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Characterizing the P2X4 receptor as a contributor to cell membrane fusion and *C. trachomatis* L2 vacuole fusion

In

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by

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DEDICATION

I dedicate this thesis to my brothers and sister, Axel Ahrens, Grant Koblis, Christopher Ahrens and Cassandra Koblis for their support and continued understanding throughout my program. Being the oldest, they have looked up to me but it is their accomplishments that have inspired me to continue to persist in my goals. Throughout my years at UC Merced, they have been completely understanding of my absent attendance throughout their achievements. I would like to thank them for their continued encouragement and patience.

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ABSTRACT OF THE THESIS

Characterizing the P2X4 receptor as a contributor to cell membrane fusion and *C. trachomatis* L2 vacuole fusion

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Cell to cell communication is important in an array of biological systems including inflammasome activation and cellular fusion. This process of communication leads to multinucleated giant cell (MGC) formation including: macrophage fusion, osteoclasts and foreign body cells (5, 35). Chronic inflammation contributes to multinucleated cells forming granulomas at sites of infection (5). The specific mechanism of membrane fusion has yet to be understood. Identified modulators of membrane fusion include the hemichannel pannexin-1 in association with purinergic receptor, $P2X_7$ (20). Preliminary results on another P2X receptor, $P2X_4$, have indicated its possible association in membrane fusion and *Chlamydia* (*C. trachomatis*) L2 vacuole fusion. Our objective is to characterize $P2X_4$'s role in the mechanism of cellular fusion and L2 vacuole fusion. We will also investigate the interaction of $P2X_4$ with pannexin-1 in both membrane fusion processes. These studies will contribute to the understanding of the role of $P2X_4$ in inflammatory and autoimmune diseases.

INTRODUCTION

MGC formation is primarily found at sites of chronic inflammation (19, 20, 23). Macrophages are critical in their wide array of biological functions including tissue repair, cytokine secretion, and immune cell recruitment (18, 19, 20). The P2X receptor family has been identified to promote macrophage fusion during inflammation and intercellular communication (19, 20). Two of the P2X receptors, $P2X_7$ and $P2X_4$, have been implicated in inflammasome activation upon ATP stimulation (16, 19). Activation of an inflammasome, a multi-protein complex, results in maturation and secretion of pro-inflammatory cytokines during an inflammatory response, and initiates cell-to-cell signaling (15, 16).

Cellular fusion leads to the formation of multinucleated giant cells during an immune response (19, 20, 23). Multinucleated giant cells have been identified in macrophages, osteoclasts, and foreign body cells (6, 19). Langhan's giant cells form from multiple fusions of macrophages at sites of infection due to bacteria or fungus (5). Components of cellular membrane fusion in MGC's have been found to be mediated by several molecules involving multiple glycoproteins mediating membrane attachment and fusion when cells come into contact with one another (15). In addition, macrophage fusion can be induced by soluble extracellular molecules such as cytokines, growth factors, and E-cadherins (11, 15, 31). It has been described that macrophage fusion requires activation by cytokines released by T lymphocytes, such as interleukin-4, interferon- γ, and tumor necrosis factor-α (16, 18). Furthermore, activation of purinergic signaling has been indicated in the promotion of fusion in MGC formation, though the entire mechanism of cellular fusion is still not understood (19, 20, 31).

Purinergic Signaling

The investigation of purinergic signaling in mammals has been found to play a major role in various biological systems. Their ubiquitous expression in mammalian cell types has accrued increasing interest in their modulation. Purinergic receptors were originally identified as being abundant in the nervous system but have been identified to be widely distributed in virtually all cell types including, kidney cells, hepatocytes, and endothelial cells (10, 17). Their regulation contributes to multiple cell processes such as apoptosis, HIV replication, and

inflammatory responses which has increasingly gained interest in the study of purinergic receptors in recent years (26, 27, 30).

Regulation of purinergic signaling is maintained by extracellular signaling molecules composed of purine nucleotides (13). Previous research on purinergic receptor activation has emphasized purine nucleotide adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine (13) as activators of purinergic receptors. Specifically, as an extracellular signaling molecule, ATP release can act as a danger signal for compromised cells (27). This release is regulated throughout many systems; including the immune, nervous, endocrine, respiratory and circulatory systems (28). Further, receptors activated by extracellular ATP, ADP and adenosine include two families of purinergic receptors: adenosine/ P1 receptors and P2 receptors which can additionally recognize uridine nucleotides, UTP an UDP (29). In contrast to P2 receptors, P1 receptor activation requires adenosine, an ATP metabolite (17). Adenosine/P1 receptors couple to G proteins upon activation (29). The adenosine/P1 receptor family includes four subtypes: A1, A2A, A2B and A3 (29). The P2 receptors are divided into two families: 1) the metabotropic G protein-coupled P2Y receptors, and 2) the ionotropic ligand gated P2X receptors (29). Previously, five subtypes of the P2Y family were characterized – P2Y1 P2Y2, P2Y4, P2Y6, and P2Y11 (29). However, recent studies have identified others, including P2Y12, P2Y13 and P2Y14 (7), making a total of eight P2Y receptors. Previous studies on P2X receptors have unanimously characterized seven subtypes denoted $P2X_1$ –$P2X_7$ (7). The various P2Y and P2X receptors have been identified in different cell types where cells may express specific P2Y receptors with P2X receptors at the same time and/or location (17). Expression of these receptors at the same or varying locations in cells can determine the specific function of cell types.

The P1 receptors are expressed in different tissue types including neuronal, kidney and mast cells (29). P1 receptors contain a seven transmembrane motif with an extracellular N-terminus and C-terminus exposed to the cytoplasmic region of the cell membrane (29). Similar to P1 receptors, P2Y receptors contain seven transmembrane-spanning motifs with their N-terminus exposed to the extracellular region of the plasma membrane and their C-

terminus remaining intracellular (17). P2Y receptors are distributed in various cell tissues with expression in monocytes, T-cells, neurons, and epithelial cells (29). In contrast to the P2Y and P1 receptors, P2X receptors are plasma membrane ion channels that allow the movement of monovalent and divalent cations Na^+, K^+, and Ca^{2+} (17). Cation movement in the P2X channels is directly regulated by activation of extracellular ATP (17). P2X receptors are comprised of two transmembrane domains (TM1 and TM2), and are distinctly characterized by a large extracellular loop responsible for ligand binding (17). A large portion of the P2X receptor family protrudes from the plasma membrane with an intracellular N-terminus and a longer C-terminus (17). It is universally understood that P2X receptors are abundant in both the central and peripheral nervous systems where they respond more quickly than P2Y receptors (29, 32). Despite their abundance in neuronal cells, P2X receptors are expressed in most cell types including fibroblasts, epithelial and alveolar cells (8, 17, 24, 33). Notably, Purinergic receptor activation in various cell types have been identified in association with membrane channels to induce membrane and protein processes.

$P2X_4$ Functions

In this project, we will focus on $P2X_4$'s contribution to membrane cellular fusion in multinucleated giant cell (MGC) formation. Earlier research identified $P2X_4$ as being widely distributed in both excitatory and non-excitatory neuronal cells whose processes include calcium wave signaling, membrane excitation, and neurotransmitter release (34). Currently, $P2X_4$, is detected to be expressed ubiquitously throughout mammalian cells (4, 27). Inhibitor experiments have indicated the following distinctions between $P2X_4$ and the other members of the P2X family: 1) it is somewhat resistant to P2X antagonists pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and suramin, and 2) it is responsive to Ivermectin's allosteric inhibition to the receptor which is not observed in other P2X receptors (22, 34). Like all P2X receptors, $P2X_4$ has an allosteric site distinct from its ligand binding site (34). Unlike the other P2X receptors, $P2X_4$'s allosteric site is the most extensively characterized and can be modulated by trace minerals, zinc and copper (34). A study using Xenopus laevis oocytes measured ATP current in the presence or absence of different metals (26). They

concluded copper to act as an allosteric inhibitor while zinc acts as an inducer suggesting $P2X_4$ to have specific metal-binding sites distinct from its ligand-binding domain (26, 34). The unique allosteric regulation employed by $P2X_4$ may provide insight into its allosteric regulation with drugs Ivermectin and suramin used to combat parasitic worms.

Pannexin-1

The pannexin hemichannels family is composed of three different members: pannexin-1, pannexin-2 and pannexin-3 (Panx1, Panx2, Panx3) (26). The Panx1 hemichannel is found in numerous human tissue types including, the brain, heart, lung and thymus. In comparison to Panx1, Panx2 and Panx3 are more restricted in their expression in tissue types (26). Panx1is the only hemichannel in the pannexin family identified to be regulated by purinergic signaling, specifically, its complex with $P2X_7$ (16, 26). ATP activation of $P2X_7$ recruits Panx1 thereby creating a K^+ efflux in the cell and activating an inflammasome (16, 26). However, Panx1 has been identified to interact with other purinergic receptors, $P2X_1$ and $P2X_4$ (36).

Chlamydia Trachomtis

Intracellular bacterial infections affect millions worldwide and are major contributors to fatal ailments including pneumonia, sterilization, blindness and heart disease (11, 12). *C. trachomatis* is the most common bacterial sexually transmitted disease (STD) (12). *C. trachmatis* is an obligate intracellular pathogen found to be prevalent in the eye, genital tract and rectum (11, 27). It is the leading cause of preventable blindness in the world with 15% of the population affected (11, 12). Prolonged infection of *C. trachomatis* can cause a variety of complications in both males and females varying from infertility in men and women to ectopic pregnancy in females (11, 12). The *C. trachomatis* strain of the *Chlamydia* family is composed of three different strains or serovars, L1, L2, and L3 of which we will focus on the L2 strain (2). The bacteria has become a world-wide pandemic with high infection rates in Northern Europe, Africa and the United States (11, 12).

C. trachomatis invades the mucosal epithelium in multinucleated cells (27). The infection triggers epithelial cell ingestion of the bacteria resulting in vacuole formation,

which is homologous to other intracellular bacterial pathogens (27). Vacuole formation allows the bacteria to evade recognition of the host immune system and provides protection for replication of the pathogen (27). A significant characteristic of *C. trachomatis* is its ability to induce macrophage formation of multinucleated giant cells (MGC) (27). Individuals infected with *C. trachomatis* have increased dramatically over the past decade. Therefore, further study into *C. trachomatis vacuole* fusion will show possible alternatives to preventing and clearing persistent infection.

A critical step to *Chlamydia*'s replication cycle is its ability to form small, vacuole inclusions (22). The inclusion separates the bacteria from the host cytoplasm and eludes fusion with the host cells lysosomes allowing replication to occur (22). During infection, *Chlamydia* invades host cells as infectious, non-replicating elementary bodies (EBs) (22). As the EBs form many tiny vacuoles they are able to differentiate into metabolically active and non-infectious reticulate bodies (RBs) (22). The RBs reside in the many vacuoles, eventually fusing together during replication in the host cell (22). $P2X_7$ has been previously characterized in *C. trachomatis* vacuole-lysosome fusion but $P2X_4$'s involvement in *C. trachomatis* vacuole-vacuole fusion has yet to be characterized (9, 27).

Upon cell to cell contact, the plasma membrane $P2X_7$ receptor has been characterized as being expressed in very high levels in macrophages, microglial and dendritic cells (8, 10). The interaction of the $P2X_7$/pannexin-1 (Pan1) complex has also been observed in cell fusion of MGC's (20). Other purinergic receptors associated with macrophage fusion have yet to be characterized. The route of expression of purinergic receptors like $P2X_4$ and $P2X_7$ are not only of significance to macrophage modulation, but also may provide significance as an approach to vaccination. The development of vaccines is important for prevention in nations with limited health care or for civilizations that cannot undergo testing for bacterial strains such as *C. trachomatis.* By characterizing vacuole and macrophage fusion pathways, C*hlamydia* replication can be better understood and aid in prevention against the bacteria's debilitating effects.

Previous Studies

$P2X_4$ and $P2X_7$ recruitment complexes have been identified in many immunological recruiting processes (10). In cell types expressing $P2X_4$, the receptor resides in the plasma membrane largely within intracellular compartments (28). Previous studies in neuronal cells indicate the interactions of $P2X_4$ and $P2X_7$ in pore formation where ATP expression was enhanced compared to $P2X_7$ alone (34). Alternatively, in a rat alveolar macrophage study, upon ATP stimulated current, $P2X_4$ receptor activation was observed in the cell's membrane while $P2X_7$ receptor activation was not (4). The authors concluded that the rat alveolar macrophages only express the $P2X_4$ receptor as there was no evidence in expression of other P2X receptors (4). The unique regulation of $P2X_4$ in comparison to the other P2X receptors may represent a novel approach into characterizing $P2X_4$ during an innate immune response. Both receptors are particularly prevalent in immune cells, such as endothelial and epithelial cells (28).

A previous study observed T-cell receptor (TCR) stimulation to be mediated by Panx1, $P2X_4$ and $P2X_1$ (36). The authors concluded TCR activation to be facilitated by the translocation of $P2X_1$, $P2X_4$ and Pannexin-1 to the immune synapse (26). $P2X_7$ in contrast, remained uniformly distributed on the cell surface upon TCR stimulation (26). It is at the interface of communication between T-cells and antigen presenting cells where $P2X_1$, $P2X_4$ and Pannexin-1 are present but not $P2X_7$. This indicates that P2X4 may have unique roles in membrane communication not present in other purinergic receptors. This could be beneficial in that $P2X_4$ is expressed ubiquitously and though it is usually complexed with $P2X_7$, it may have additional roles. Characterizing $P2X_4$'s role in cellular fusion would be a novel approach to many biological applications. Provided these previous studies, we predict $P2X_4$'s association with pannexin-1 will show endogenous regulation of macrophages. Furthermore, given the homology shared between $P2X_7$ and $P2X_4$, we will investigate $P2X_4$'s involvement with Pannexin-1 in cell fusion.

Pannexin and Purinergic Signaling

Currently, extensive research has focused on the regulation between pannexin hemichannels and purinergic receptors (20). The involvement of Pannexin hemichannels function in ATP release and expression in various cell types has intrigued interest in its association with purinergic receptors. The pannexin family of single-membrane hemichannel's include three members: Panexin-1, Panexin-2, and Panexin-3 (Panx1, Panx2, and Panx3) (26). Panx2 and Panx3 are confined to more specific cell types including neurons, chondrocytes and osteoblasts (26). Conversely, Panx1 hemichannel expression has been identified in various cell types and responses (26). Panx1 regulation has been indicated in calcium wave propagation, vascular tone mucociliary lung clearance and can act as a tumor suppressor in gliomas (26). Despite Panx1's cellular advantages, the opening of the Panx1 hemi-channel has been linked to detrimental effects such as cell death and seizures under epileptic conditions (26). Notably, Panx1 channel opening can also promote HIV-1 viral infections, a mechanism similar to the one employed by P2 receptors (3, 26). Panx1's interaction with purinergic receptors upon ATP stimulation has prompted investigation into their co-regulation in biological functions.

Cumulative Research

Co-regulation between Panx1 and Panx2 has been identified in Xenopus oocytes resulting in a reduced current in Panx1 upon electrophysiological stimulation (21, 26). The authors speculate the reduced current in their respective channels to be attributed to the interaction between Panx1 and Panx2 resulting in a mis-fold or mis-traffic in their channels (21, 26). Some studies conclude less co-regulation to occur between Panx1 and Panx3 hemichannels with no functional change observed in either channel (26). Signaling co-function in Panx2 and Panx3 has yet to be identified suggesting they do not co-oligomerize in vivo and are not expressed in the same cell types (26). Further, Panx1 hemichannel has been the only channel in the Pannexin family to be repeatedly identified to undergo recruitment by purinergic receptors in response to ATP stimulation (20, 21, 26).

Current studies have investigated the interactions between Panx1 and purinergic receptors in a wide array of

molecular processes. In contrast to the co-regulation between pannexin hemichannels, Panx1 is widely accepted to interact with P2 receptors in cellular responses (20, 21, 26). Panx1's interaction with P2X receptors has been implicated in apoptosis and cellular immune responses. In addition to P2X simulation, Panx1 channel activation can occur through P2Y purinergic receptors. Ca^{2+} signaling is regulated upon activation of Panx1 channels through P2Y receptors in the cytoplasm (26). An earlier study measuring apoptosis observed Panx1 to form the pore component of the $P2X_7$ "death complex" in Xenopus oocytes (26). Co-expression of $P2X_7$ and Panx1 in Xenopus oocytes revealed cell death (21). Apoptosis initiation was induced by direct hemichannel opening of Panx1 interaction with $P2X_7$ upon increased exposure to ATP (21). These results were not observed upon the introduction of Panx1 alone (loco). In addition, cell death was not initiated with the co-expression of Panx1 and the P2Y receptor (21). The authors conclude that upon ATP stimulation, $P2X_7$ requires the recruitment of Panx1 pore for apoptosis initiation (21). Ongoing studies continue to explore the recruitment of Panx1 by purinergic receptors and where it has been identified in other cellular processes including inflammasome activation.

Purinergic Receptors in inflammasome activation

Vital to purinergic signaling is Panx1's pore association with $P2X_7$'s signaling complex in the release of ATP in multiple cell types. ATP release induced by Panx1's pore complex with $P2X_7$ has become an essential component in inflammasome activation (20, 26). $P2X_7$ stimulation upon ATP binding recruits Panx1 and triggers a K^+ efflux releasing the pro-inflammatory cytokine interleukin (IL)-1 β and initiating inflammasome activation (18). Concurrently, upon stimulation with ATP, is the activation of caspase-1 and ATP release (26). Inflammasome activation can eventually lead to pyroptosis or programmed cell death different from apoptosis.

Toll-like receptors are single membrane receptors expressed in dendritic cells and macrophages used in the identification of conserved microbial molecules. Toll-like receptor independent inflammasome activation is initiated by regulation of Panx1 though, without the stimulation of $P2X_7$ (25). During an inflammatory or immune response, macrophages expressing $P2X_7$ activate the receptor through

ATP binding and recruit Panx1 resulting in further release of ATP (20). ATP release in stressed cells acts as a danger signal and recruits more macrophages to the site of inflammation ultimately resulting in cellular fusion (20). More recently, $P2X_4$ has been associated in the inflammatory response and plays a unique role in macrophage fusion (20).

Cellular Fusion Studies

It is universally recognized that macrophages, osteoclasts and foreign body cells fuse to form MGCs (5). Specifically, pathogens such as mycobacteriums, including *Mycobacterium tuberculosis*, can trigger MGC formation. Macrophage fusion in infected granuolomas are better referred to as Langhans' giant cells. Prosthetics have also been shown to induce MGC formation, referred to as foreign body cells (1, 5). Synctium formation, or multinucleated cells from multiple unicellular cells, is critical in the transmission of viral particles such as Influenza and HIV (35). Common factors associated with MGC formation include adhesion proteins, dendritic cell-specific transmembrane protein (DC-STAMP); a fusion factor, and macrophage fusion factor (1). However, the direct fusion mechanism employed for MGC formation is not fully understood. Purinergic signaling and Pannexin pore formation have been identified in MGC formation and can provide a pathway into fusion amongst microbial infection (20).

As previously mentioned, Panx1 and P2 receptors play a role in viral infections. Studies on the release of ATP have been identified in Panx1's pore association with $P2X_7$'s signaling complex. Release of ATP by Panx1's interaction with P2Y has also been observed as a component in HIV-1 infected, CD4+ cells (30). The T-cell surface receptor CD4+ interacts with HIV-1 envelope glycoprotein complex (Env) expressing cells and prompts the release of ATP by Panx1 hemichannels into the extracellular space (26). The released ATP in the extracellular milieu acts on the P2Y2 purinergic receptor and activates the kinase Pyk2 in T lymphocytes (26). Activation of the Pyk2 kinase depolarizes the membrane and triggers membrane-to-membrane fusion allowing cell-to-cell transmission of the virus (26). The authors observed Panx1, P2Y2, and Pyk2 to be all physically recruited to the

site of contact between membrane fusion of Env-containing CD4 cells (30). They concluded Panx1 and P2Y2 to be integral in the transmission of the virus to induce infection (30).

Further investigation into purinergic signaling as a component of HIV-1 transmission can contribute to the cellular fusion mechanism, "contagious apoptosis" in HIV-1 envelope expressing cells (3). "Contagious apoptosis" or cell-to-cell fusion and transmission of an apoptotic signal was identified in a previous study between healthy CD4+ cells and infected HIV-1 Env cells (3). Co-culture of Env+ cells expressing an initial apoptotic pathway with healthy CD4+ cells formed syncytium, a type of multi-nucleated cell (3). The syncytium enabled transmission of the lethal apoptotic signal from Env+ cells to healthy CD4+ cells resulting in apoptosis of the multinucleated cell complex (30). The wide array of mechanisms underlying cellular fusion have yet to all be identified.

$P2X_4$ and $P2X_7$ have been implicated to promote cellular membrane fusion in osteoclasts, lysosomes and alveolar epithelial cells (26, 24, 29). The $P2X_4$ and $P2X_7$ receptor subunits also share the largest percentage of similar amino acid sequences; 48.6 % similarity in humans and 49.8% similarity for rat (10)**.** In a study using transfected human embryonic kidney (Hek 293) cells expressing $P2X_4$ and $P2X_7$, the receptors were found to form functional heteromeric receptors (14). The authors observed a 2-fold increase in biotinylated $P2X_4$ receptors at the plasma membrane in the presence of $P2X_7$ (14). Their results revealed sensitivity to inhibitors/activators, Ivermectin and 2', 3'-o-(2,4,6-trinitrophyl) adenosine 5-triphosphate (TNP-ATP) upon coexpression of both receptors, compared to no sensitivity observed with $P2X_7$ alone (14). The results suggest $P2X_4$ and $P2X_7$ to display both a structural and functional interaction between the two receptors (14).

Pore fusion has also been identified in $P2X_4$. Previous studies indicate $P2X_4$ receptors are expressed on exocytotic vesicles in alveolar epithelial (24). Fusion-activated Ca^{2+} entry in alveolar epithelial cells requires membrane fusion found to be facilitated by the $P2X_4$ receptors (24). Ca^{2+} entry mediated by $P2X_4$ is a critical step to exocytosis of the cellular membrane (24). Additionally, $P2X_4$ mediates exocytosis in lysosomes, suggesting a role for $P2X_4$ in membrane fusion (28).

Purinergic signaling's role in cellular fusion has been characterized with only a few receptors currently showing fusion promotion. Quite recently, however, additional purinergic receptors such as $P2X_4$, have been implicated to promote cellular fusion in biological processes. Examination into $P2X_4$ as a contributing component of cellular fusion could aid in microbial therapy.

Innovation

Many studies have verified $P2X_7$ in biological processes including apoptosis, renal regulation and inflammasome activation (26). In comparison to the other five types of P2X receptors, $P2X_7$ shares the greatest subunit homology with the $P2X_4$ receptor (10). Both receptors' proximity to one another on human chromosome 12 is closest at only 24 Kb apart (10). In addition, $P2X_4$ and $P2X_7$ are present in the same tissues (10). Earlier, our lab investigated the interaction between $P2X_4$, $P2X_7$ and Panx1 in reactive oxygen species (ROS) production upon ATP exposure in *Porphyromonas gingivalis (P.gingivalis)* infected ginigival epithelial cells (16). Our lab's preceding research identified the $P2X_4$ receptor to be involved in NLRP3 inflammasome activation with the recruitment of Panx1 (16). We found, in the presence of ATP, purinergic receptor, $P2X_4$ associates with the $P2X_7$/Panx1 pore complex during a *P. gingivalis* infection in gingival epithelial cells (16). Previously, reactive oxygen species (ROS) production via $P2X_7$ was identified in the activation of NLRP3 inflammasome and caspase-1 (16). This motivated our investigated into another P2X receptor's role in ROS generation, $P2X_4$. Our findings revealed, upon ATP release, ROS assembly can be induced through a $P2X_4$/ $P2X_7$ /Panx1 complex (16). Additionally, inhibition or deletion of either $P2X_4$, $P2X_7$ or Panx1 did not induce secretion of IL-1 β in the inflammasome in *P. gingivalis* infected cells treated with ATP (16). Our lab concluded that ROS production is stimulated by the $P2X_4$/ $P2X_7$ /Panx1 complex and that $P2X_4$ can act as a component of inflammasome activation in response to a pathogen (16). Therefore, inflammasome activation can be triggered through the activation of different purinergic receptors, specifically $P2X_7$ and $P2X_4$. Downstream of ROS production, is the activation of the NLRP3 inflammasome, suggesting $P2X_4$ as a modulator of inflammasome

activation in response to pathogen invasion (16). Previously, $P2X_4$ had not yet been identified in the ATP-mediated activation of an inflammasome (16). It is this relationship between $P2X_4$, $P2X_7$, and Panx1 that has prompted us to study the interaction between $P2X_4$, and Panx1 in cell membrane fusion. $P2X_4$'s involvement in macrophage fusion has yet to be characterized and can provide novel insight into the fusion mechanisms of innate immune responses.

Unique to $P2X_4$'s activation is 1) its sensitivity to Ivermectin, and 2) reversible inhibition of *C. trachomatis* infection with micromolar amounts of ATP. These functions are characteristic of $P2X_4$ and are not observed in other P2X receptors (27, 34). Ivermectin is a clinically approved drug used for the treatment of parasitic worms Onchocerca volvulus in humans and Onchocerca cervicalis in horses (22). Our lab's previous research on Ivermectin in human cells found *C. trachomatis* infection epithelial cells to be inhibited upon treatment of micromolar concentrations of Ivermectin (22). Previous studies have indicated Ivermectin to allosterically inhibit $P2X_4$ (22, 34). Though all P2X receptors exhibit allosteric inhibition, none are responsive to Ivermectin like $P2X_4$ (22, 34). This may suggest a potential role for this receptor in chlamydia infected cells not seen in other P2X receptors.

METHODS AND MATERIALS

Cell Culture

Overexpressed $P2X_4$ Hek cells, overexpressed $P2X_7$ Hek cells and Hek -293 wild type cells were grown in tissue culture flasks, reaching 75-85% confluency before experimentation. The cells were maintained in Dulbecco's Modified Eagle Medium/ F-12 (DMEM) with 10% fetal bovine serum, 100 U/ml streptomyocin and 100 U/ml penicillin. The cells were grown in a humidified incubator at 37 □C and 5% CO_2.

Quantification of Cellular Fusion

Overexpressed $P2X_4$ Hek cells, overexpressed $P2X_7$ Hek cells and Hek -293 wild type cells used for immunofluorescence imaging were grown in 24 well plates with a coverslip added to each well. 24 hours after the cells were plated, they were fixed for 10 minutes with 100% cold methanol. Cells adhered to the coverslips were then washed three times with phosphate buffered solution (PBS). After fixing the cells, they were immersed in an Anti-β actin antibody stain (green) with PBS, and .2% triton for 10 minutes. After 10 minutes the coverslips were washed with PBS. The coverslips were then stained with PBS, .2%triton and Hoechst stain for another 10 minutes. After staining, the coverslips were washed with PBS and then mounted and sealed on coverslip slides. The Anti-β actin antibody mix was administered to view the actin cytoskeleton. At each time point, Hoechst's stain and β-actin staining were merged to provide evidence of cell fusion in $P2X_4$ and $P2X_7$ overexpressing Hek cells.

Overexpressed $P2X_4$ Hek cells, overexpressed $P2X_7$ Hek cells and Hek -293 wild type cells used for Guava analysis were grown in 12 well plates, with 50,000 cells plated in each well with DMEM media. At 12, 24 and 48 hours post plating, cells were first detached with 200 uL of Ethylenediaminetetraacetic acid (EDTA). After detachment the cells were transferred to a 96 well plate and spun down in a high speed centrifuge at 800 revolutions per minute (rpm) for 5 minutes. The resulting supernatant was removed and cells were washed with 200uL of PBS. Permeabilizing buffer and 1uL of red and green fluorescence dyes were added to the wells and placed in an incubator at 37 □C for 20 minutes. Cells were then

centrifuged again at 800 rpm's for 5 minutes. The supernatant was removed and the cells were re-suspended in 300 uL of staining buffer in sample tubes. Both the red and green fluorescence are shown separately and fused at the 12, 24, and 48 hour time points.

C. trachomatis Expansion

HeLa cells were grown to 75%-80% confluency in DMEM media containing 10% FBS, and .5% gentimiacin in large petri dishes. Before infecting the HeLa cells with *C. trachomatis*, a media change was administered. HeLa cells were infected *C. trachomatis* for 36 hours. Plates were swirled for every 15 minutes during the first hour of infection and 30 hours post infection to distribute elementary bodies (EBs). At 48 hours post infection, the cells were scraped off the dish with a rubber police/cell lifter and pipetted into two 50 mL vials. The tubes were then spun down in the low speed centrifuge for 5 minutes at 1200 rpm and 4 □C. The supernatant was removed and placed in high speed centrifuge tubes. The tubes were transferred to the high speed centrifuge and spun at 14,000 rpms for 5 minutes at 4 □C. The supernatant was disposed and the pellets were re-suspended in DMEM.

C. trachomatis Experiments

The western blot demonstrating $P2X_4$'s expression in vacuole fusion was performed by first collecting cell lysates. Protein extracted from cell lysates were ran on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and probed with Anti-$P2X_4$ antibody. Actin was used as an equal loading control. Proteins were transferred from the gel onto a nitrocellulose membrane. To prevent interactions between the antibody and membrane, blocking or non-specific binding of non-fat dry milk with Tris-Buffered Saline (TBS) was employed. The probed membrane or detection was undergone in a two-step process involving the primary Anti-$P2X_4$ antibody, and a secondary antibody. After the blots with the primary antibody were incubated overnight at 4 □C, the membrane was rinsed and exposed to a secondary antibody for two hours at room temperature. At the end of the two hours, a substrate was added to induce fluorescence. Unbound probes were further washed away and the western blot was detected.

HeLa cells used in immunofluorescence images were grown in 12 well plates each well containing a coverslip with DMEM media prior to infection. Before infection of *C. trachomatis* in HeLa cells, media was changed and quantification of *C.trachomatis* was done via multiple dilutions over 36 hours. 48 hours after infection, infected HeLa cells adherent to coverslips were fixed in methanol for 10 minutes. Coverslips were then washed with PBS. After, coverslips were submerged in a mixture containing an Anti-Chlamydia antibody stain (green) with Hoechst, .2% Triton and PBS for 10 minutes. Following the 10 minutes, coverslips were washed with PBS and then submerged in an Anti-P2X4 antibody stain (red) with .2% Triton and PBS for an additional 10 minutes. Coverslips were then washed with PBS and mounted to microscope slides.

RESULTS

As expected, immunofluorescence images of $P2X_4$ overexpressed Hek cells showed cell membrane fusion similar to our positive control, $P2X_7$ overexpressed Hek cells after 24 hours in the merge columns (Fig. 1). The overexpressed $P2X_4$ Hek cells show considerable fusion compared to the negative control, wild-type Hek 293 cells upon merging the actin structure (β-actin) and nucleus' of the cells (Hoechst) (Fig.1). Guava analysis of $P2X_4$ overexpressed Hek cells, also revealed similar results to $P2X_7$ overexpressed Hek cells at 48 hours (Fig. 2-3). Figure 2 is an example of our raw Guava data of overexpressed $P2X_7$ Hek cells after 48 hours. The first graph of $P2X_7$ overexpressed cells represent cells stained with red fluorescence and the second graph represents cells stained with the green fluorescence (Fig. 2). The third graph demonstrates cells in the red quadrant, green quadrant and in the upper right quadrant (Fig. 2). The upper right quadrant reveals the cells that merged expressing a combined red and green fluorescence color (Fig 2). Quantification of our Guava analysis revealed Hek 293 wild-type cells to have less cell fusion compared to overexpressing $P2X_4$ Hek cells (Fig. 3). Figure 3 includes our raw data of overexpressed $P2X_7$, $P2X_4$, and Hek wild-type cells. We observe at the 12, 24, 48 hour time-points, a continual increase in the percentage $P2X_4$ overexpressed Hek cells and $P2X_7$ overexpressed Hek cells in a 50,000 cell sample (Fig. 3). In comparison, Hek 293 wild type cells showed a minimal increase in the percentage of cells throughout the 48 hours (Fig. 3). Moreover, $P2X_4$ overexpressed Hek cells are found to be involved in cell membrane fusion displaying similar characteristics to $P2X_7$ overexpressed Hek cells involvement in cell membrane fusion

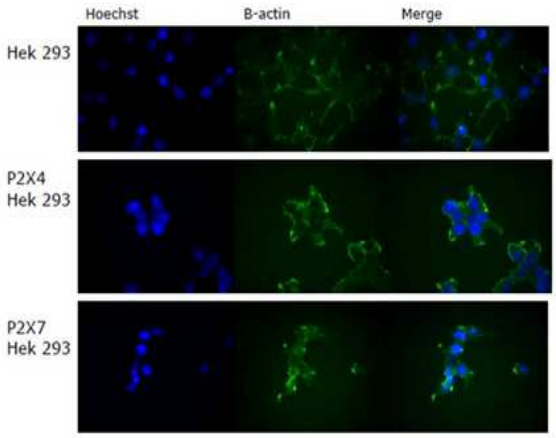

Figure 1: Immunofluorescence of Hek 293 wild-type cells, overexpressed P2X4 and P2X7 Hek cells. Each cell type was stained with Hoechst to view the nucleus and β- actin is stained in green to observe the cytoplasm. Images were merged to view cellular fusion in each cell line.

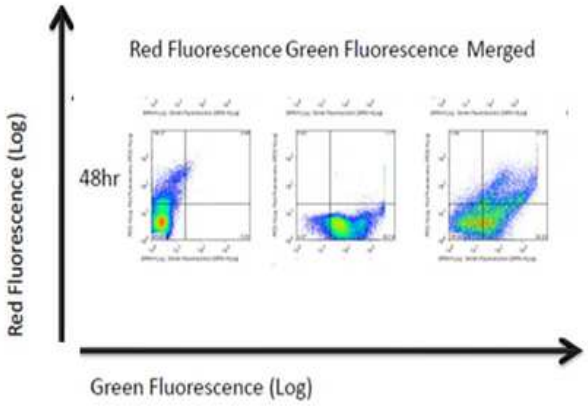

Figure 2: Fusion assay of P2X7 overexpressing Hek cells at 48 hours.

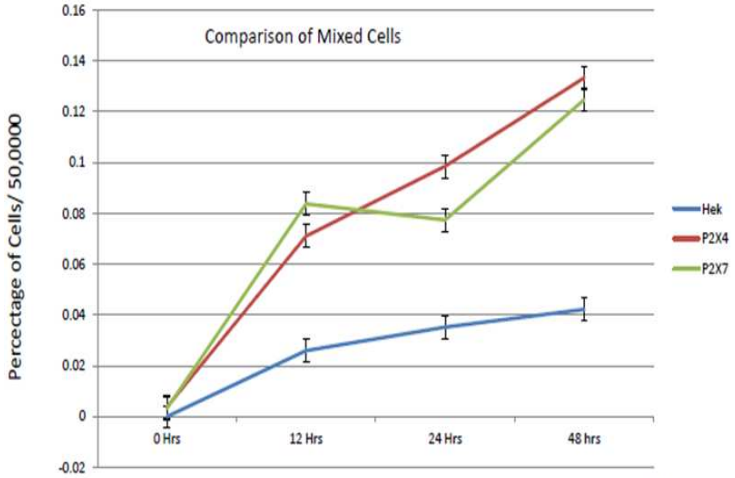

Figure 3: Quantification of cell fusion assay of wild-type Hek 293 cells, (blue), overexpressed $P2X_4$ Hek cells (red) and overexpressed $P2X_7$ Hek cells (green) at 0,12, 24, and 48 hours. Error bars are standard deviations of each time point in individual cell lines.

Aim 2: $P2X_4$'s involvement in C. trachomatis L2 vacuole fusion

Our results indicate that the $P2X_4$ receptor is a possible participant in vacuole fusion during a *C.trachomatis* infection (Fig.4-5). A Western blot of $P2X_4$, demonstrates an increase in the $P2X_4$ receptor during C. trachomatis infection at 24 hours post infection (Fig. 5). This is in contrast to our earlier findings of $P2X_7$, whose expression does not increase with micromolar concentrations of ATP during a *C.trachomatis* infection. Immunofluorescence images of HeLa cells infected with Chlamydia show an increase in the $P2X_4$ receptor (red) when merged with *C.trachomatis* vacuoles (green) and the cell's nucleus (blue) compared to the control HeLa cells 48 hours post infection (Fig 5). The control HeLa cells showed no fusion compared to the infected HeLa cells when merged over a 48 hour time point (Fig 5). As predicted, $P2X_4$ shows to be localized on Chlamydia vacuole membranes and is associated with the promotion of Chlamydia vacuole fusion (Fig. 5).

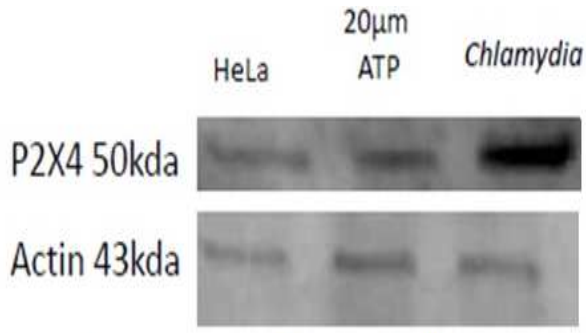

Figure 4: Western demonstrating the increase in P2X4 receptor during a *C. trachomatis* infection at 24 hpi.

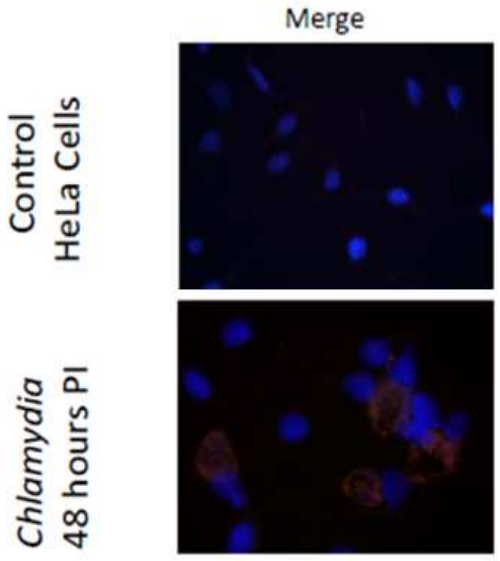

Figure 5: Immunofluorescence with endogenous P2X4 associating with *C. trachomatis* inclusion. The nucleus is stained with Hoechst, P2X4 receptor is shown in red and *C.trachomatis* vacuole is stained green.

DISCUSSION

Aim 1: $P2X_4$ in Membrane Fusion

The direct mechanism underlying cellular membrane fusion in MGC formation has yet to be fully understood. In addition to cadherines, adhesion molecules and snares observed in cellular fusion, purinergic receptors have also been identified to promote membrane fusion in macrophages (19). Our collaborator Dr. Francesco Di Virgilio, investigated the expression of the purinergic receptor $P2X_7$ in multinucleated cells. Their results indicate $P2X_7$ to be highly expressed in macrophages and to act as a promoter of membrane fusion between macrophage cells (19). Additionally, Di Virgilio's lab investigated $P2X_7$'s interaction with Panx1 hemichannel in MGC formation (20). They concluded Panx1's pore forming channel to be associated with $P2X_7$ and a requirement for macrophage fusion (20). These results have verified purinergic signaling as a component in macrophage membrane fusion and MGC formation.

Previous studies on the purinergic receptor $P2X_4$, have characterized its involvement in T-cell and inflammasome activation and pathogen inhibition (16, 28, & 35). In neuronal cells, $P2X_4$ is closely associated with pannexin-1 hemichannel on the cell membrane promoting Ca^{2+} entry and ATP release (35). Our results indicate that *$P2X_4$* does behave similarly to *$P2X_7$* in the promotion of cell fusion (Fig. 3-5). Our hypothesis of $P2X_4$ is based on these previous findings. We hypothesized the purinergic receptor $P2X_4$, contributes to cell membrane fusion in the formation of multinucleated giant cells. The overexpressed *$P2X_4$* Hek cells and overexpressed *$P2X_7$* Hek cells demonstrated increased cellular fusion compared to the wild-type Hek cells (Fig. 1-3). Similar to the overexpressed $P2X_7$ overexpressed Hek cells, $P2X_4$ overexpressed Hek cells showed a similar percentage in membrane fused cells (Fig. 2-3). Thus indicating $P2X_4$ to be involved in membrane fusion.

Characterization of $P2X_4$ as a regulator of cellular fusion would be a novel approach into identifying the mechanism of cellular membrane fusion in MGC formation. Our results suggest that $P2X_4$ is a modulator of cellular fusion (Fig. 1-3) and in combination with our previous results on inflammasome activation, we predict $P2X_4$ could interact with Panx1. Further, we would like to examine $P2X_4$'s

interaction with Panx1 in cellular fusion during an inflammatory response.

Aim 2: Characterize the $P2X_4$ receptors contribution to C. trachomatis L2 vacuole fusion

We had previously indicated inhibition of *C.trachomatis* infection through the ligation of $P2X_7$ with millimolar concentrations of ATP (28). Ligation of the $P2X_7$ receptor upon millimolar amounts of ATP resulted in fusion of chlamydial vacuoles to lysosomes and inhibited infection (28). The cell secretes millimolar concentrations of ATP in response to physically compromised cells and micromolar concentrations of ATP in response to stressed or dying cells (28). When exposing $P2X_7$ to micromolar amounts of ATP, inhibition of *C. trachomatis* was not observed. However, extracellular micromolar concentraions of ATP activated $P2X_4$ receptors and inhibited *C.trachomatis* replication (28). Micromolar concentrations of ATP in human cells are more indicative of inflammation caused by infection (28). In addition, our study indicated reversible inhibition of *C.trachomatis* infected epithelial cells via stimulation of $P2X_4$ (28). Our results implicated $P2X_4$ to play a role in multinucleated cells and in vacuole-vacuole membrane fusion (28). Provided our results, we hypothesized activation of the purinergic receptor to $P2X_4$ contribute to vacuole fusion during *C. trachomatis* infection. Collectively, our results imply $P2X_4$ to be associated on *C. trachomatis* vacuoles and thus as a modulator of chlamydia infection (Fig. 4-5).

Our results can provide further insight into the receptors modulation of other microbial pathogens. In reference to chlamydia induced infection, $P2X_4$ is abundant in vaginal epithelial cells and in a cancer cell line of human epithelial cancer cells, it was the most abundant purinergic receptor expressed (28). $P2X_4$ has yet to be characterized in this manner and may provide a unique aid in microbial or viral therapy.

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