 uM ATP (C). (D), ROS measurements taken as mean fluorescence intensity based on ImageJ software analysis. Experiment was done in triplicate. * $p<0.05$; ** $p<0.01$, compared to untreated cells.

changes in the cells after ATP stimulation was done by collecting mRNA before six hours of incubation since that was the time in which there was a significant decrease in cell count. After optimizing these time points for ATP treatment, we began experiments to measure changes in ROS, caspase-1 activation, and gene expression due to ATP and the various gene manipulations of interest.

### ROS production by ATP stimulation is dose dependent.

ROS measurements by DCF (Green) are outlined in the Methods and Materials section. ATP at a concentration of 3mM produced significantly more ROS in the cytosol compared to treatment with a concentration of 100uM. The amount of extracellular ATP in the cells seems directly proportional to the amount of ROS produced (Fig. 4). Through this result, we can conclude that ATP does generate oxygen radicals, but we cannot conclude that it activates the inflammasome until we see the same treatment activating caspase-1.

### Caspase-1 activation by ATP stimulation

It has been shown previously that the caspase-1 activation in GECs is a result of ATP stimulation [18]. To confirm this, we did a Western blot on control cells, cells treated with 3mM ATP, and cells treated with 20uM nigericin to see capase-1 activation. Clearly, the activated caspase-1 (the p20 band), was found in both ATP and nigericin treatment lanes and with greater intensity than that of the control (Fig. 5). We next wanted to find out if the caspase release was time dependent.

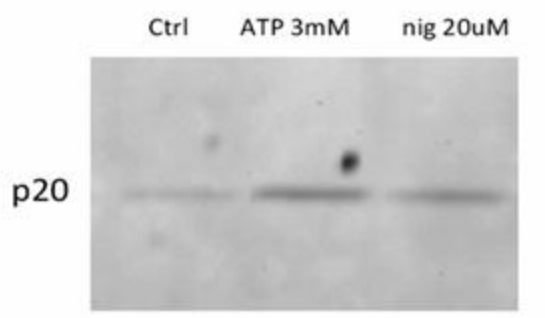



Fig. 5 GECs showing activated caspase-1 in the cellular extract. Wild type GECs were treated with ATP for 3 mM while nigericin was used as a positive control. After 3 hours, bands indicate cleaved p20 subunit.

Caspase-1 activation was assayed by Western blot for 3mM ATP treatment at 5, 15, 30, 60 and 180 minute time points. Caspase activation indicated by the cleaved p20 subunit has a much stronger signal at 60 and 180 minutes on the cell extract as well as supernatant (Fig. 6).

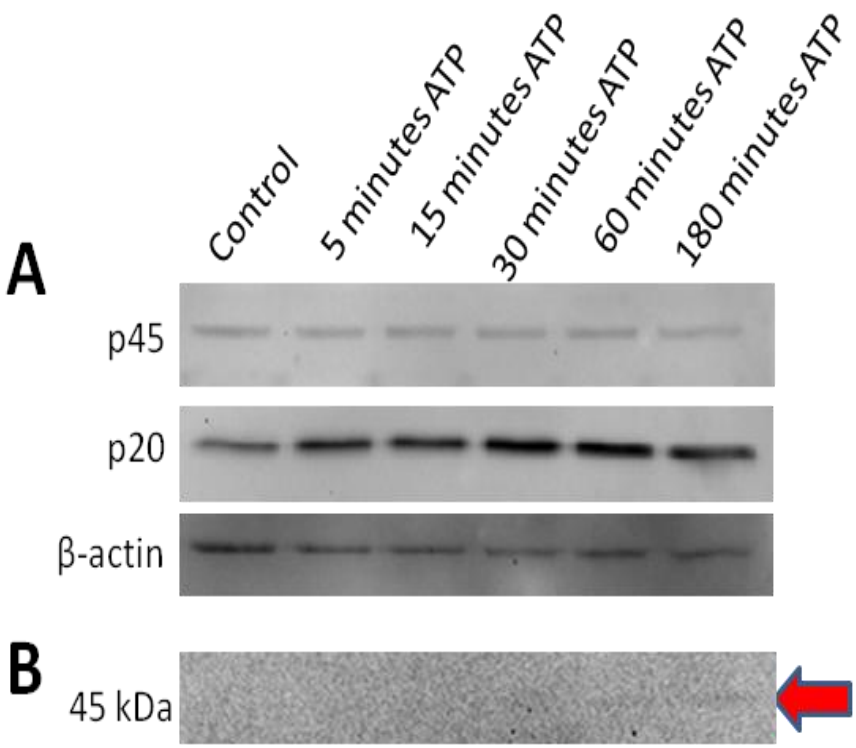



Fig. 6 A time course of caspase-1 activation in GEC cellular extracts (A), and supernatant (B). Arrow indicates procaspase p45. ATP was administered at 3mM concentration. Beta actin was used as a control.

Although the control with no ATP treatment shows a signal as well, we predict a possible explanation for this is that caspase-1 may be activated in wild type cells since they are naturally undergoing the processes of apoptosis or routine homeostasis. Therefore we cannot ignore the possibility that caspase-1 is not activated in cells unstimulated with ATP. Procaspase-1 at 45 kDa was also detected in the supernatant at a very small amount after 180 minutes of ATP incubation (Fig. 7), which may have originated from dead cells that had detached. The result of small bands in the supernatant brings to our attention to investigate whether cleaved caspase-1 is secreted into the supernatant or degraded within the cell.

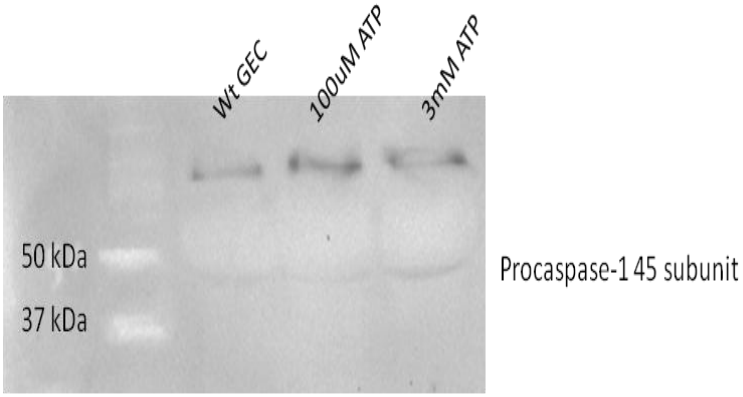



Fig. 7 Western Blot of supernatant of GECs stimulated with ATP for 3 hours. A very faint band is seen at 45 kilodaltons.

FLICA

Preliminary results of caspase-1 activity using the fluorochrome inhibitor of caspase (FLICA) was used to determine ATP induced caspase-1 activation at 5 and 15 minutes post treatment (Fig. 8). A green signal indicates that activated caspase-1 within the cell has been bound irreversibly to an inhibitor with a fluorescent moiety. The result shows that

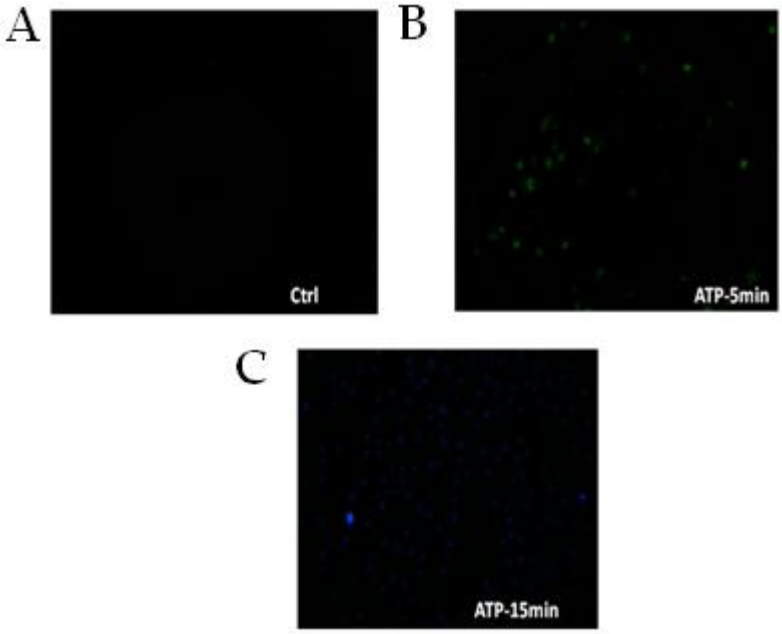
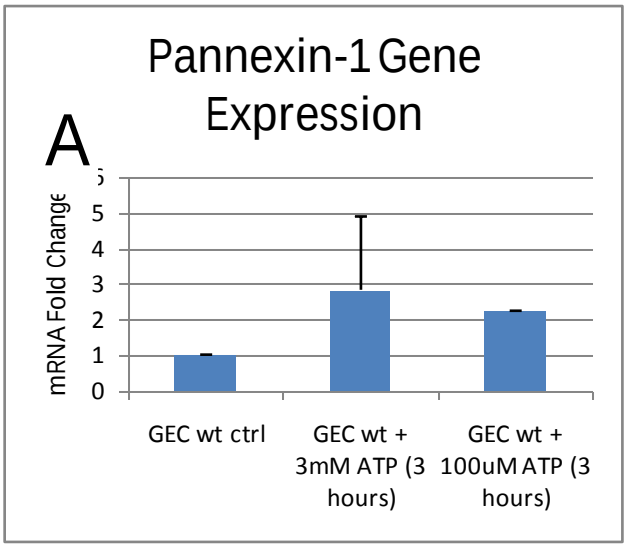


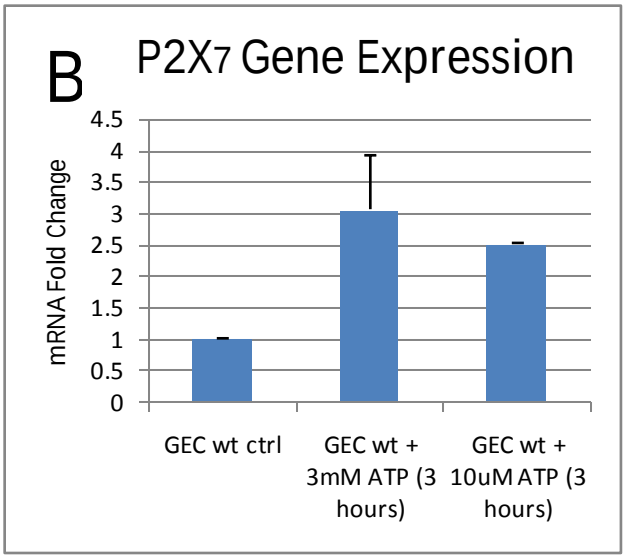

Fig 8. FLICA assay on GECs stimulated with 3mM ATP for 0 minutes (A), 5 (B) and 15 (C) minute time points. Green fluorescence indicates activated caspase-1 within the cell, while blue staining is 4',6-diamidino-2-phenylindole (DAPI) representing nuclei.

while caspase-1 is activated at 5 minutes, within 15 minutes, there is no signal at all. This may indicate that caspase-1 could be secreted, or degraded intracellularly. However, the experiment was only done once, so it must be replicated to ensure the disappearance of FLICA stained caspase-1 activity after 5 minutes of ATP treatment.

## Purinergic receptor mRNA expression changes due to ATP stimulation

As previously reported, purinergic receptors are up-regulated in response to nerve damage in microglial cells [29], we wanted to determine whether stimulation with ATP was enough to increase the expression of $P2X_4$, $P2X_7$ or pannexin-1. We show here that in response to 3mM and 100uM ATP, each of these genes are up-regulated in a dose dependent manner, indicating that they serve a role in ATP-induced immune response (Fig 9). Because these three genes are up-regulated, it is worthwhile to investigate exactly what role



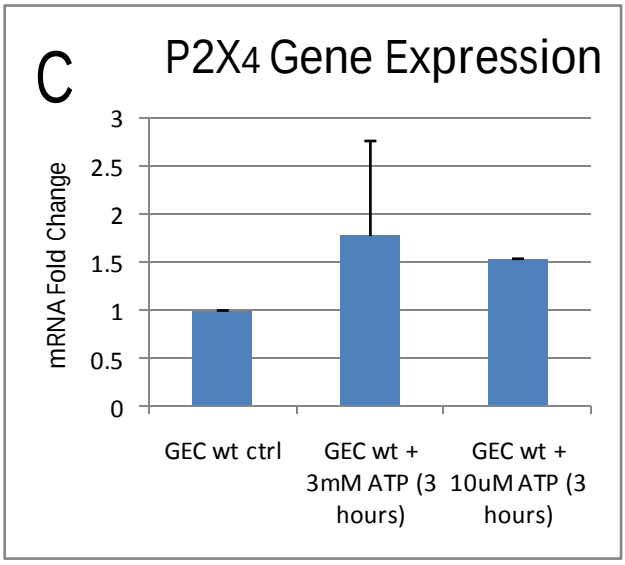




Fig. 9 Quantitative polymerase chain reaction done on mRNA of wild type GECs with and without ATP stimulation for pannexin-1 (A), $P2X_7$ (B) and $P2X_4$ (C) genes. Fold change of mRNA was normalized to wild type expression.

they play in activation of the inflammasome.

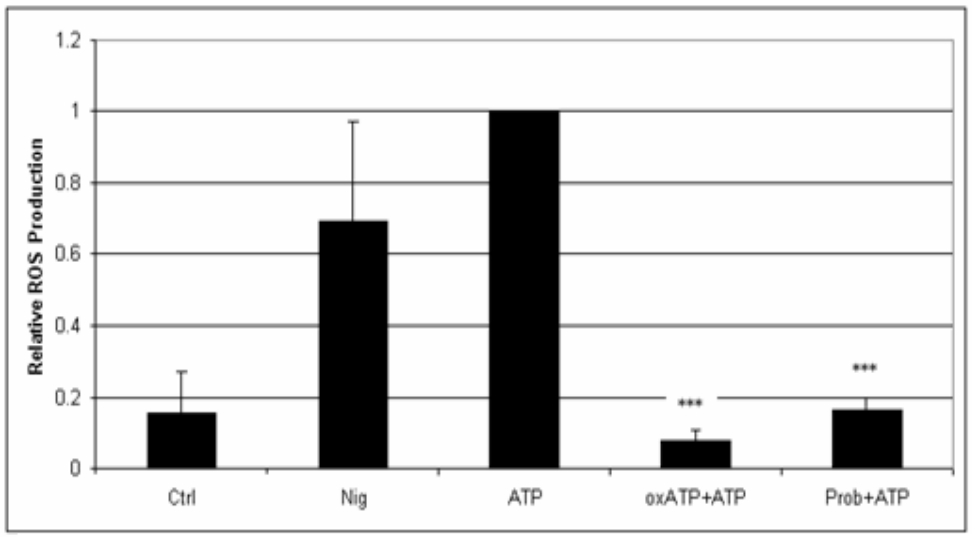



Fig. 10 GECs treated with nigericin, ATP, oxATP, and probenecid. Cells were then imaged on the fluorescent microscope and mean fluorescence intensity was measured for ROS production. oxATP+ATP and Prob+ATP experiments were done in triplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to untreated cells.

ROS production is dependent on $P2X_7$ and $P2X_4$.

ROS production in GECs seems to be $P2X_7$ dependent. ATP stimulates greater levels of ROS than nigericin (positive control). Cells pretreated with $P2X_7$ antagonist oxidized ATP (30 minutes) followed by 3 mM ATP for one hour and probenecid (10 minutes) followed by 3 mM ATP for an hour showed a dramatic decreased production of ROS within the cell (Fig. 10).

We next sought to make stable knockdown GEC cell lines of $P2X_4$ $P2X_7$. After infection with lentiviral particles with targeted knockdown of these two

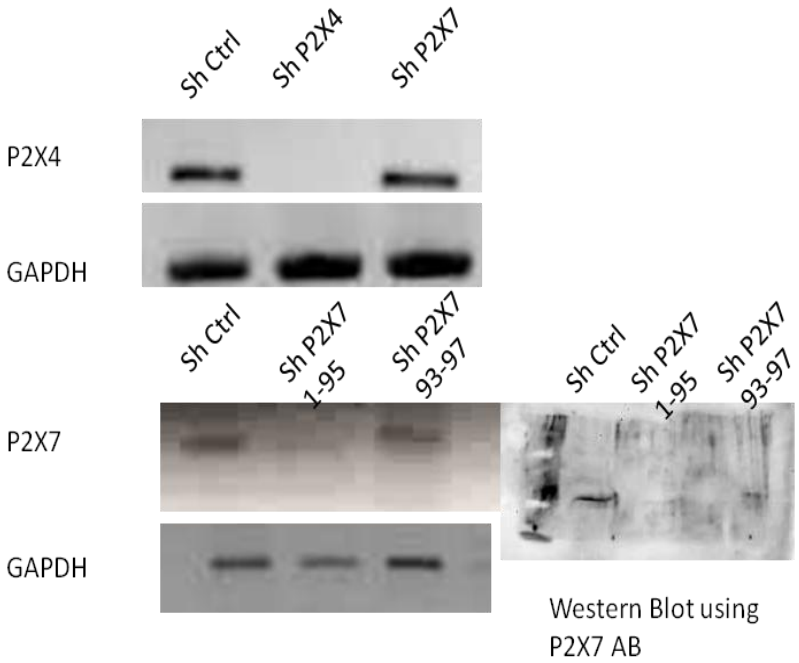



Fig. 11 Left panels, Agarose gels of cDNA in $P2X_4$ and $P2X_7$ knockdown cell lines, verifying knockdown of these receptors in GECs. cDNA was hybridized with P2X4 and P2X7 primers to detect band intensity. Right panel shows Western blot of cell extract of $P2X_7$ knocked down cells using Santa Cruz $P2X_7$ antibody.

genes, we made sure $P2X_4$ and $P2X_7$ gene expression levels were in fact lower than normal by regular PCR with PCR products viewed on a 2% agarose gel (Fig 11). The results show that the band intensities for short hairpin (sh) $P2X_4$ and $P2X_7$ was less intense compared to the sh controls. For $P2X_7$, The numbers 1-95, and 93-97 indicate different sequences of

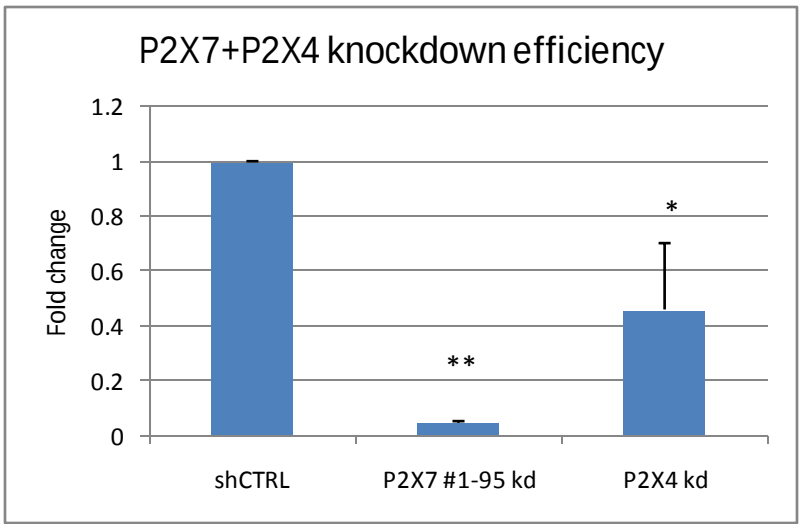



Fig. 12 $P2X_4$ and $P2X_7$ knockdown efficiency assayed by quantitative PCR in two different targeted lentiviral shRNA sequences. Gene expression was normalized to GAPDH housekeeping gene and compared to the Sh Control. * $p<0.05$; ** $p<0.001$

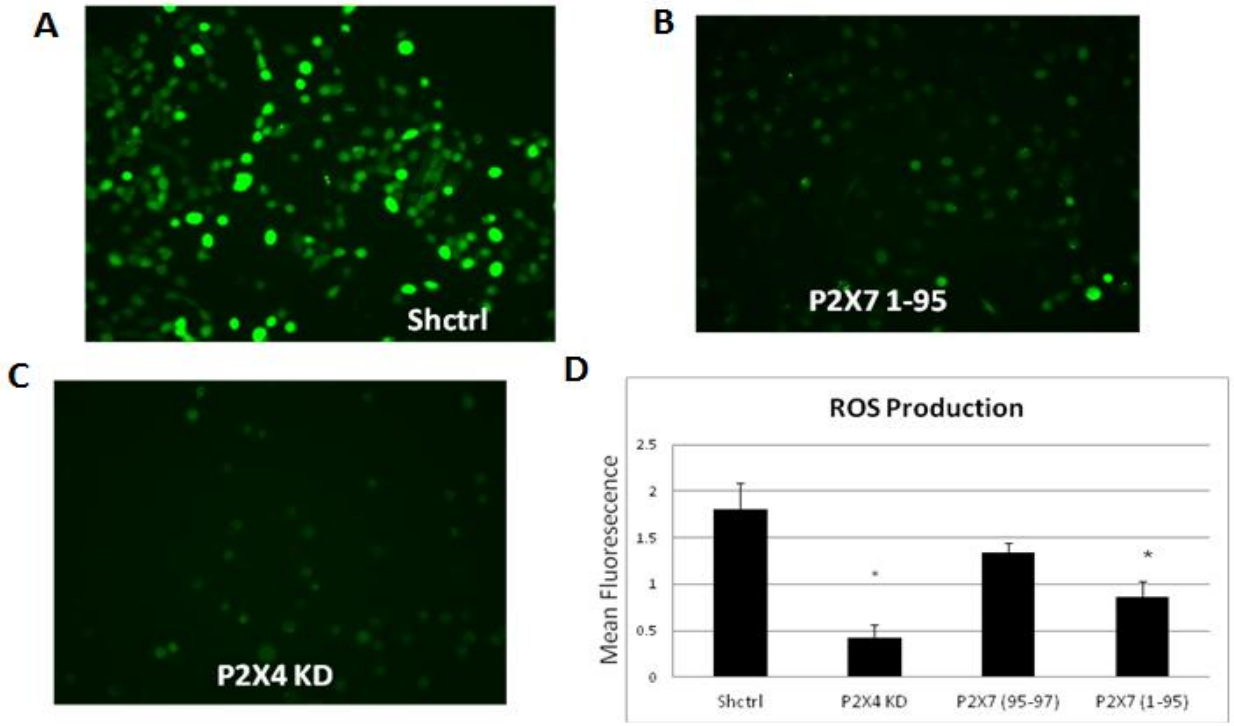



Fig. 13 ROS production images of mean fluorescence intensity in ShControl GEC stimulated with 3 mM ATP for 3 hours (A), P2X7 sequence 1-95 knockdown GECs (B), and P2X4 knockdown GECs (C). Mean fluorescence measurement of Sh Control, $P2X_7$ and $P2X_4$ knockdown GECs stimulated with 3 mM ATP for 3 hours (D). Experiment was done in triplicate. * $p<0.05$, compared to ATP treated control cells.

$P2X_7$ that were purchased to compare knockdown efficiency. Additionally, western blot of P2X7 knockdowns was done to show that $P2X_7$ was knocked down in the protein level as well as the gene expression level. Regarding $P2X_4$ knockdown technique, only one sequence of lentiviral particle targeted knockdown was purchased. Although a band was not seen in the $shP2X_4$ DNA sample hybridized with $P2X_4$ primers, there should have at least been a faint band since the lentiviral approach does not completely knock out the gene. Therefore, further confirmation and quantification of knockdown was performed with qPCR (Fig. 12). The qPCR analysis showed that the $P2X_7$ #1-95 cell line had a 19.72 fold decrease in mRNA while the P2X4 cell line had a 2.17 fold decrease in $P2X_4$ mRNA compared to the control that had a nonspecific knockdown target.

Following confirmation of knockdown of expression, we performed the same microscopy imaging to measure mean fluorescence of ROS production. Fig. 13 shows a clear decrease of ROS production of $P2X_7$ and $P2X_4$ knockdown cell lines compared to the control GECs. $P2X_4$ exhibited a 4.3 fold decrease in ROS production following ATP treatment while $P2X_7$ #95-97 and #1-95 showed a 1.21 and 2.12 respectfully fold decrease compared to the control.

Lastly, we wanted to see the effects of antagonists of P2X receptors, namely 5-(3-Bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one (5-BDBD) and Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS) (Fig. 14).

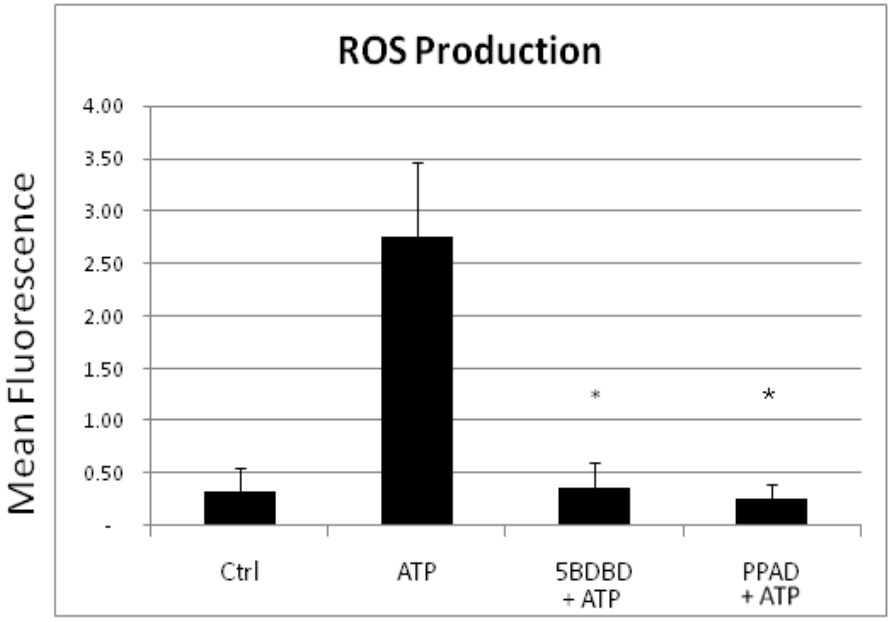



Fig. 14 ROS mean fluorescence measurements of wild type GECs treated with ATP, 5-BDBD+ATP, or PPADS+ATP. Experiment is representative of 3 different trials. * $p<0.05$ compared to untreated cells.

5-BDBD and PPADS were incubated with GECs for 10 minutes until treatment with 3mM ATP for 1 hour. These drugs were able to inhibit activity of the purinergic receptors based on the reduced amount of ROS being produced. The average mean fluorescence for PPADS and 5-BDBD treatment were comparable to the control, while ATP treatment alone had more than a 7-fold increase of signal.

## ROS production is dependent on pannexin-1

We next investigated the role of pannexin-1 in ROS production. As mentioned before, high extracellular potassium ($K^+$) is able to open pannexin-1 channels leading to caspase-1 activation in primary neurons. Probenecid, a pannexin-1 channel blocker was shown to inhibit activation of caspase-1 in astrocytes stimulated with KCl in the cell media (10). In Fig. 10, we were able to show that probenecid treatment for 10 minutes followed by 3mM ATP treatment for 1 hour showed a dramatic decreased production of ROS within the cell. Next, we wanted to create knockdown GECs with a lower expression of pannexin-1. After infection with lentiviral particles with targeted knockdown for three different sequences of the pannexin-1 gene, we made sure the gene expression levels were in fact lower than normal by qPCR (Fig. 15). Numbers 46, 48 and 36 indicate the different sequences purchased from Sigma. Both sequences 46 and 48 showed a 7.98 and 3.03 fold decrease respectively in mRNA level compared to the Sh control, while sequence 36 was at comparable levels. Because of this, we decided to discontinue the culturing of sequence 36 for further experiments.

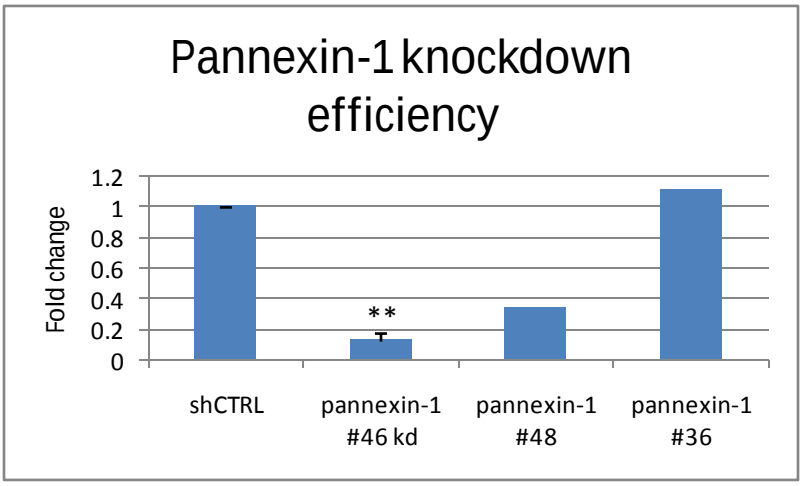



Fig. 15 Pannexin-1 knockdown efficiency assayed by quantitative PCR in three different targeted lentiviral shRNA sequences. Gene expression was normalized to GAPDH housekeeping gene and compared to the Sh Control. * $p<0.05$; ** $p<0.001$

Following confirmation of knockdown of expression, we performed the same microscopy imaging to measure mean fluorescence of ROS production. Fig. 16 shows a clear decrease of ROS production in pannexin-1 knockdown cell lines compared to the control GECs. We decided to use the Hoechst stain on these pictures to stain the nucleus blue color; however

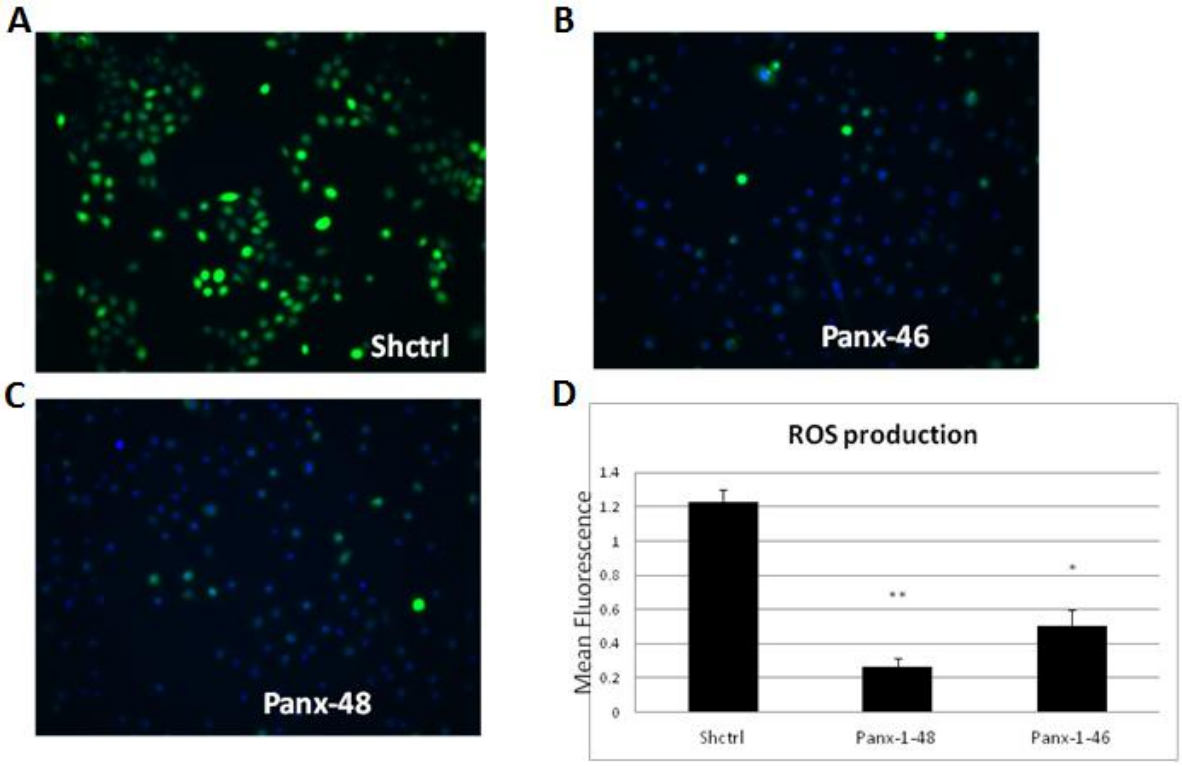



Fig. 16 Sh Ctrl, Pannexin-1 sequences 46 and 48 following 3 mM ATP treatment were imaged by fluorescent microscopy for ROS production (A-C) and the mean fluorescence of these images was measured by ImageJ software (D). Experiments were done in triplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to untreated cells.

the Hoechst stain seemed to be very toxic to the cells in a short amount of time, so we used the brightfield imaging instead for other microscopy experiments. The fact that pannexin-1 seems to have a role in producing ROS within GECs leads us to believe that it may have a role in activating the inflammasome. The next step would be to see if pannexin-1 will initiate the cleaving role of caspase-1.

Caspase-1 activation in GECs by ATP stimulation is P2X4,7 and pannexin-1 dependent

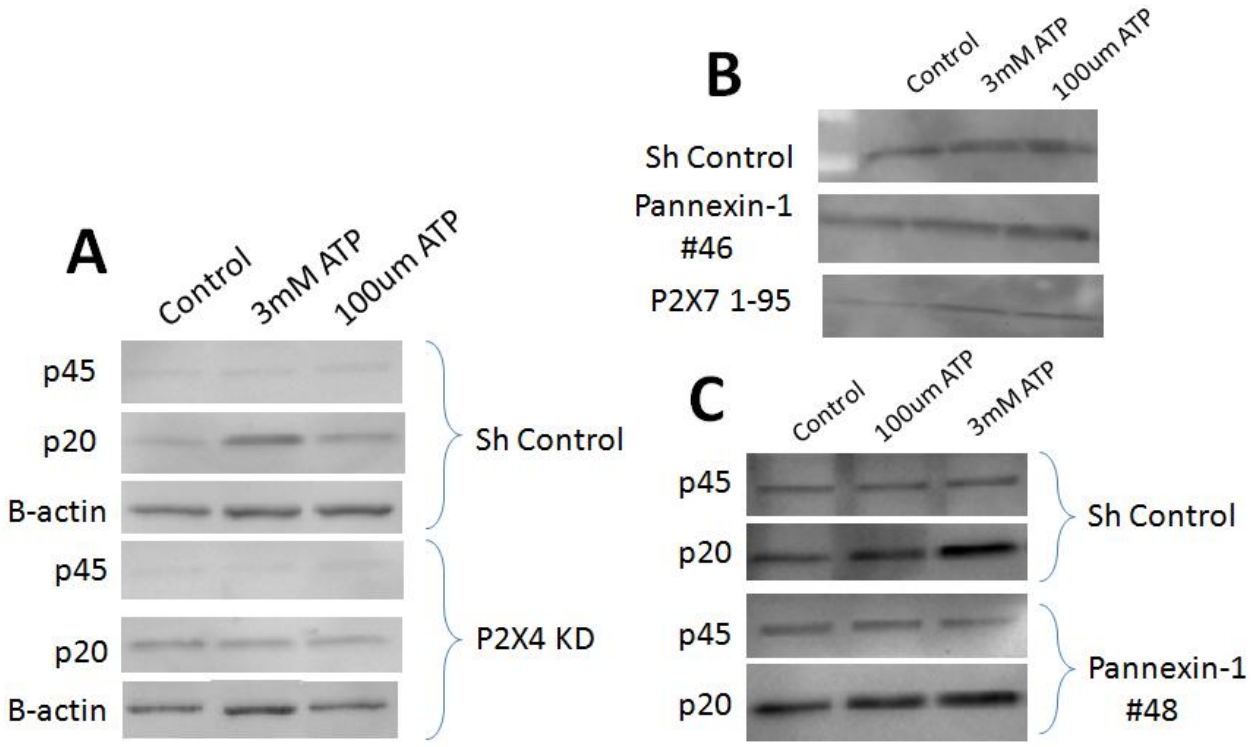



Fig. 17 Western blots of GEC cell extracts for caspase-1. P2X4 knockdown (A), P2X7 (B) and Pannexin-1 (C) cell lines are shown treated with or without ATP for 3 mM or 100 uM concentrations and compared to the control.

To determine if caspase-1 activation is dependent on $P2X_4$, $P2X_7$ and pannexin-1, we ran caspase-1 Western blots on ATP treated wild type GECs and compared the band intensities to those of ATP treated GECs that are knockdown in $P2X_4$, $P2X_7$ and pannexin-1 (Fig. 17). So far, our results are inconclusive as we keep getting various intensities in our bands that do not correspond to the amount of ATP treatment. More careful replicates of the data must be obtained to have a more consistent and definite conclusion. Pannexin-1 seems to not have a strong influence in the activation of caspase-1, as the band intensities are not very different compared to the control cells. However, P2X7 clearly has a lower amount of activated caspase-1 compared to Sh control.

Specific antagonists were then administered to the GECs that are supposed to block activity of purinergic receptors as well as pannexin-1 (Fig 18). 5-BDBD was used at a concentration of 150 uM for 10 minutes, followed by 3 mM ATP for an hour. PPADS was incubated with the cells at 100 uM for 10 minutes, followed by 3 mM ATP for an hour. Oxidized ATP was used at a concentration of 0.3 mM for 30 minutes followed by 3 mM ATP, while probenecid was used at 1 mM for 10 minutes, followed by 3 mM ATP. Each of these drugs showed a decreased activity of caspase-1 compared to the 3 mM ATP treatment by itself. This result shows that the pannexin-1 channel and $P2X_4$ and $P2X_7$ receptors have a role in mediating caspase-1 in response to danger. Because of this, the affects of ATP on heteromer formation of these three genes must be analyzed.

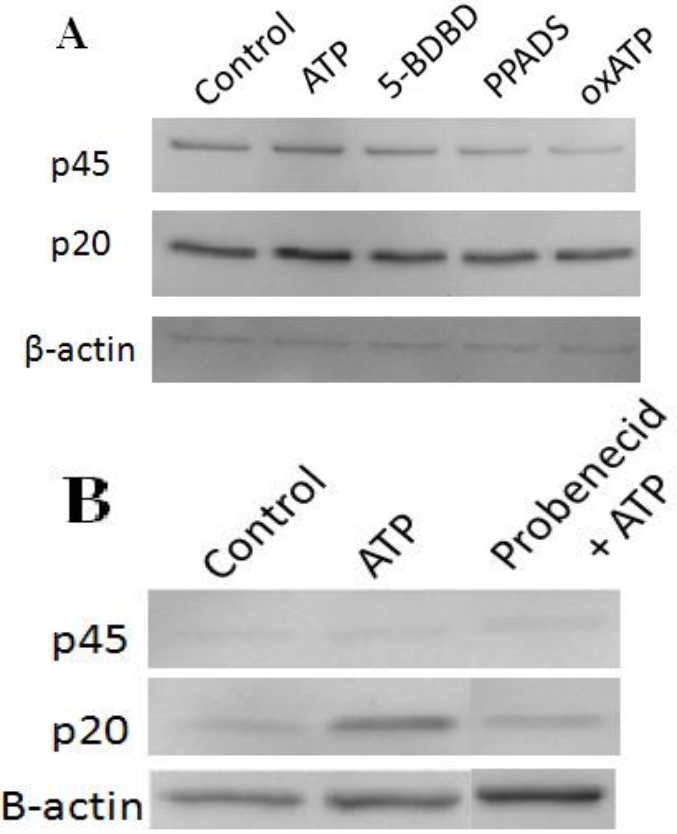



Fig. 18 Western blots of cell extracts of wild type GEC treated with the drugs 5-BDBD, PPADS, oxATP (A) and probenecid (B). Treatment with drugs were compared to untreated control and treatment with 3 mM ATP.

## Heteromer formation of $P2X_4$ and $P2X_7$

Verification of expression of recombinant fusion protein in GEC was done using V5 and $P2X_4$ antibodies (Fig. 19). Hybridization of overexpressed $P2X_4$ cell extracts with V5 antibody showed clear bands at the approximate molecular weight of the $P2X_4$ protein. Once this was confirmed, we proceeded to perform the co-immunoprecipitation on wild type and overexpressed $P2X_4$ GECs. Since this was the first time we used the Dynabeads kit, we first used about $3.6 \times 10^7$ of each cell line and brought the protein complex down with anti-$P2X_4$ and

anti-V5 coupled with magnetic Dynabeads. Complexes were then run on a 12% PAGE gel and hybridized with anti-$P2X_7$. Fig. 20 shows a very faint band just under 75 kDa indicating that $P2X_4$ and $P2X_7$ do in fact interact in unstimulated wild type cells. The well between the total cell extract and the Dynabeads conjugated with Anti P2X4 / V5 is the cell debris that was left behind following lysis of cell pellet to ensure that lysis was efficient and that proteins were present in the supernatant. Although this is just a preliminary result, we expect the overexpressed P2X4-V5 protein to have a thicker band. This supplements the possibility that it is a natural response of GECs to form heteromer complexes between purinergic receptors, whether they may be for launching an immune defense or maintaining homeostasis.

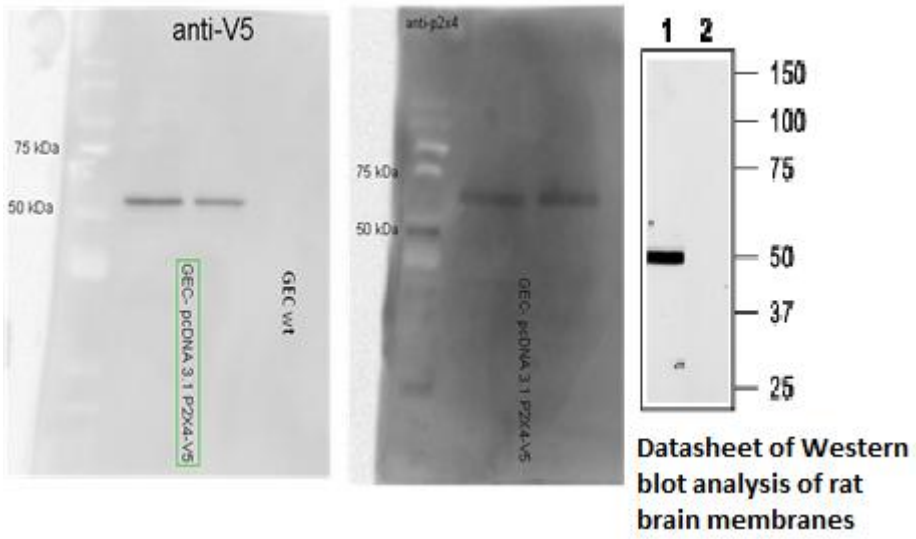



Fig. 19 Western blot analysis of stable cell lines transfected with pcDNA 3.1-V5. Bands indicate that the V5 and P2X4 antibodies hybridized at similar approximate molecular weights, showing that a V5 tag has been attached to a recombinant plasmid and is being stably expressed in GECs.

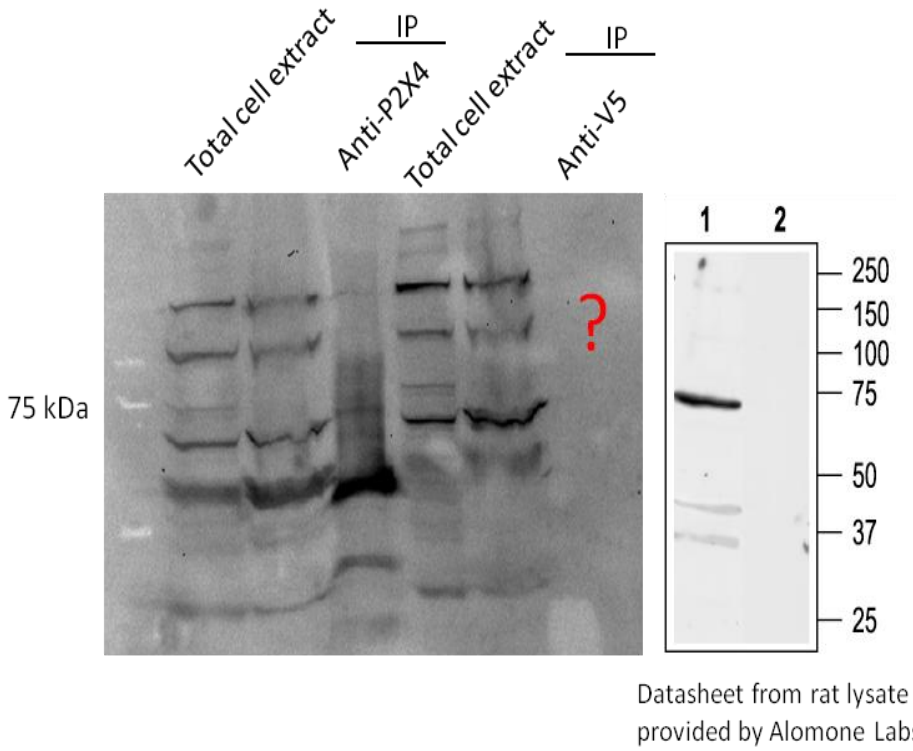



Fig. 20 Co-immunoprecipitation of wild type GEC and those transfected with pcDNA3.1-P2X$_4$-V5. Anti-P2X$_4$ was used to bring the protein complex down after cell lysis, followed by blotting with anti-P2X$_7$. Datasheet of the P2X$_7$ antibody from Alomone Labs is provided on the right panel for comparison.

# Discussion:

In accordance with previous findings, we found that GECs express both ionotropic and metabotropic P2X and P2Y purinergic receptor families, as well as pannexin-1. P2X 2, 3 and 5 are most likely not expressed, as they had very faint bands below 100 base pairs. These small bands most likely suggest that the primers dimerized instead of hybridizing with GEC complementary DNA. An alternate rationale for these light bands could be attributed to an alternate form or splice variant of the mRNA, or the primers may have degraded over time, so further PCR amplification must be done using alternate primers specific for those P2X genes.

Measuring the amount of ROS production in the GECs turned out to be troublesome and required a different method of measuring compared to cervical epithelial, HeLa cells. Flow cytometry was used for measuring ROS production in HeLa cells quite successfully in our lab in that there were no artifacts obscuring the results. On the other hand, using flow cytometry to measure the ROS production in GECs did not produce usable results. The fluorescence emitted by the cells, due to an excess of spontaneous ROS production, was so great in the control that the fluorescence emitted by the cells with different treatments could not be compared against them. There were no consistent differences between the negative controls (ctrl, oxATP+ATP, prob+ATP) and the positive controls (nig, ATP); the control seemed to show greater ROS levels than the nigericin and ATP, while the oxATP and probenecid pretreatments also showed high levels of ROS, thus making the results unreliable. We attribute this spontaneous ROS production to the cellular stress caused in the flow cytometry preparation process. The GECs are tightly adherent cells, so they are probably greatly stressed during trypsin treatment to detach the cells. While GECs are not usually in suspension and are normally closely adjacent to their neighbors, there is a certain amount of cell-to-cell signaling in the normal state. So when they are put in suspension for the flow cytometer, this in itself could potentially initiate stress responses in the form of ROS production. For this reason we found an alternative method for measuring ROS production by using live-cell fluorescence microscopy. This method greatly reduced the amount of stress inflicted on the cells. However, a different kind of problem emerged from this process. We noted that if there were GECs touching one another on the tissue culture plate (from which we took our pictures, at about 90% confluency); they tended to

fluoresce excessively when stimulated by the laser. Only when the cells were spread apart ( ≤70 % confluency) did they act as expected. This, admittedly, doesn't give us much insight into how these cells would act in physiological conditions as cells in the gingival epithelium are always very tightly packed, however these cells are also immortalized, and not the primary cells you would find inside your mouth, which could help explain this artifact. Another explanation for the fluorescence artifact's amplification is due to the limitations of the dichlorodihydrofluorescein diacetate probe itself. Once this molecule is hydrolyzed intracellularly to the carboxylate anion, it is retained inside the cell to scavenge oxidants. Intracellular hydroxyl radicals ($^{\cdot}OH$), compounds formed from peroxidase, peroxynitrite decomposition or heme interaction with $H_2O_2$, $^{\cdot}NO_2$, hypochlorous acid and carbonate anion radicals are all able to oxidize DCFH into DCF giving off the fluorescent signal. The source of artifacts may come from the intermediate radical, $DCF^{\cdot -}$, which is formed from oxidation of DCFH. This intermediate can rapidly react with $O_2$ to form superoxide $O_2^{\cdot -}$. The dismutation of $O_2^{\cdot -}$ yields additional $H_2O_2$, which can establish a redox-cycling mechanism leading to artifactual amplification of fluorescent intensity.

Determination of the optimal ATP treatment time for GECs was done to see if there was a shorter time frame in which we could treat the cells that would result in less cell death. ROS production is transient, yet shorter treatment times were assayed to see if they were just as effective in obtaining a strong fluorescent signal. We determined that 5 minutes stimulation with ATP gave varying results that were not statistically significant, most likely due to the preparation time taken before imaging could be captured. Another factor that may have given fluctuation across the trials was because of the cognate cell processes that serve to neutralize toxic ROS. Since ROS production and neutralization is an evolutionarily conserved mechanism, enzymes such as superoxide dismutase, catalase and glutathione cysteine ligase most likely played a role in regulating the amount of ROS that can be detected by the microscope. Furthermore, evidence has shown that cells will employ an autophagy type mechanism to remove ROS generating mitochondria in the cell in order to avoid cellular damage [56]. While the 30 min ATP condition showed significant ROS production, there was a less dramatic signal compared to other time points. The choice for the optimal ATP treatment time was then

narrowed down to either 15 minutes or 1 hour. The 15 min showed greater variance of ROS production across trials than the 1 h hour treatment, and since our nigericin control treatment was already set at 1 hour; it seemed to be our best optimal time for doing ROS measurements.

GEC viability was then surveyed in response to different time points of ATP stimulation. We wanted to have a time frame in which we can do experiments without losing too many cells that would affect downstream applications. In order to observe any mRNA changes that happen in the cell due to ATP treatment, it is important to collect RNA while the cells are still alive. We therefore used a simple Trypan blue viability test of cells stimulated with ATP at 0, 3, 6, 9, 12 and 24 hours. 0 and 3 hours of stimulation resulted in a viability above 90%, while all time points 6 hours and later resulted in below 85% viability. We therefore chose 4 hours as sufficient time for any mRNA synthesis in response to ATP without resulting in too much cell death. Although Trypan blue is not the most accurate way of measuring apoptotic rate, it was also confirmed visually by light microscope in that a portion of the cells had disappeared by 6 hours stimulation. This method of calculating cell death is not sufficient enough to tell us the rate of apoptosis induced by ATP, but it should be accurate enough for the purpose of measuring gene expression.

We next sought to find out if ATP stimulation produced ROS in a dose-dependent manner. This was important for us to make sure that stimulation of GECs with ATP directly influences the production of ROS. Our results show that 3 mM ATP had much higher ROS response in the cells compared to a treatment with 100 uM, showing a direct relationship between the amount of ATP present in the extracellular space and the amount of ROS created.

The control of caspase-1 activation was determined solely by the stimulation of ATP. As hypothesized, ATP should be enough to stimulate ROS production and subsequent inflammasome activation to cleave procaspase-1 into its active form, which would be ready to survey the cell and cleave any inflammatory cytokines to expedite their maturation and secretion into the extracellular space to combat infection. Our results clearly show that ATP can activate a more robust caspase-1 compared to the unstimulated control. Nigericin, which disrupts membrane potential and transports monovalent cations such as $K^+$ across cell

membranes is already a known potent caspase-1 activator, yet the 3mM ATP was enough to produce a thick p20 caspase-1 band even compared to 20 uM of nigericin positive control.

Whether or not caspase-1 is released in a time-dependent manner was determined at ATP treatment time points of 5, 15, 30, 60 and 180 minutes. The result seems to show there is no significant difference between caspase activation at 5, 15 and 30 minutes while a much greater amount of cleaved active p20 subunit was seen after 60 minutes of stimulation. It is normal to get a small amount of caspase-1 in untreated cells because cells are routinely undergoing apoptosis, producing background in the Western blots, however it should not be a dense band.

There is also the problem of determining whether activated caspase-1 is secreted into the supernatant of cells or being degraded within the intracellular space. This question must be answered since outside literature has shown in many instances that caspase-1 is secreted into the medium for immune cell types in suspension such as bone marrow derived macrophages and THP-1 monocytes. However, active caspase-1 subunits of p10 or p20 do not seem to be secreted into the supernatant in any adherent epithelial cells. Although we were able to detect light bands in the supernatant at late time points, these were probably from dead cells floating around in the supernatant. At this point, more experiments must be done to verify that GECs do not secrete caspase-1. Precipitating caspase-1 from the supernatant has proved to be challenging since the active subunits are small in size, not present in large amounts, and may be cleaved rapidly by other proteases in the vicinity.

To determine whether purinergic receptors $P2X_4$ and $P2X_7$ and pannexin-1 were up-regulated in response to ATP treatment, we extracted RNA samples at 4 hours post ATP treatment and measured the fold change of these three genes. $P2X_4$, $P2X_7$ and pannexin-1 were in fact up-regulated in a dose dependent manner in response to ATP treatment, indicating that they may possibly be involved in ATP activated inflammasome complex and ROS production. Whether or not they are directly responsible for triggering the formation of the complex, they definitely have a response to extracellular ATP.

In order to verify that the ROS production via ATP stimulation was truly $P2X_7$ dependent, the $P2X_7$ specific antagonist, oxATP was used pre-ATP treatment. There have been no studies

showing that oxATP binds to any of the other purinergic receptors. Also, an oxATP treatment before treatment with ATP did not yield any significant difference in ROS production when compared to the untreated control, but exhibited much lower fluorescence compared to GECs treated with ATP alone. Thus, we can more safely say that oxATP does indeed block the affects of ATP in a $P2X_7$ dependent manner, and that $P2X_7$ is required for sensing extracellular ATP as a danger signal for the immune response.

On top of the ROS production being $P2X_7$ dependent, there are also the effedcts of the pannexin-1 channel to consider. The pannexin-1 receptor having been found to physically associate with the $P2X_7$ receptor [55] would put forth the possibility of one receptor being vital in the physiological function of the other. Probenecid, while being associated with dye-uptake, has been shown to inhibit specifically pannexin-1, while showing no effect on the connexin channels. This suggests that probenecid is at least specific to pannexin families, even though connexins are structurally and functionally similar to pannexin families [54]. In the same study, it was shown that probenecid attenuated the release of ATP into the extracellular medium possibly acting as a danger signal. It has been found that the pannexin-1 channels are highly permeable to ATP, and that probenecid stimulates this release of ATP into the extracellular milieu [54]. When we used probenecid to block pannexin-1 channels, we saw a clear decrease in the amount of ROS being produced compared to ATP treatment alone. The rationale for this result is that the pannexin-1 pores are supposed to create larger pores that complement the P2X7 channels. Blocking these pores altogether may inhibit the purinergic channel component as well, preventing cytosolic signaling.

Lastly, the effects of 5-BDBD, an inhibitor with specific binding affinity to $P2X_4$ to block the receptor's current was experimented on. PPADS was also used for experimentation on GECs, although this chemical is non-selective for the purinergic P2X family. Both of these inhibitors did produce a lower ROS production compared to ATP treatment alone based on the mean fluorescence intensity. This result further verifies that $P2X_4$ and possibly other purinergic P2X receptors are responsible for the mediation of ATP-induced inflammasome activation.

Stable knockdown cells for $P2X_4$, $P2X_7$ and pannexin-1 were generated to provide more data for us that ATP stimulation does affect ROS production and caspase-1 activation in GECs.

Experiments done in triplicate indicate that with lower amounts of these receptors and hemichannel by short hairpin RNA, ROS signal by microscopy is in fact lower. The ROS response however is not completely abolished since there must be other mechanisms by which ATP is stimulating ROS. Additionally, since the cell lines were not completely knocked out of the three genes, there will always be a few of the receptors present on the cells that can induce the inflammasome response. Caspase-1 immunoblots of these three knockdown cell lines when treated with ATP and compared to the control must still be experimented on, as band intensities fluctuate throughout the samples, giving inconsistent data. Pannexin-1 knockdown seems to not have any influence on caspase-1 activation, since the band intensities are comparable to the shControl. A possible explanation for this is that even without fully expressed pannexin-1 channels in GECs, cells are able to activate caspase-1 through other mechanisms. Following ligation of ATP to purinergic receptors, it is possible that the cells do not depend on pannexin-1 to mediate inflammatory responses. Although pannexin-1 knockdowns exhibit a decreased the amount of ROS being produced, the channel may not play a large enough role to activate the inflammasome. $P2X_4$ and $P2X_7$ knockdown however shows a modest decrease in signal. When GECs were treated with the drugs 5-BDBD, probenecid, oxATP, and PPADS before treatment with 3 mM of ATP, caspase-1 was shown to have a less amount of activation compared to GECs treated with 3 mM of ATP alone. With this result, it is safe to say that blockage of pannexin-1 channel or $P2X_4$ or $P2X_7$ receptors by specific antagonists will induce less caspase-1 in GECs.

When looking at heteromer formation between $P2X_4$ and $P2X_7$, co-immunoprecipitation of the wild type protein complex was pulled down with $P2X_4$ antibody. Since we previously concluded that $P2X_4$ has a very low basal expression level in GECs, we cultured wild type GECs to about 90% confluency in two T150 flasks to obtain $3.7 \times 10^7$ cells, or 50.2 mg total cell pellet weight. Since receptor proteins are usually low in number, we also conjugated the Dynabeads with 20 ul of $P2X_4$ antibody, or 6 ug. After hybridization with $P2X_7$ antibody, very faint bands were shown just under 75kDa, which is what the datasheet for the antibody indicates as a $P2X_7$ signal. This result shows promise, as $P2X_4$ $P2X_7$ heteromer formation is now shown in a variety of cells including GECs. It will be interesting to explore these possible physiological reasons

behind heteromer complex formation, and how it may affect the mechanism behind inflammasome function. The presence of many bands above and below the $P2X_7$ band may result for many reasons. Although the polyclonal $P2X_7$ antibody was indicated as applicable for co-immunoprecipitation, it may not have been specific enough for our purposes. Additionally, when antibody conjugated beads were incubated with cellular extract samples, they were then boiled, denaturing the $P2X_4$ antibody coupled to the beads. And since the $P2X_4$ and $P2X_7$ antibodies were both derived from the same rabbit species, it is likely that the large dark smear at 60 kDa is from the denatured heavy chain of the $P2X_4$ antibody.

Overexpressed pcDNA3.1-P2X4-V5 GECs were also subject to the same co-immunoprecipitation protocol using anti-V5 to pull down the protein complex. However, GECs transfected with this plasmid grow much slower and are more fragile compared to wild type cells. Because of this, we were only able to recover about 32 mg of cell pellet compared to the 50.2 mg used in the wild type control. With fewer cells and less monoclonal V5 antibody, the $P2X_7$ band was comparable in intensity to the wild type, indicating that these cells do in fact overexpress the $P2X_4$ gene. Sadly, the results show that the Dynabeads coupled with anti-V5 were unable to hybridize with $P2X_7$ in the cellular extract. It is useful to note however, that in lane 5 of the blot, cell debris following lysis and precipitation of the proteins showed no band at the $P2X_7$ specific molecular weight, indicating that lysis of the pellet was efficient, and that any of the P2X7 proteins from the cells were in fact incubated with the Dynabeads. A possible explanation for the failed result is that the V5 antibody never was able to conjugate with the Dynabeads. Therefore, a new V5 antibody must be tested in this experiment, and should give more conclusive results.

Epithelial cells in the oral cavity are the first line of defense in combating pathogenic colonizing bacteria. It is important to study the innate immune system of GECs because they are usually the initial site of microbe invasion. The P2X receptors and pannexin-1 channel are largely uncharacterized in GECs, yet they must contribute to infection control and inflammasome activation. Extracellular ATP released from stressed or necrotic cells will ligate to purinergic receptors, serving as a danger signal for other components of immune system and initiate a prompt innate immune response by triggering secretion of IL-1b, generating ROS,

inducing apoptosis and killing of intracellular pathogens. It is very important to study the purinergic receptors and pannexin channel in their role of ROS production and inflammasome activation because these signaling events can be manipulated by intracellular bacteria in order for them to successfully colonize host cells. If any pathogens are able to bypass these apoptotic mechanisms, they will be able to survive longer. Therefore, continuing examination must be done on P2X4, P2X7 and pannexin-1 proteins and how they can be engineered in the future to avoid intervention of their signaling pathways by foreign pathogens.

# Future Studies:

In the future, we would like to do further experiments using various inhibitors specific for the purinergic receptors and pannexin-1. 4-[(2*S*)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester (KN-62), a potent non-competitive antagonist at the $P2X_7$ receptor [51] may be used in our studies to further confirm if ATP stimulated caspase-1 and ROS production are $P2X_7$ dependent. 2',3'-*O*-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt (TNP-ATP) is another high-affinity antagonist for P2X receptors that displays 1000-fold selectivity over $P2X_2$, $P2X_4$ and $P2X_7$ and has been shown to inhibit ATP evoked currents [52]. Another experiment that can be done involves potassium efflux, and determining whether it is a major factor in activating the inflammasome. Previous reports show that LPS-primed Bac1 macrophages that were stimulated with 1mM ATP or 1 uM nigericin for 5 minutes in test medium containing 130 mM KCl as the major extracellular salt resulted in completely blocked ability of ATP or nigericin to induce in vitro processing of caspase-1 and IL-1β [53]. Similar experiments can be done in GECs, to determine how great of a role $K^+$ efflux affects inflammasome activation.

Additionally, it will be beneficial to see what the effect of knocking down all three of the genes of interest in order to see how drastic the change would be on ROS and caspases-1 activity. Since knocking down individual genes was only able to abrogate some of the ROS and caspase-1, double or triple knockdown may completely stop the activation of inflammasome.

Also, since P2X receptors share some homology, we must confirm that knockdown with one gene on GECs is not affecting expression of one of the other purinergic receptors. Similarly with inhibitors, we must be careful that the inhibitors are not acting on multiple receptors or other off-targets.

In regards to co-immunoprecipitation, it would be worthwhile investigating interactions between $P2X_4$, $P2X_7$ and pannexin-1. Co-immunoprecipitation of neuronal lysates indicates that panexin-1 associates with components of the multiprotein inflammasome complex, including $P2X_7$ [38]. It would be interesting to see if there is a functional interaction between $P2X_4$ and pannexin-1 or $P2X_7$ in gingival epithelial cells. Lastly, more data must be generated to compare interactions between $P2X_4$ and $P2X_7$ showing that their interaction is increased after

ATP stimulation. This would suggest that heteromerization is the preferred assembly pathway under stressful physiological conditions.

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