Characterizing the Role of IL-1β in Macrophage Clearance of *Citrobacter rodentium* Infection

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

Inflammatory Bowel Diseases (IBD) are a group of conditions characterized by chronic inflammation and a dysregulated immune response of the gastrointestinal tract. The etiology is unknown; however, research has indicated a complex relationship between environmental stimuli, genetic predisposition, and changes in the microbiome. The nod-like receptor protein complex 3 (NLRP3) is part of a cytosolic microbial sensor complex called the inflammasome, involved in the maturation and secretion of the proinflammatory cytokine IL-1β. Some individuals with IBD have a mutation in the NLRP3 gene that causes a hypoproduction of IL-1β, contrasting many other immune-mediated diseases involving the inflammasome. *Citrobacter rodentium* is a mouse pathogen used as a model for IBD as it causes IBD-like colitis in mice. In previous work, we showed that extraneous IL-1β improved the ability of macrophages to phagocytose *C. rodentium* in *Nlrp3*$^{-/-}$ mice and that clearance of infection is dependent on the NLRP3 inflammasome. In my PhD, I investigated the involvement of the NLRP3 inflammasome in the ability of macrophages to clear *C. rodentium* infection. Furthermore, I investigated how macrophages interact with epithelial cells during infection and how anaerobic bacteria isolated from IBD patients can activate this complex. My **hypothesis** was that inflammasome activation is required for macrophages to phagocytose and kill *Citrobacter rodentium*.

My experimental approach was to infect the mouse macrophage cell lines, J774A.1 and RAW 264.7, with *C. rodentium in vitro*. Gentamicin protection assays were used to determine intracellular bacterial survival. Nigericin and extracellular ATP

were used to stimulate the inflammasome, with YVAD and KCl used as inhibitors. Reactive oxygen species (ROS) expression was analyzed using the fluorescent dye DCFDA with ROS inhibition by DPI, NaC, or Acetovanillone. Gene expression was analyzed using qPCR and proteins were analyzed using Western blot, ELISA, and a Proteome profiler. Immunofluorescence was done using spinning disk confocal microscopy. Epithelial cell line CMT-93 was used in transwells to understand epithelial interaction with macrophages during infection. Tight junction permeability was measured using trans-epithelial electrical resistance with a volt-o-meter. Anaerobic bacteria were collected from patient ileal washes and cultured in an anaerobic chamber using brain heart infusion media. Human monocyte cell line THP-1 was differentiated using phorbol 12-myristate 13-acetate into macrophages, which were then infected with the anaerobic bacteria.

Nigericin decreased intracellular bacterial survival in RAW 264.7 macrophages; however, these macrophages do not express the ASC protein and thus cannot activate the NLRP3 inflammasome. Therefore, I proposed a model for alternative pathway activated by Nigericin for killing intracellular pathogens. In addition, extracellular ATP decreased intracellular bacterial survival in J774A.1 macrophages through ROS generation. ATP activation caused an increase in cytosolic maturation of IL-1β but no change in secretion. ROS generation induced by ATP-activated inflammasomes was found to be mediated by the mitochondria. Furthermore, supernatant from ATP-activated macrophages was able to induce inflammasome activation and ROS production in naïve macrophages. ATP also caused a cytokine shift towards an anti-inflammatory state after the infection was

cleared. In the presence of epithelial cells, these macrophages facilitated increased tight junction recovery. Macrophages were recruited to the epithelial apical membrane during infection and showed expression and production of ZO-1 and claudin 1 tight junction proteins. *NLRP3* gene expression was increased in epithelial cells after exposure to extracellular ATP during infection but there was no change in secretion of IL-1β.Anaerobic bacteria isolated from patients with Crohn disease increased secretion of IL-1β and induced ROS generation compared to those isolated from individuals that did not have IBD.

This thesis describes the complex nature of the NLRP3 inflammasome within macrophages and its interaction with epithelial cells, in the context of the gastrointestinal tract. My *in vitro* studies reinforce the importance of understanding pathways involved in enteric pathogen clearance by macrophages. Improving our understanding of the molecular pathways within macrophages will aid in regulating and clearing enteric pathogens and possibly explain the unique role of inflammasomes in the gut, in the context of IBD, and why this is different from other settings. This understanding has the potential for development of new therapies targeting the uncontrolled immune activation due to defective bacterial clearance, as seen in IBD.

PREFACE

The research conducted for this thesis was performed under the direct supervision of Dr. Eytan Wine at the University of Alberta.

Chapter 2 has been submitted to Frontiers Immunology and is currently under review. Heather Armstrong and I are co-first authors on the manuscript. We both were involved in experimental design, data analysis, and manuscript composition. Richard Chan was responsible for experimental design and data analysis. Eytan Wine was the supervising author.

Chapter 3 of this thesis has been published as *Bording-Jorgensen, M., Alipour, M., Danesh, G., Wine, E. "Inflammasome Activation by ATP Enhances Citrobacter rodentium Clearance through ROS Generation". Cell Physiol Biochem. 2017;41(1):193-204. doi: 10.1159/000455988. Epub 2017 Jan 23*. I was responsible for experimental design, data analysis, and manuscript composition. Ghazal Danesh. assisted with data collection and analysis. Misagh Alipour contributed with manuscript edits and experimental design. Eytan Wine was the supervising author.

Chapter 4 and 5 have not been published yet (both to be submitted in the coming months). I was responsible for experimental design, data analysis and manuscript composition. Heather Armstrong assisted with data collection, analysis, and manuscript editing. Jeremy Jerasi and Dawson Lafleur were responsible for the Western blots. Emma Zwaigenbaum and Vivian Fung assisted with the epithelial cell work. Eytan Wine was the supervising author.

Chapter 6 has not been published yet. I was responsible for experimental design, data analysis and manuscript composition. Heather Armstrong assisted with data collection. Misagh Alipour was involved in collecting anaerobic bacteria from the patients. Ghazal Danesh assisted with experimental design, data collection, and analysis. Jeremy Jerasi and Dawson Lafleur were responsible for the Western Blots. Eytan Wine was the supervising author.

ACKNOWLEDGEMENTS

This work would not have possible without the support of my friends and family. To my parents Earl and Doreen who have encouraged me throughout my academic pursuit. My sister Krista and my brothers Brent and Jode along with their spouses. To my nieces and nephews Sera, Jayden, Isabell, Alliyah, Collin, Seth and Ethan who have encouraged me to pursue my teaching aspirations.

I would like to thank past and present members of the Wine lab and CEGIIR for their support over the years. To Deenaz, who started as my supervisor during my undergraduate project and then became a dear friend and colleague when I started graduate school. Misagh, who was a great mentor and friend. Rossi, thank you for your friendship and mentorship throughout my graduate program. Heather for your friendship and for aiding me in getting papers published. Rae and Rebecca for your friendship both in and out of the lab. To my students Ghazal, Vivian, and Emma for all your hard work in the lab.

Thank you, Eytan Wine, for allowing me the great pleasure of joining your lab, for being an amazing supervisor as well as mentor. Your guidance throughout my academic journey allowed me to excel in this field. Saswati and Judy, thank you both for giving me the opportunity to teach and discover my passion for it. My committee members Mario Feldman, Emmanuelle Cordat, Nicolas Touret and Harrissios Vliagoftis for your guidance throughout my program.

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LIST OF ABBREVIATIONS

A/E	Attaching/Effacing
ALR	Aim2 like receptor
AMP	Antimicrobial peptide
ASC	Apoptosis-associated speck-like protein
ATP	Adenosine triphosphate
CD	Crohn Disease
CFU	Colony Forming Unit
DAMPs	Danger Associated Molecular Pattern's
DCFDA	2,7 dichlorofluoresceine diacetate
DPI	Diphenyleneiodonium
DSS	Dextran sodium sulphate
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
GI	Gastrointestinal
IBD	Inflammatory Bowel Diseases
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KC-GRO	Kerotinocyte hemoattractant/ human growth-related oncogene
KCl	Potassium Chloride

LEE	Locus of Enterocyte Effacing
LPS	Lipopolysaccharide
M	Microfold
MAMPs	Microbe Associated Molecular Pattern's
M-CSF	Macrophage colony-stimulating factor
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NLRP3	Nod-like receptor family pyrin domain containing 3
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOX	NADPH oxidase
NT	Non-Treated
PRM	Pattern Recognition Molecule
PYD	Protein pyrin domain
ROS	Reactive Oxygen Species
T3SS	Type 3 Secretion System
TEER	Transepithelial Electrical Resistance
TLR	Toll-like receptor
Th	T helper
TNBS	2,4,6-trinitrobenzene sulphonic acid

TNFα	Tumour necrosis factor α
UC	Ulcerative colitis
YVAD	Ac-Tyr-Val-Ala-Asp chloromethylketone

Chapter 1

Introduction

CHAPTER 1. INTRODUCTION

1.1 GASTROINTESTINAL TRACT IN HEALTH AND DISEASE

1.1.1 GUT ANATOMY AND PHYSIOLOGY

The Gastrointestinal (GI) tract consists of an upper and lower distribution, with the upper portion being from the mouth to the duodenum and the lower being the small intestines, including the duodenum, and the colon. The major functions of the GI tract are the breakdown of food, absorption of water and nutrients to provide energy for all cellular functions, immune development, hormone production and secretion, and the removal of xenobiotics [1]. These processes are very complex and require a multitude of eukaryotic cell types and organ systems including neurological, vascular, musculoskeletal, immune, and the lymphatic systems to maintain homeostasis. Furthermore, there are other organs involved in gut functions, such as the pancreas, gallbladder, and liver, which will not be discussed here. In addition to these organ systems there is also the microbiome that, in recent literature, has shown to be instrumental in human development; microbial alterations can influence the progression of multiple diseases [2-5].

The interactions between the immune system and the microbiome are being investigated but there are still many unanswered questions. Separation of the microbes in the gut lumen and the nutrient rich environment of the host is orchestrated by a mucus layer and a single layer of eukaryotic epithelial cells. The

microbiome will be discussed later as this interesting dynamic has been shown to be involved in normal development, homeostasis, and disease. The focus of this thesis is on the lower GI tract and the functions and diseases associated with it.

1.1.2 SMALL INTESTINES

The small intestine is subdivided into three main parts: most proximal is the duodenum, then the jejunum, finally the distal section is the ileum. This is where the breakdown and absorption of nutrients from food occurs. The layers of the small intestine include the serosa, muscularis, submucosa, and mucosa. Serosa layer consists of a single layer of epithelial cells and is most distant from the lumen. Muscularis consists of two layers of smooth muscle and nerves (typically placed between these 2 layers) that work together to coordinate peristalsis in a proximal to distal direction [6]. The submucosa comprises connective tissue that includes nerves, blood vessels, and lymphatic, and is rich in immune cells. Finally, the mucosa is the innermost layer, which is built from a single layer of epithelial cells and contains villi that protrude into the lumen and crypts that invaginate the submucosa, to increase surface area and maximize absorption. Epithelial cells in the small intestine have a rapid turnover rate with renewal from stem cells located at the base of the crypt [6]. **Figure 1.1** illustrates the different cell types comprising the small intestine: enterocytes, microfold (M) cells, goblet cells, Paneth cells, tuft cells, enteroendocrine, and cup cells. Enterocytes are the absorptive cells and the rest (excluding M and cup cells) are secretory. Goblet cells secrete mucin, which lines the intestinal wall.

Enteroendocrine cells are responsible for hormone secretion that regulates various intestinal functions. Paneth cells are responsible for regulation of the microbiome, maintenance of neighboring stem cells, as well as the secretion of antimicrobial factors into the mucin layer [7]. M cells are specialized epithelial call and are found above specialized areas called Peyers Patches, which cover the lymphoid follicles and function as the interface between luminal content and immune cells; these allow for immune education and stimulation. Cup cells are similar to M cells but do not transfer pathogens to the immune system. Lastly, tuft cells are the least abundant cell type in the intestines and they serve as chemosensory cells and are directly related to type 2 immune responses.

The duodenum is the shortest section of the small intestine, but it is important for regulation of the enzymes required for digestion. The pH of the stomach is very acidic and as gastric juices flow into the duodenum, specialized cells called S cells secrete the protein secretin. In order to neutralize the acid, secretin stimulates the liver to produce bile and the pancreas to secrete bicarbonate. Bile also allows for the breakdown and absorption of fats and has a significant impact on the microbiome through the GI tract [8]. Jejunum is primarily responsible for the absorption of sugars, fats, and amino acids.

The ileum has a large role not only as serving the link between the small and large intestine but also for immune function. Peyer's patches are only found in the ileum and their main role is to facilitate interactions between the lumen and the host immune system. This aggregation of lymphoid follicles allows for the discrimination between commensal bacteria and pathogens. The M cells in the Peyer's patch

facilitate the transcytosis of luminal bacteria to the underlying immune cells, which allow for sampling and antigen presentation [9]. These immune cells can be macrophages, dendritic cells, B cells, and T cells. M cells can direct transcytosis through endocytosis, pinocytosis, phagocytosis, and Immunoglobulin (Ig) A receptor recognition. IgA is abundant in the lumen and can bind to both commensal and pathogenic bacteria [10]. Since M cells enable the movement of luminal material into the mucosa, several pathogens such as *Listeria monocytogenes, Salmonella enterica* Typhimurium, and *Shigella flexneri* use this to their advantage to invade the epithelial layer [11-13].

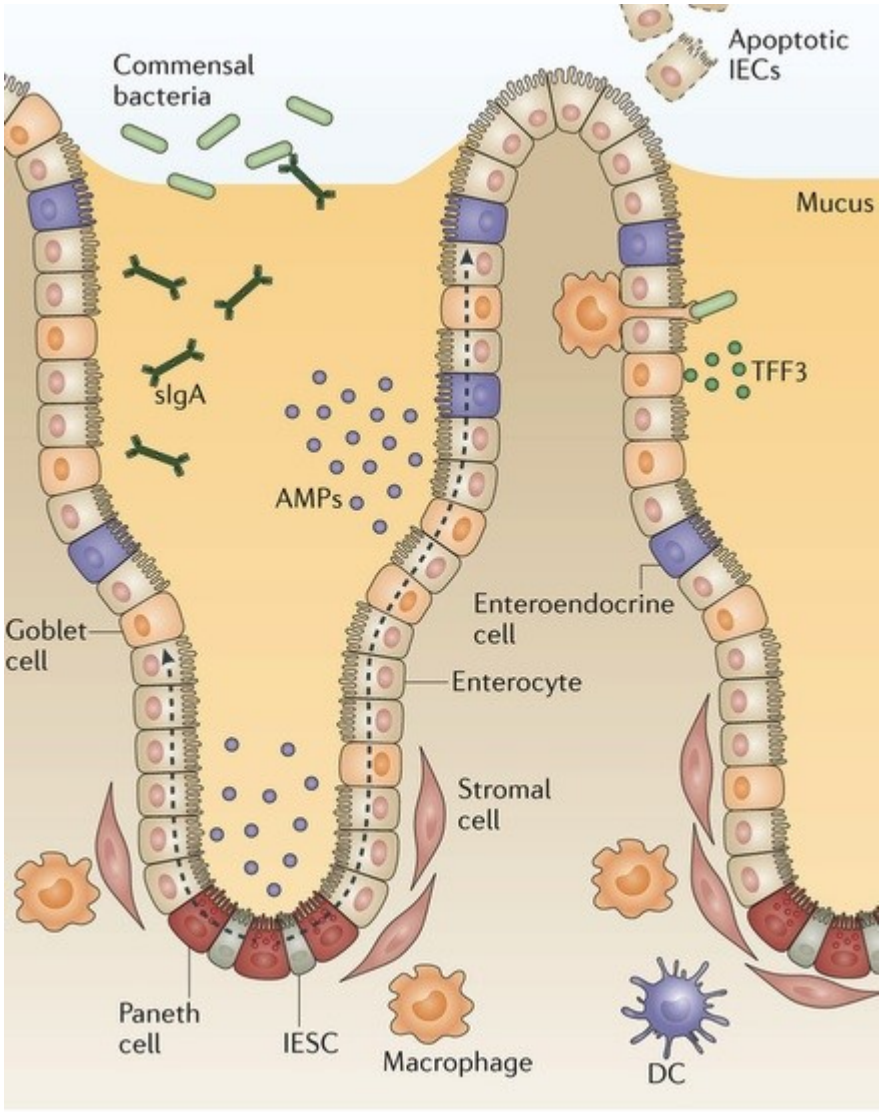

Fig 1.1. Schematic of small intestine mucosal layer. Intestinal epithelial cells form a monolayer that acts as a physical barrier and separates the luminal microbes from the mucosal immune system. The dashed arrows illustrate the movement of the differentiated cells from the crypt (stem cells) up the villous (differentiated cells), except Paneth cells, which remain in the crypt. Paneth cells and Goblet cells secrete mucous and antimicrobial peptides to prevent microbes from interacting with the epithelial barrier. Modified from Peterson *et al* [14].

1.1.3 LARGE INTESTINES

The large intestine is also subdivided into various sections. Immediately following the ileum is the cecum. which is also connected to the appendix. Next are the ascending colon, transverse colon, descending colon, sigmoid colon, and ending with the rectum. The large bowel is much shorter than the small intestine with a wider lumen and its main functions are the reabsorption of water from indigestible material, nutrient absorption (much less than the small bowel), and the movement of waste for excretion in stool. The appendix is blindly connected to the cecum next to the ileocecal junction; the function of the appendix is still not completely understood. Another important difference between the small and large intestines is the mucus layer. In the small intestines, the mucus layer is a thinner single layer and has a rapid turnover rate. In the large intestine, the mucus layer is comprised of two layers with the outer layer sloughed off. The inner mucus layer is thicker with many antimicrobial properties such as Ig and antimicrobial peptides (AMP) making it impermeable to commensal bacteria [15]. Pathogens of the large intestine need to get through this mucus layer to infect the underlying epithelial cells. Bacterial density increases through the intestinal tract with the highest possible concentration found in the colon at 10^{11} colony forming units (CFU) per gram of stool [16].

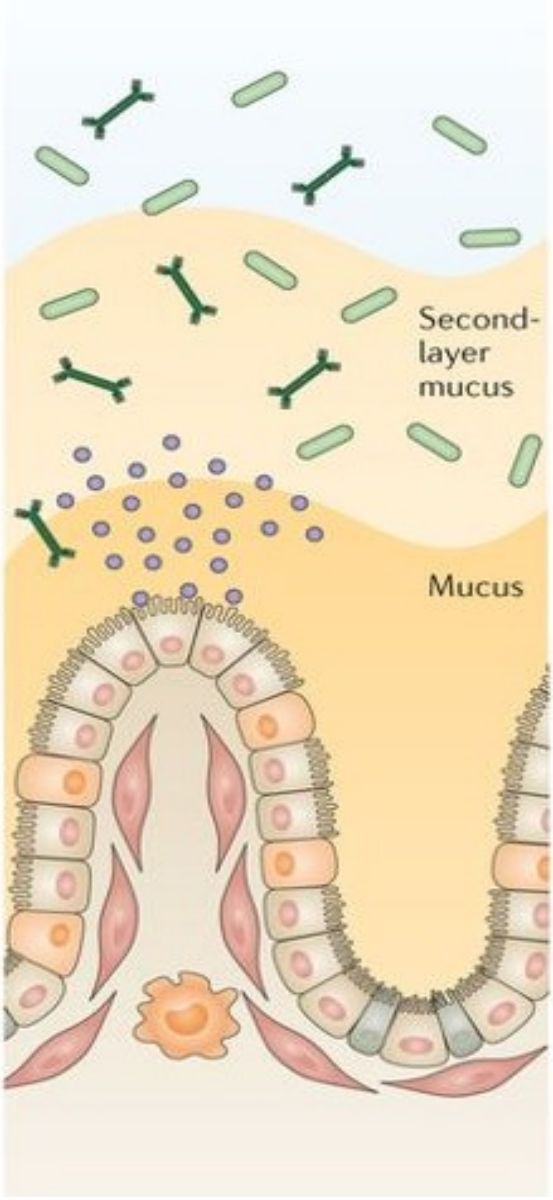

Fig 1.2. Schematic of the colonic mucosa. The differentiated epithelial cells of the large intestine are goblet cells and enterocytes. There are two distinct mucous layers within the colon with the second layer being sloughed off. Bacteria are associated with the outer mucous layer along with secretory IgA. The inner layer contains many antimicrobials. Modified from Peterson *et al* [14].

1.1.4 INFLAMMATORY BOWEL DISEASES

Inflammatory Bowel Diseases (IBD) are a group of chronic debilitating diseases for which the etiology is unknown and there is no cure. Once thought to be a Western/developed world disease, prevalence has recently been increasing in developing countries, including Asia. The prevalence of IBD is the highest in Canada and Northern Europe and may be plateauing, but there is a concerning rise in incidence in the pediatric population, especially in Canada [17]. The two main categories of IBD are Crohn disease (CD) and ulcerative colitis (UC), where patients with CD can have inflammation anywhere from the mouth to the anus whereas UC is restricted to the large intestine. CD is presented throughout the GI tract as a discontinuous, transmural inflammation whereas UC is a continuous ulceration with mucosal inflammation. Although the etiology is unknown, evidence suggests involvement of an inappropriate immune response to the normal intestinal microbiome, with predisposition due to host genetics, and a clear role for environmental factors [18].

Currently, there have been over 200 genes associated with susceptibility for IBD, most of which are involved in the host immune response and interaction with microbes, such as the first and still most potent susceptibility gene nucleotide-binding oligomerization domain-containing protein 2 (NOD2) [19, 20]. The inappropriate immune response in CD is associated with a T helper (Th) 1 and Th17 cytokine profile including an increase in interleukin (IL) 1β, IL-12 and tumour necrosis factor (TNF) α and a decrease in IL-10 [21]. In contrast, UC is characterized as a Th2 and Th17 mediated response with increases in IL-4, IL-5, IL-13 and IL-23 [21] Disruption

of the epithelial barrier leads to luminal antigen infiltration and subsequent inflammatory response; however, it is unknown whether this is a cause or effect of IBD [22]. Treatments for IBD depends on which subset of IBD the patient has, the severity of the disease, and the chance of success. Immune modulation is the most common treatment and if this fails then usually the only option is surgery to remove the inflamed sections. This is particularly important for UC as there is an increased risk of colon cancer with these patients if inflammation is not well controlled [23].

As the etiology is unknown there are multiple models used to study both the onset and progression of IBD. Mouse models of IBD can either be infectious, chemically-induced, immune-mediated, or various knockouts to the susceptibility genes. One of the more commonly used infectious model utilizes the Gram-negative murine specific pathogen *Citrobacter rodentium,* which induces IBD-like colitis; however, in contrast to IBD, once the infection has been cleared, the animal no longer shows symptoms [24]. Non-infectious murine models utilize various chemicals to disrupt the barrier and stimulate an immune response, such as dextran sodium sulfate (DSS), trinitrobenzene sulfonic acid (TNBS), acetic acid, or oxalazone [25]. There are numerous knockout models available to study IBD including NOD*2* and *IL-10*. IL-$10^{-/-}$ is interesting in that these mice develop colitis spontaneously whereas most knockouts still require either chemical or infection to initiate the disease, illustrating the important role IL-10 has as an anti-inflammatory cytokine [26]. As the microbiome has gained interest over recent years, gnotobiotic or germ-free mice have been used to identify potential microbes involved in the pathogenesis of IBD. This is difficult as most microbes in the GI tract are unculturable in a laboratory setting due to their low

oxygen tolerance or fastidious nature. In addition to choosing the model required for a research question, the strain of the mouse has also been observed to determine if a chemical or infectious treatment will work. For example, C57BL/6 and DBA/2 mice are resistant to TNBS and do not develop colitis; as well, some strains are more susceptible to *C. rodentium* infection [24].

1.2 INTESTINAL IMMUNE SYSTEM

1.2.1 GENERAL IMMUNOLOGY

Immunology is divided into two major categories being innate and adaptive immunity. Innate immunity is a genetically-encoded, rapid response to an antigen through mechanisms that exist prior to infection, whereas adaptive immunity is the recognition of an antigen through specific receptors/antibodies and exhibits memory for the same antigen [27]. Epithelial cells, macrophages, natural killer cells, dendritic cells, neutrophils, eosinophils, basophils, and mast cells are all part of the innate immunity whereas adaptive immunity mostly involves T and B cells. These two sides of immunity are not exclusive and require extensive communication and co-stimulation from other immune system factors to be effective [27]. Activation of the innate immune system leads to the production of the inflammatory mediators' cytokines, chemokines and signalling lipids, which can be either pro or anti-inflammatory.

Recognition of microbial antigens can be through receptors such as Toll-like receptors (TLR's) and nucleotide-binding oligomerization domains (NOD). NOD1 and NOD2 are innate immune sensors that recognize microbial factors within the cytosol. The most well known microbial associated molecular pattern (MAMP) that NOD receptors recognize are fragments of peptidoglycan produced by both Gram-positive and Gram-negative bacteria. Recognition of a MAMP by NOD2 induces oligomerization and recruitment of the RIPK2 serine/threonine kinase [28]. This leads to activate of the IκB-Kinase and MAPK pathways, which induce gene regulation and production of innate immunity products such as cytokines and chemokines. TLR's comprise a family of 10 receptors in humans and 12 in mice localized to the cell surface or intracellular compartments that are responsible for recognizing a wide variety of MAMP's. TLR4 is localized to the surface of the cell membrane and is the most relevant to this thesis as it recognizes lipopolysaccharides of Gram-negative bacteria. Activation of TLR4 recruits the TIR domain adaptor MYD88, which then activates the same pathways as NOD2 (IκB-kinase and MAPK) [29].

Cytokines and chemokines are proteins synthesized by immune cells that serve as immune modulators for various functions such as endothelial adhesion, proliferation, and maturation of additional immune cells. Cytokines act through receptors and can shape the adaptive immune system through activation of T- helper (Th) cells; examples are IL-12 and IL-4, which activate Th1 and Th2 responses, respectively. With respect to IBD, CD is considered a mostly Th1 mediated response associated with macrophages, whereas UC is a Th2 response associated with natural killer cells; however, this distinction is certainly simplistic and more recent research

highlights the complexity of immune dysregulation in IBD [30]. Chemokines are like cytokines but are usually a smaller polypeptide and their main function is chemotaxis (recruitment of immune cells, following a concentration gradient); however, other molecules that are not defined as chemokines can also have chemotactic activity.

This thesis focuses on macrophages and epithelial cells, therefore, most of the discussion will be on innate immunity. However, it is important to recognize that macrophages and their interactions with pathogens will directly affect the adaptive system. Mucosal immunity is an example of the direct interaction of innate and adaptive immunology without the presence of infection. For example, dendritic cells and resident macrophages will recognize both commensal and pathogenic bacteria in the lumen; antigens will then be presented to B cells which secrete IgA in response. Secretory IgA is then actively transported across the epithelial barrier into the mucus layer where it can opsonize other bacteria for engulfment. Other important aspects of mucosal immunology are AMP secreted by the GI epithelial cells as part of the innate immune system. These are oligopeptides that target a broad spectrum of microbes which usually target the bacterial membrane or interfere with DNA or protein synthesis [31].

1.2.2 INFLAMMASOMES

Inflammasomes are a group of multimeric protein complexes that respond to various stimuli including danger and microbial molecules. There are two families of proteins classified as inflammasomes: nod-like receptors (NLR) and AIM2-like receptors (ALR) [32]. Canonical NLR's consist of 3 important domains - a leucine-rich repeat, NACHT domain, and pyrin domain (PYD). When activated, the inflammasome complex recruits the adapter protein apoptosis-associated speck-like protein consisting of a CARD domain (ASC), which recruits caspase 1 (**Fig 1.3**) [33].

Inflammasome activation is diverse and can be initiated by many stimuli. For example, recognition of lipopolysaccharide (LPS) by TLR4 from Gram-negative bacteria initiates a signalling cascade, leading to nuclear factor kappa B (NFκB) activation. NFκB is a transcription factor that initiates the transcription and translation of the inflammasome proteins, pro IL-18, and pro IL-1β (Signal 1). Oligomerization of the NLRP3 inflammasome, upon activation, leads to the assembly of the adapter ASC and the recruitment of pro caspase 1, which undergoes autocleavage where it can lead to the maturation of IL-18 and IL-1β (Signal 2) (**Fig 1.4**) [34]. IL-1β can interact with the IL-1 receptor to initiate a positive feedback loop by acting as signal 1; this feedback loop is tightly controlled by IL-1 receptor 2, which acts as a decoy receptor as well as the released IL-1β receptor antagonist that binds to IL-1β outside of the cell.

There has been some recent evidence suggesting that inflammasome activation can also lead to a form of programmed cell death called pyroptosis through activation

of gasdermin D; however, the exact mechanism of inflammasome activation has not been fully investigated. Pyroptosis is characterized by cell swelling, membrane disruption, and eventual release of the cytoplasm caused by the pore formation of gasdermin D [35]. Gasdermin D activation requires caspase 1, 4, or 5 in humans and 1 and 11 in mice. The mechanism by which gasdermin D forms the pore is largely unknown; however, there is some evidence suggesting that it binds to phosphoinositide PI(4,5)P2 in the inner leaflet of the cell membrane [35].

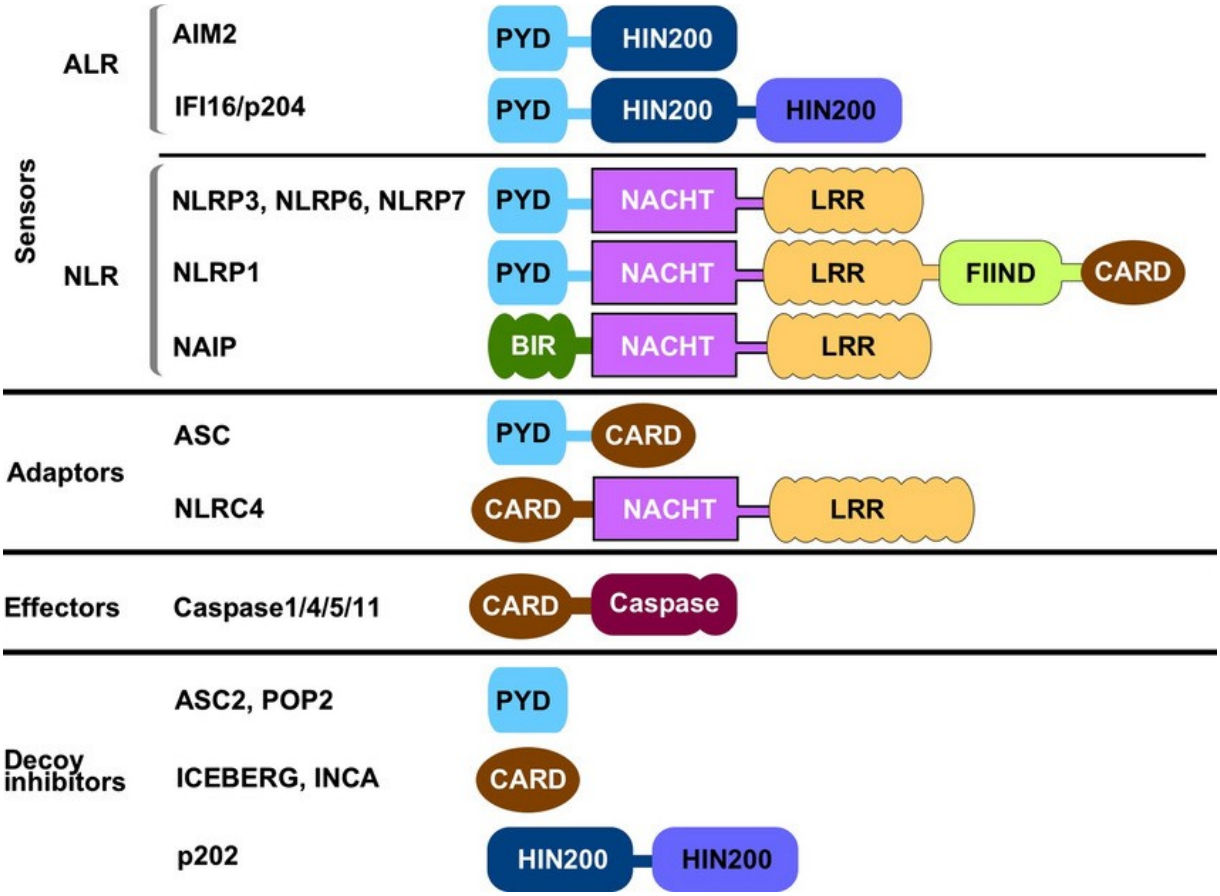

Fig 1.3. Inflammasome domains and structure. There are two distinct sensors that form inflammasomes, nod-like receptors (NLR) and AIM2-like receptors (ALR). These will recruit their respective adaptors, which activate the effectors [33].

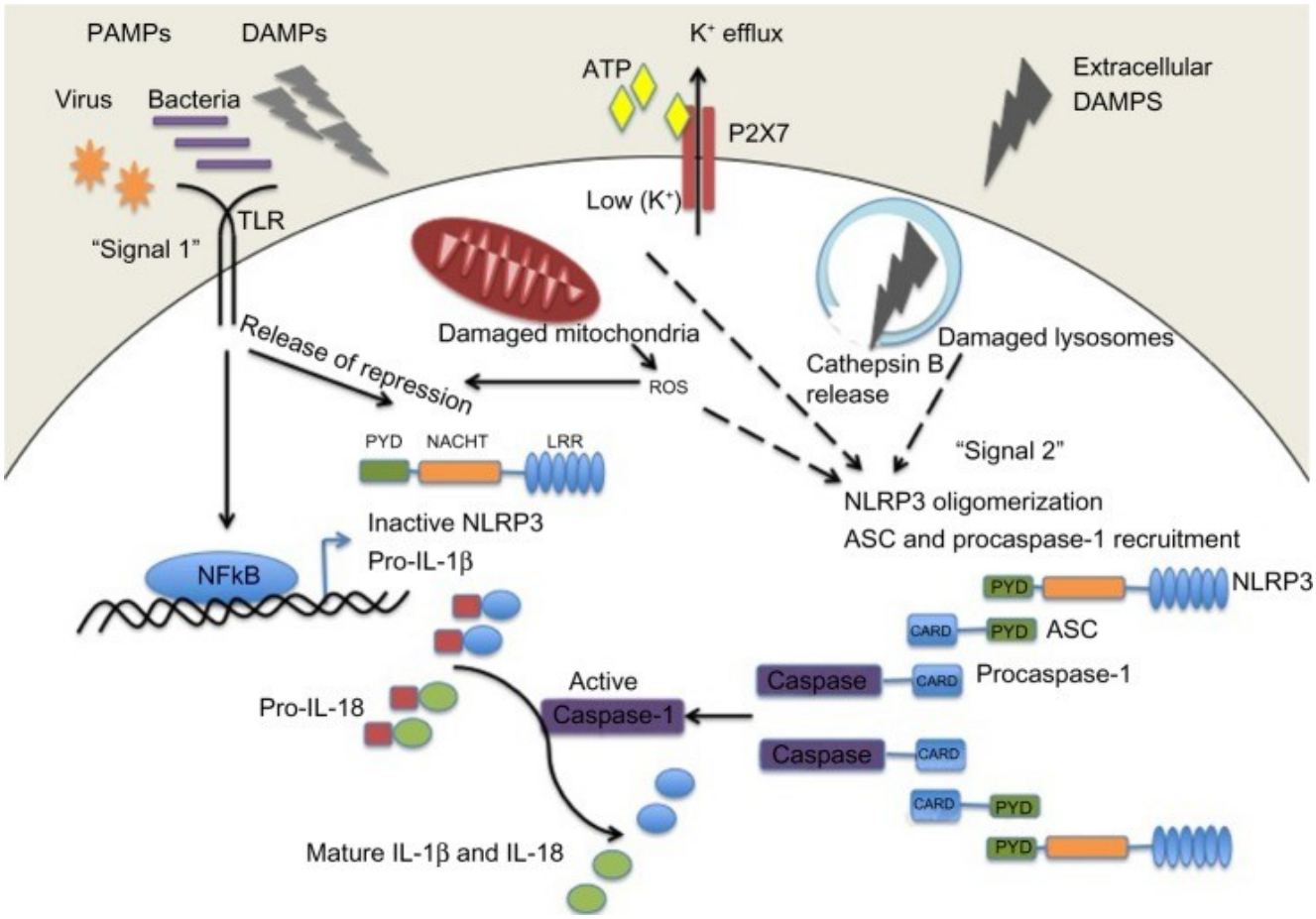

Fig 1.4. Inflammasome Activation Pathways. Activation of 'signal 1' by bacterial or viral PAMPS, or by danger signals can prime the inflammasome through NFκB signalling and expression of inactive NLRP3, pro IL-1β, and pro IL-18. A second signal through extracellular DAMP's such as ATP, damaged lysosomes, or damaged mitochondria will initiate 'signal 2' to activate the inflammasome complex. This activation leads to the cleavage of caspase 1 into its active form, which will then cleave pro IL-18 and pro IL-1β into their mature forms [34].

The PYD domain is found at the N-termini of proteins, amino acid residues 1-95 in human and mouse, involved in apoptosis and inflammation mediating the protein-protein interactions between NLRP3 and ASC [36]. The crystal structure of the NLRP3 PYD domain revealed that it shares a conserved hydrophobic core with other PYD involved in the death pathway [37]. This protein contains 6 helices, which form an anti-parallel bundle fold placing the N and C-termini on the same side of the molecule [37]. Analysis of the crystal structure revealed that the surface of the NLRP3 PYD domain contains conserved hydrophobic regions, which may mediate the interaction with the PYD domain of ASC [37].

NACHT is a family of nucleotide phosphatases found in animal, fungal, and bacterial proteins [38]. These proteins contain conserved regions corresponding to ATP/GTPase activity, Mg^{2+} binding site, various amino acid residues not seen in other NTPase proteins, as well as 4 other motifs [38]. Interestingly, this family of proteins can have either pro-apoptotic or anti-apoptotic properties depending on its NTPase activity [38]. The NLRP3 NACHT protein consists of three helical domains, an ATP binding site and hydrolysis site, with a NACHT-associated domain connected to the C- terminus; however, a crystal structure for NACHT has yet to be solved so most information is inferred from structure predictions and modeling [39].

LRR's are pattern recognition molecules consisting of between 20-30 amino acids with a conserved 11- amino acid core arranged as a β-helix used as a binding scaffold [39]. Their function in NLR's is largely unknown but data suggests they may be involved in signal sensing or activation [39].

Caspases are cysteine-aspartic proteases, transcribed as pro-proteins that require cleavage of a 20 amino acid residue to be catalytically active [40]. Caspase 1 is involved in canonical inflammasome activation whereas caspase 4/5 (Human) and caspase 11 (Mouse), these are functional orthologs, are found in noncanonical activation. Caspases can be categorized based on their initiating or effecting protein-protein interactions. Caspase 1,4,5, and 11 are all part of the initiating family and bind to the CARD domain [40]. The activated caspase will bind to four amino acid residues in their respective substrates (pro IL-18 and pro IL-1β) and usually cleave an aspartic acid [40].

IL-18 and IL-1β are proinflammatory cytokines that require processing by a secondary signal for maturation and secretion. They are both part of the IL-1 family; however, they have different receptors and elicit different inflammatory pathways. The IL-18 receptor consists of two chains, one for ligand binding and the other a core receptor, with both required for signalling [41]. IL-18 signalling leads to the upregulation of other proinflammatory cytokines IFNγ, TNFα, and IL-8, with IFNγ being the main protective role in bacterial infections due to increased nitric oxide production in macrophages [42]. IL-18 does not always require inflammasome activation as Fas ligand stimulation can also stimulate maturation and secretion; in addition, several tissues such as the epithelial cells in the gastrointestinal tract constitutively express the IL-18 precursor [43].IL-18 binding protein is constitutively expressed and secreted with a high affinity to IL-18 but is not related to the IL-18 receptor; this protein is regulated at the gene level through negative feedback by IFNγ [43]. IL-1β is one of the most potent proinflammatory cytokines and plays a

significant role in preventing microbial infections, as indicated by patients on anti IL-1β medication being at risk of severe infections [44]. This protein is transcribed as a 31 kDa precursor with the active form being 17 kDa, with no signal peptide so the secretion is poorly understood. IL-1β is not present in the Golgi or the endoplasmic reticulum but rather is transcribed on free polyribosomes associated with the cytoskeleton [45]. There have been multiple hypotheses about how it is secreted, with most evidence suggesting it depends on the initial inflammasome stimuli. IL-1β can be found in the cytosol as well as localized within lysosomes or microvesicles, which can provide for release other than pyroptosis [45]. The IL-1 receptor is part of the TIR family as they have an intracellular TIR domain but differ from Toll-like receptors by having an extracellular IgG domain [46]. Activation of this receptor by IL-1β requires the addition of the IL-1 receptor accessory protein, which is recruited once IL-1 receptor binds to IL-1β; this binding then activates the signalling cascade that leads to NFκB activation. In addition, there is a decoy receptor called IL-1 receptor II, which can bind to IL-1β but lacks the TIR domain and thus the accessory protein does not bind. As IL-1β is increased in many inflammatory conditions, there have been a few anti IL-1β drugs synthesized. Anakinra is a recombinant form of the IL-1 receptor, which can bind to either IL-1β or IL-1α preventing activation of their receptor. Canakinumab is an IgG monoclonal antibody and Rilonacept is a fusion protein consisting of IgG and the extracellular portions of the IL-1 receptor [47].

1.2.3 NLRP3 INFLAMMASOME

The NLRP3 inflammasome is the most extensively studied inflammasome as it has implications in many diseases. This inflammasome can be activated by a plethora of stimuli including microbes, extracellular ATP, toxins such as Nigericin, uric acid crystals, glucose, hyaluronan, asbestos, and many more [48]. Most conditions associated with this complex are sterile inflammation coupled with a hyperproduction of IL18/1β, such as gout, metabolic syndrome, rheumatoid arthritis, and Familial Mediterranean Fever. IL-1β has been shown to be central to these diseases and many others as knockout mouse models of the inflammasome are protected against disease development [34]. Inflammasomes have been found in many cell types including macrophages neutrophils, dendritic cells, and epithelial cells. NLRP3 has also been linked to IBD; however, there is much debate as to whether it is protective or not. *NLRP3*$^{-/-}$ mice show increased bacterial adhesion and disease severity than wildtype mice and this can be reversed with the administration of exogenous IL-1β [49]. Patients with IBD have an elevated expression of NLRP3 and increased caspase 1 activity [50]. Recently though, it has been shown that IL-18 can be protective in IBD depending on whether it is in the epithelia (protective) or lamina propria (inflammatory) [34].

Adenosine triphosphate (ATP), a DAMP with low extracellular levels under homeostatic conditions, can be released by damaged cells, secreted by neutrophils, or directly from intestinal bacteria during infection or necrosis [51]. The extracellular ATP sensor purinergic receptor $P2X_7$ is a known activator of the NLRP3 inflammasome through reactive oxygen species (ROS) production and potassium

(K^+) efflux as well as an increase in intracellular calcium (Ca^{2+}), although the precise mechanism of activation is not known [52-54]. $P2X_7$ recruits pannexin 1, which forms a pore in the cell membrane, allowing for efflux of K^+, another activator of the NLRP3 inflammasome [55]. Inhibition of either the purinergic receptor or K^+ efflux prevents NLRP3 complex formation (Signal 2). YVAD is a short polypeptide that binds to procaspase-1 preventing its autocleavage. Glyburide prevents K^+ efflux downstream of the $P2X_7$ receptor but upstream of NLRP3 [56]. Recent research has shown the mitochondria may be involved in ATP-induced NLRP3 inflammasome activation. Data suggests extracellular ATP may affect inner membrane transporters, but the mitochondria needs to be functioning for caspase-1 activation and subsequent IL-1β processing [57]

Nigericin is a toxin derived from *Streptomyces hygroscopicus*. It is described as a potassium ionophore, which facilitates H^+/K^+ anti-port across cell membranes [58]. This transport of K^+ from the cytosol to the extracellular space has been shown to activate the NLRP3 inflammasome through signal 2. The mechanism of inflammasome activation through Nigericin is unknown.

1.2.4 NLRP6 INFLAMMASOME

NLRP6 shares the conserved domains with NLRP3: nucleotide-binding domain, LRR, and the N-terminal pyrin domain. This inflammasome is found in multiple haematopoietic cells including macrophages, dendritic cells, and neutrophils. It is also highly expressed throughout the gastrointestinal tract in epithelial cells and

plays a role in maintaining intestinal health [59]. NLRP6 has shown to regulate mucin production, in particular MUC2, from goblet cells possibly though autophagy [60]. MUC2 is the dominant mucin found in the inner mucus layer; depletion of this allows for microbes to contact with the epithelial barrier. Similar to NLRP3, NLRP6 also induces the secretion of IL-18 in response to microbes; IL-18 from NLRP6 has been shown to be required for secretion of AMP by increasing the levels of active IL-22 [60, 61]. Decreased AMP in the lumen can lead to dysbiosis of the microbiome as well as increased susceptibility to infection. Patients with IBD have decreased expression of NLRP6 and this is associated with decreased mucin production and possibly a link to dysbiosis [62].

1.2.5 MACROPHAGES

Macrophages are an integral part of innate immunity as phagocytes, antigen presenting cells, and directors of inflammation. These cells are grouped into two main categories, M1 and M2. M1 are classically described as being proinflammatory, mediating tissue damage, whereas M2 are anti-inflammatory and related to wound healing [63]. These cells are unique in that they can switch between these two forms effectively, from clearing an infection to helping repair the damaged tissue. However, depending on the situation, each of these classifications can be beneficial or harmful. In the case of bacterial infections, dominance of M1 will allow for clearance but if the dominant form is M2 then it is associated with bacterial persistence [63]. Regulation between these are determined by cytokines, such as interferon (IFN) γ and macrophage colony-stimulating factor (MCSF), secreted from other immune cells. In

addition to circulating monocytes that will differentiate into macrophages upon entry to tissue, there are resident macrophage present within the major organ systems: Kupffer cells are found in the liver, microglia in the brain, alveolar macrophages in the lung, kidney macrophages, heart macrophages, peritoneal macrophages, Langerhans cells in the skin, red pulp in the spleen, and intestinal macrophages [64]. Some of these tissue macrophages originate prenatally, are not from a monocytic origin, and are self renewing; macrophages in the GI are not of this origin; data suggests they originate from monocytes and have a limited lifespan [65]. Monocytes originate from the bone marrow during hematopoiesis into Ly6c cells that circulate in the blood; after receiving a signal they will migrate into the tissue and undergo differentiation into the tissue macrophage or an inflammatory state, depending on the signal. In the GI tract, regulatory T cells constitutively express IL-10, which induces differentiation into tissue macrophages and tolerance to commensal bacteria [65, 66]. In IBD there is an accumulation of proinflammatory infiltrating macrophages, which are responsible for secreting cytokines, expressing ROS, and leading to chronic inflammation [66].

1.2.6 REACTIVE OXYGEN SPECIES

Macrophage phagocytosis of microbes and their killing is mediated partly through respiratory burst. This is the conversion of molecular oxygen to oxygen free radicals by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) and subsequent dismutation to hydrogen peroxide by superoxide dismutase [67]. It is

still unclear as to how much ROS is present within the phagolysosome and how it kills the bacterium. NOX2 is the primary NADPH found in macrophages but is not the only means of defense; these cells also secrete defensins, AMP, reactive nitrogen species, lactoferrins, and proteases [68]. However, NOX2 is required as some individuals with chronic granulomatous disease have a non-functional NOX2, which results in uncontrolled infections. ROS are not only found in the phagosome but can also be generated by the mitochondrial electron transport chain, xanthine oxidase, peroxisomes, and the endoplasmic reticulum [68]. Mitochondrial ROS is produced under normal physiological conditions through complex I and III but is scavenged by mitochondrial superoxide dismutase and glutathione peroxidase, which produces water t [69, 70]. Other than immune function, ROS are also important for some protein modifications, which increases their susceptibility to proteolysis, and regulates gene expression, cell growth, and apoptosis [71]. However, if ROS is not balanced it can lead to serious consequences for the cell and cause some diseases. Increased ROS production has been associated with chronic obstructive pulmonary disease, IBD, DNA-damage leading to cancer, neurodegenerative disorders, and angiogenesis [71].

1.3 MICROBIOME AND ENTERIC INFECTIONS

1.3.1 MICROBIOME

The Human Microbiome Project was an extensive study aimed to identify the commensal microbes associated with the human host from the skin, mucosal surfaces (nasal, gastrointestinal tract, lungs), mouth, and urogenital tract [72]. Data from this project and others stated that microbes outnumbered human cells 10:1; research since then has suggested this number to be closer to 1:1 when red blood cells are considered, as previous calculations only included nucleated cells [16]. The human GI microbiome contains all domains of life (*Bacteria, Eukaryota,* and *Archaea*), which starts at birth from environmental sources; differences are seen in babies born naturally versus caesarian however this is not permanent [73]. The microbiome changes throughout our life, with early dominance of *Bifidobacterium* spp and adulthood dominated by *Bacteroides* and *Firmicutes* at the phylum level [74]. Throughout the GI tract, the microbial community changes depending on physiological differences including nutrient availability and composition, host immune activity, oxygen levels, and pH [74]. Studying the microbiome is difficult as the majority of gut microbes are fastidious obligate anaerobes making them unculturable in most laboratory settings; therefore, most of those microbes identified are through metagenomic studies. Immune tolerance to commensals is not fully understood due to this inability to culture, with most of our knowledge of bacterial adherence to epithelial cells coming from pathogens (usually aerobic). Commensal bacteria have several benefits to the host such as providing nutrients like vitamin K and short chain

fatty acids, as well as adherence to the mucus layer or epithelial cells providing a barrier from potential pathogens [74]. Drastic changes in the composition of the microbiome, as compared to healthy individuals (termed dysbiosis), have been associated with diseases such as IBD, celiac disease and diabetes. Dysbiosis in IBD includes a decrease in overall bacterial diversity, loss of *Clostridia* and *Bacteroides* with an increase in *Enterobacteriaceae* [62, 74, 75]. Diet appears to have the largest influence over the microbiome composition relating to diversity and richness [76]. *Faecalibacterium prausnitzii* belongs in the Firmicutes phylum and has been found through meta analyses to be one of the most abundant microbes in the human intestines [77]. This bacterium has been shown to both provide energy to intestinal epithelial cells through production of short chain fatty acids and induces anti-inflammatory effects [78]. In IBD, particularly UC, there is a significant decrease in abundance of *F. prausnitzii* that may partially explain immune dysregulation and loss of epithelial integrity [78].

Fusobacterium nucleatum is another bacterium with an association to IBD, this one being negative. This bacterium is normally found in the oral cavity with known roles in periodontitis and prenatal issues such as preterm birth with low colonization rate of the gastrointestinal tract; however, evidence has shown it has the capability of thriving within the colorectal tumour environment that UC patients are at risk of developing [79]. Although *F. nucleatum* may be found within cancerous tissue, there is not enough evidence to determine whether it is causing tumour formation or if the environment allows it to outcompete other microbes [79]. Although the NIH-funded Human microbiome project has ended, as of 2017, there are still many unanswered

questions. It is unclear what the normal healthy microbiome is, as it appears to be based on the individual to some degree, and how changes can influence disease. In addition, it is still unclear how to treat individuals with diseases such as IBD where we do not know if the microbiome changes are a cause or effect of the disease.

1.3.2 CITROBACTER RODENTIUM PATHOGENESIS

C. rodentium is a Gram-negative bacterium and murine pathogen that colonizes the mouse colon through attachment to epithelial cells via an adhesion/effacing (A/E) lesion. This bacterium belongs to the Enterobacteriaceae family with similar genetic material and pathogenicity to Enterohaemorrhagic *Escherichia coli* (EHEC) and Enteropathogenic *E. coli* (EPEC) including the locus of enterocyte effacement (LEE) pathogenicity island, responsible for A/E lesions [80]. This island encodes a type 3 secretion system (T3SS) and several effector proteins, including the translocated intimin receptor (Tir) [80]. Tir is injected into host cells where it integrates into the host cell membrane allowing for interaction with intimin; this then triggers actin polymerization leading to increased tight junction permeability and pedestal formation [80]. *C. rodentium* also expresses a T6SS that suggests it may use this to outcompete commensals and other pathogens to allow colonization [80]. There have been a few other potential virulence factors identified such as pili, fimbriae, and adhesion molecules but their function is still unknown [80].

Infection by *C. rodentium* is self limiting with the clearance being 2-3 weeks; however, those bacteria being shed are more infectious than non host-adapted, with a higher expression level of the T3SS genes [80]. *C. rodentium* induces changes in the microbiome of the infected mouse with decreases in *Bacteroides* and an increase in *Firmicutes* [80]. Colitis in these mice simulates IBD and is the most commonly used infection model to study this disease, but it is only a model and does not represent the disease entirely. Clearance of this pathogen requires robust innate and immune defense. Upon attachment, epithelial cells undergo a stress response that causes secretion of defensins, AMP, and ROS, as well as cytokines and chemokines [81]. Antigen presenting cells such as recruited monocytes/macrophages activate their NLRP3 inflammasome through TLR recognition and secrete IL-1β and IL-18, which we and others have shown to be important for clearance [49, 82]. Some other cytokines are important for protection in this infection, including IL-22 and IL-23. IL-22 is part of the IL-10 family which promotes mucosal healing by enhancing the expression of gap junction proteins [81]. As this is a self-limiting disease, it is often referred to as IBD-like colitis. However, it provides a model for understanding both the immune system and the microbiome during infections. IBD is not considered an infectious disease but there is evidence suggesting a role for pathobionts, which may share pathogenicity with *C. rodentium*.

Previous work in the Wine lab infected wildtype and *Nlrp3*$^{-/-}$ mice with *C. rodentium.* We then injected exogenous recombinant IL-1β into these mice and observed the colonization and clearance of the pathogen. *Nlrp3*$^{-/-}$ mice showed higher colonization rates, with increased dissemination to other organs, cytokine and

macrophage levels were increased, and a loss of epithelial integrity; all of this was reversed when given exogenous IL-1β. Interestingly, disease severity increased in wildtype mice given exogenous IL-1β illustrating the importance for immune balance to prevent detrimental effects from inflammation. In addition, isolated peritoneal macrophages from *Nlrp3*$^{-/-}$ showed increased phagocytosis of *C. rodentium* when given IL-1β [49]. This research shows the macrophage inflammasome to be instrumental in clearing infection and regulating immune balance, which is important for individuals with IBD.

1.4 HYPOTHESIS AND AIMS

The link between the inflammasome and IBD has been controversial as many models show IL-1β to be increased and thus contributing to the disease. However, it has been shown that expression of NLRP3 and IL-1β are increased but secretion is decreased. *C. rodentium* infection is increased in *NLRP3* knockout mice but when IL-1β is injected, the infection is cleared. Interestingly, wildtype mice who are given exogenous IL-1β have a worse infection, suggesting that the benefits and detriments of the inflammasome require a delicate balance.

The NLRP3 inflammasome can be activated by a plethora of stimuli and it is associated with many diseases, however the mechanism of its activation is not fully understood. Extracellular ATP has already been shown to aid in the clearance of *E. coli* and *Staphylococcus aureus* during peritonitis but not for enteric infections. In addition, the role of macrophages in this clearance is not well characterized as other immune cells (such as neutrophils) are also activated. Furthermore, the role of the

NLRP3 inflammasome in epithelial cells is completely unknown. The inflammasome is a microbial sensor found in many cells and thus the intestinal microbiome must be able to influence it, which may be critical for immune tolerance and is dysregulated in IBD.

The objective of this thesis was to determine if manipulation of the inflammasome could improve macrophage ability to clear an infection, and thus actually improve potential outcomes in IBD models, and to define involved mechanisms. I started using the RAW 264.7 mouse macrophage cell line and nigericin as the inflammasome activator; however, I then observed that this cell line does not express the ASC protein and, therefore, the NLRP3 complex cannot form. At this point, I changed to the J774A.1 mouse macrophage cell line and used ATP as the activator as this is more physiologically relevant.

My **hypothesis** is that inflammasome activation is required for macrophages to phagocytose and kill *Citrobacter rodentium* and that this will benefit the host by reducing immune activation, in relation to IBD

My specific aims are:

1. To determine whether addition of the inflammasome activators ATP and Nigericin affects macrophage engulfment and clearance of *C. rodentium.*

2. To define the mechanism by which ATP-induced inflammasome activation increases ROS production during bacterial infection in macrophages.

3. To characterize the inflammasome-activated macrophage, *C. rodentium* interplay in the presence of epithelial cells.

Chapter 2

Alternative Activation of the NLRP3 Inflammasome in Macrophages Increases Bacterial Killing

CHAPTER 2. ALTERNATIVE ACTIVATION OF THE NLRP3 INFLAMMASOME IN MACROPHAGES INCREASES BACTERIAL KILLING

Submitted to Frontiers Immunology June 2018 (under review)

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2.1 ABSTRACT

Background: Altered microbiota has been associated with a number of diseases including inflammatory bowel diseases, diabetes, and cancer. This dysregulation is thought to relate the host inflammatory response to enteric pathogens. Macrophages play a key role in host response to microbes and are involved in bacterial killing and clearance. This process is partially mediated through the potassium efflux-dependent, cytosolic, PYCARD-containing inflammasome protein complex. Surprisingly, we discovered an alternative mechanism for bacterial killing, independent of the inflammasome/PYCARD.

Methods: Using the inflammasome-deficient Raw264.7 macrophages, which lack PYCARD, we found that the potassium efflux activator nigericin improves bacterial phagocytosis and killing. Macrophage response to nigericin was examined by gene profiling and subsequent qPCR, which demonstrated altered expression of a series of genes involved in the caspase-1/IL-18 bacterial killing pathway.

Results: Based on our results we propose a model of bacterial killing unrelated to inflammasome activation in macrophages.

Conclusions: Improving our understanding of the molecular pathways driving bacterial clearance within macrophage cells will aid in the development of improved immune-targeted therapeutics in a number of diseases.

2.2 INTRODUCTION

Dysbiosis, or altered microbial composition and function, is characteristic of a number of diseases including inflammatory bowel diseases (IBD), obesity, diabetes, autism, and cancer [83], likely mediating a dysregulation of the host immune response [62, 84, 85]. Detailed analysis of the intestinal inflammatory response to enteric pathogens may be key to understanding disruption of homeostasis and disease progression and discovering novel therapies.

Interestingly, bacterial DNA can stimulate activation of host innate immune cells through recognition of pattern-recognition molecules (PRMs) by microbe-associated molecular patterns (MAMPs) [86-88]. PRMs are located within innate immune cells such as dendritic cells, macrophages, and neutrophils, along with epithelial cells. Examples of transmembrane PRMs are toll-like receptors (TLRs) and C-type lectins (CLRs); cytosolic PRMs include members of the NOD (Nucleotide Oligomerization Domain)-like receptor (NLR) family and DNA sensing DAI and AIM2 complexes [89]. Macrophages are especially pivotal for a functional immune response as they are involved in bacterial killing and clearance by engulfing and

eradicating invading pathogens [90]. We have previously demonstrated that bacterial clearance by macrophages can be improved through the stimulation of the multiprotein complex known as the inflammasome [82]. Better bacterial clearance was associated with a reduced pro-inflammatory response, suggesting a potential mechanism for the link between increased susceptibility to IBD and inflammasome dysfunction [82, 91].

A number of inflammasomes have been identified, including NLRP1, NLRP2, NLRP3, and NLRC4 [34]. The best characterized inflammasome is NLRP3, in which NLRP3 forms a complex with PYCARD [also known as the adapter protein apoptosis-associated speck-like protein (ASC)] and procaspase-1 [92]. Assembly of this complex results in self-cleavage of procaspase-1 to active caspase-1, leading to the activation of the proinflammatory cytokines interleukin (IL)-1β and IL-18 [89, 93-95]. NLRP3 activation involves a two-step process including a priming signal, which includes recognition of MAMPs (*e.g.*, bacteria or lipopolysaccharide) by cell surface TLRs, followed by a second step involving NLRP3 complex assembly and activation [34, 96-99]. Activation of the inflammasome is driven by agonists such as adenosine triphosphate (ATP), which indirectly interact with NLRP3 via stimulation of $P2X_7$ nonselective K^+ or Ca^{2+} channel receptors [89].

While inflammasome modulators such as Nigericin are described as NLRP3-specific [100], their indirect effects led us to hypothesize that they may be able to regulate inflammation and bacterial killing via inflammasome-independent mechanisms. Nigericin is a toxin derived from *Streptomyces hygroscopicus*. It is described as a potassium ionophore, which facilitates H^+/K^+ anti-port across cell

membranes, thereby activating NLRP3 by causing potassium efflux. In this *in vitro* study, we examined the effects of nigericin, an NLRP3 activator, on Raw 264.7 macrophages, which lack PYCARD/ASC and therefore are incapable of NLRP3 activation. As Raw 264.7 cells are a murine cell culture model, we implemented the mouse pathogen *Citrobacter rodentium* for our *in vitro* experiments. *C. rodentium* shares many virulence factors such as formation of attaching/effacing (A/E) lesions with the commonly studied human intestinal *Escherichia coli* strains Enterohaemorrhagic *E. coli* (EHEC) and Enteropathogenic *E. coli* (EPEC), resulting in transmissible colonic hyperplasia, colitis, and bloody diarrhea [101, 102]. Our results demonstrate that the NLRP3 agonist, nigericin, promotes killing of *C. rodentium* and induction of an inflammatory response through pathways unassociated with the NLRP3 inflammasome complex, independent of ASC. Understanding this previously unrecognized mechanism of bacterial clearance within a macrophage cell system will aid in our ability to identify new methods of altering the immune response in a number of human diseases driven by altered host-microbe interactions, such as IBD.

2.3 MATERIALS AND METHODS

Cell culture

Raw 264.7 cells were obtained from the American Type Culture Collection (ATCC; Maryland, United States) and cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 5% penicillin/streptomycin and incubated (37℃, 5% CO_2) until confluent for a maximum of 20 passages.

J774A.1 cells were obtained from the ATCC and cultured in DMEM containing 10% FBS for a maximum of 25 passages.

C. rodentium (strain DBS100) was provided as a gift by Dr. Philip Sherman (University of Toronto) and cultured aerobically in lysogeny broth (LB) at 37℃.

Standard Bacterial Growth Curves

Raw 264.7 cells were seeded at $1.5x10^5$ cells per well in a 24 well plate and cultured overnight at 37℃ and 5% CO_2. Cells were treated as indicated below with ATP (Sigma), Ac-Tyr-Val-Ala-Asp-Chloromethylketone (YVAD; Sigma), nigericin (Sigma), or Gentamicin (Fischer Scientific) to determine associated toxicity or growth inhibition. Cells were manually counted using a haemocytometer and trypan blue exclusion technique to determine cell number at the indicated time points.

C. rodentium liquid LB cultures were seeded and grown overnight at 37℃. Growth density was measured at OD_{600} nm on a spectrophotometer and serial dilutions were cultured on LB agar overnight. Colony counts were performed on each plate and used to calculate colony forming units (CFU)/mL from each dilution.

C. rodentium liquid LB cultures treated with nigericin (20 μM) were seeded and grown overnight at 37℃. Cells were diluted to an OD_{600} nm of 0.1 (representing $8x10^7$ CFUs/mL) and treated with nigericin at time 0h. Growth density was measured at OD_{600} nm on a spectrophotometer at indicated time points and compared to untreated growth.

Gentamicin Protection Assay

Raw 264.7 cells were seeded into a 24-well plate overnight at a density of $2x10^5$ cells per well. Medium was removed and replaced with serum-free DMEM prior to beginning treatment. Cells were then treated with either YVAD (10 μM) and/or nigericin (20 μM), as detailed in **Fig 1A**. Treatment with YVAD occurred 1h prior to *C. rodentium* inoculation and proceeded for 3h total. Raw264.7 macrophages were inoculated with *C. rodentium* (MOI 1:10) 1h after YVAD (when appropriate) for 2h total. Nigericin treatment or ATP control (2.5 mM) was added 1.5h post-infection and proceeded for 30min total. Both non-infected and infected, no treatment (NT) controls were included. Medium was removed, and 20 μg/mL of gentamicin was added to each well for 3h to kill any bacteria not engulfed by the macrophages (gentamicin does not penetrate the eukaryotic cell membrane). Raw 264.7 cells were lysed with 1% TritonX-100 for 30 minutes to enumerate intracellular/engulfed bacteria. Lysates were plated on LB agar in serial dilutions and colonies were manually counted the following day to calculate intracellular CFU/mL.

ELISA

Raw 264.7 cells were seeded into a 24-well plate overnight at a seeding density of $2x10^5$ cells per well. Medium was removed and replaced with serum-free DMEM prior to beginning treatment. J774A.1 cells were included as a positive active-NLRP3 control. Cells were then treated with YVAD (10 μM) 1h prior to *C. rodentium* inoculation and proceeded for 3h total, as above. Raw 264.7 macrophages were inoculated with *C. rodentium* (MOI 1:10) for 2h total. KCl (45 mM) was added at the time of infection. Nigericin (20 μM) treatment or ATP control (2.5 mM) was added 1.5h post-infection and proceeded for 30min total. Both non-infected and no treatment (NT) controls were included. Supernatants were collected and centrifuged at 14000g for 10 min to remove any bacteria, cells, or debris. Protease inhibitor (1:100; Sigma Aldrich) was added to the supernatant and secreted IL-1β was measured using an ELISA following manufacturers protocol (R&D Systems).

Phagocytosis assay

Raw 264.7 cells were prepared as described for the gentamicin protection assay. In order to dissociate phagocytosis from bacterial invasion of Raw 264.7 cells, we used inert beads to measure effects on phagocytosis alone; 1:10 (Raw 264.7 cells: beads) of 2 μm conjugated beads (Polysciences Inc.) were added to the culture for 2h. Cells were treated as previously described and fixed using 4% paraformaldehyde, then stained with β-actin primary antibody (1:40; Abcam) and DAPI (1:1000; Thermo Fischer). Slides were examined using an Olympus IX-81 microscope with a

Yokagawa spinning disk confocal head, 60X oil immersion lens with a 1.42 numerical aperture, and a Hamamatsu EMCCD camera. Images were taken with equal exposure time without saturation and analyzed with Volocity imaging software (PerkinElmer). Illustrations were formatted, for noise reduction and increased sharpness, using Image J (National Institute of Health, Maryland, USA). Phagocytosis was measured by the number of beads inside the macrophages divided by the total number of macrophages in each randomly selected image. 10 photos of each treatment were taken from duplicate repeats.

RNA isolation and gene expression analysis

Raw 264.7 cells were cultured overnight prior to treatment as indicated. RNA was isolated using 1mL trizol as previously described [103]. Inflammasome gene array (QIAGEN) was run according to manufacturer's instructions. RT-qPCR was performed as previously described [103] to validate findings using the primers highlighted in **Table 1.3.1**. Both biological and technical replicates were performed on all reactions using β-Actin and Rer1 as housekeeping genes. Data were analyzed using CFX Manager Software Version 3.0 (Bio-Rad Laboratories, Inc.). Statistical significance was evaluated by the Student's unpaired *t*-test with Welch's correction using GraphPad Prism 4.0 (GraphPad Software, La Jolla, USA).

Table 2.1 Primer Sequences used in qPCR of RAW 264.7 murine macrophages

PRIMER	Forward	Reverse
IFN-γ	TTCTTCAGCAACAGCAAGGC	CCTTTTCCTCAGCGACGACT
IL-12	GATGACATGGTGAAGACGGC	AACTACTACTGGGACACGGA
Ciita	TGCAGGCGACCAGGAGAGACA	GAAGCTGGGCACCTCAAAGAT
Mapk11	CAGAAGGACCTCAGCAGTGTCT	GTACTGGCTGAAGTATGCGTGG
Cd40lg	GAACTGTGAGGAGATGAGAAGGC	TGGCTTCGCTTACAACGTGTGC
tab2	CATTCAGCATCTCACAGACCCG	CTTTGAAGCCGTTCCATCCTGG
Nlrp4e	CTCTGTCCAAGGCTTTGTGCCA	TGGGTCAAGGTTTTGTTCCGCC
Tnfsf11	GTGAAGACACACTACCTGACTCC	GCCACATCCAACCATGAGCCTT
Nlrp12	GGAAGAGACAGCAGACTCGAGAATCTTTTTCATC	GATGAAAAAGATTCTCCTCTCTGCTGTCTCTTCC
NLRP3	TGCTCTTCACTGCTATCAAGCCCT	ACAAGCCTTTGCTCCAGACCCTAT
IL-10	ATAACTGCACCCACTTCCCA	GGGCATCACTTCTACCAGGT
IL-1β	TGGAAAAGCGGTTTGTCT	ATAAATAGGTAAGTGGTTGCC
TNFα	ATGAGCACAGAAAGCATGA	AGTAGACAGAAGAGCGTGGT
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Rer1	GCCTTGGGAATTTACCACCT	CTTCGAATGAAGGGACGAAA

Statistical Analysis

Groups were compared using paired Student's *t*-test (two-tailed) analysis in Microsoft Excel. A P value of < 0.05 was considered as significant in all cases.

2.4 RESULTS

Optimization of bacterial growth and cell toxicity

Response of Raw 264.7 cells to select non-toxic doses of ATP, YVAD, nigericin, and gentamicin was examined by culture growth curves to select ideal doses for experiments (**Fig 2.1**). Raw 264.7 cells were seeded at $1x10^5$ cell per well in 24 well plates and grown overnight before beginning treatment at indicated doses. Effects of each treatment on cell proliferation and death were calculated by manual counting at indicated time points using a haemocytometer (expressed as number of cells/well) and trypan blue exclusion (shown as % of cell death). Doses with minimal effect on cell growth or survival (similar to dose zero) were selected for further study including 2.5 mM ATP, 10 μM YVAD, 20 μM nigericin, and 20 μM gentamicin. There are several limitations to the use of Trypan blue exclusion such as the lack of specificity between apoptosis or necrosis and the ability of Trypan blue to penetrate live cells.

Growth rates of *C. rodentium* in LB medium in overnight culture was evaluated by standard curve based on OD_{600} readings performed at 24 h post inoculation, compared to manual colony counts performed on serial dilutions of culture grown on

LB plates (**Fig 2.2A**). A trend line was fitted to the scatter plot and followed the formula [y = 2.7537x + 0.374], which was then used to determine the concentration of *C. rodentium* within culture, based on the solution optical density.

Given the potential toxicity of high doses of Gentamicin to Raw 264.7 cells, we optimized treatment duration. To examine the effects of gentamicin on microbial killing of *C. rodentium* cultured with Raw 264.7 cells, overnight cultures were prepared and infected as indicated in the methods section. To ensure the selected dose of gentamicin (25 µg/mL) resulted in effective microbial killing of extracellular *C. rodentium,* we plated culture supernatant on LB agar prior to lysing cells (data not shown). No growth of *C. rodentium* was found on LB agar following 24 h incubation. Cells were treated with gentamicin and lysed at 2, 4, 6, and 24 h and culture lysates were plated on LB agar. Following overnight growth, colonies were manually counted and expressed as CFU/mL (**Fig. 2.2B**). Results demonstrated that CFU/mL decreased by approximately 75% between 2 h and 4 h. While there was no significant change in CFU/mL between 4 h and 6 h, a further reduction in CFU/mL occurred between 6 h and 24 h. Subsequent experiments utilised gentamicin for 2 h and/or 4 h to ensure that only *C. rodentium* cultures taken up by macrophages and not extracellular microbes were being examined.

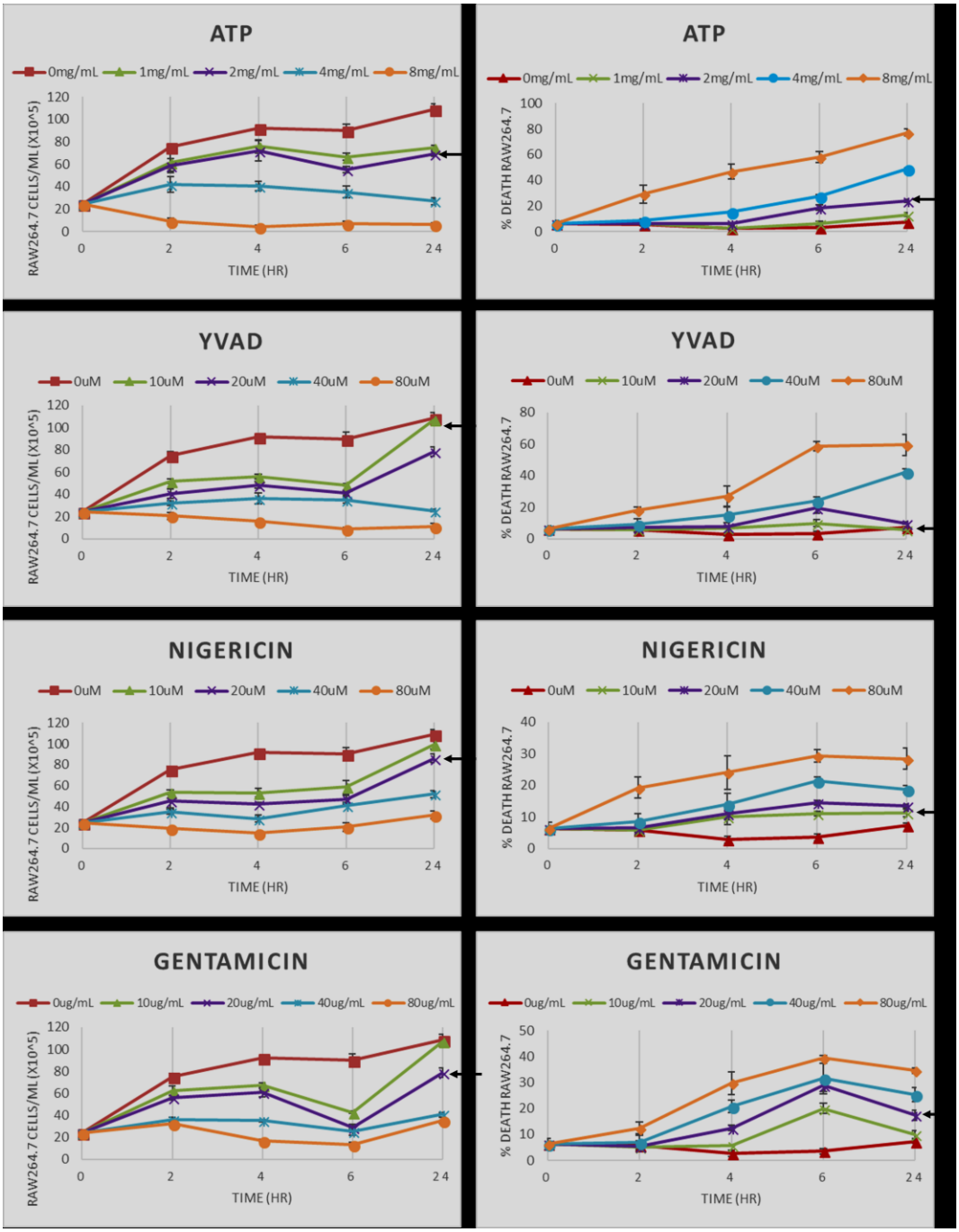


Fig 2.1. Growth response of Raw264.7 cells to ATP, YVAD, nigericin, and gentamicin. Manual counting using haemocytometer at indicated times using trypan blue exclusion at various concentrations. Cell growth (cell/mL) was interpreted by examining live cell counts only at each time point; % death represents the percentage of dead cells counted by trypan blue exclusion as a percentage of total cells. N=3.

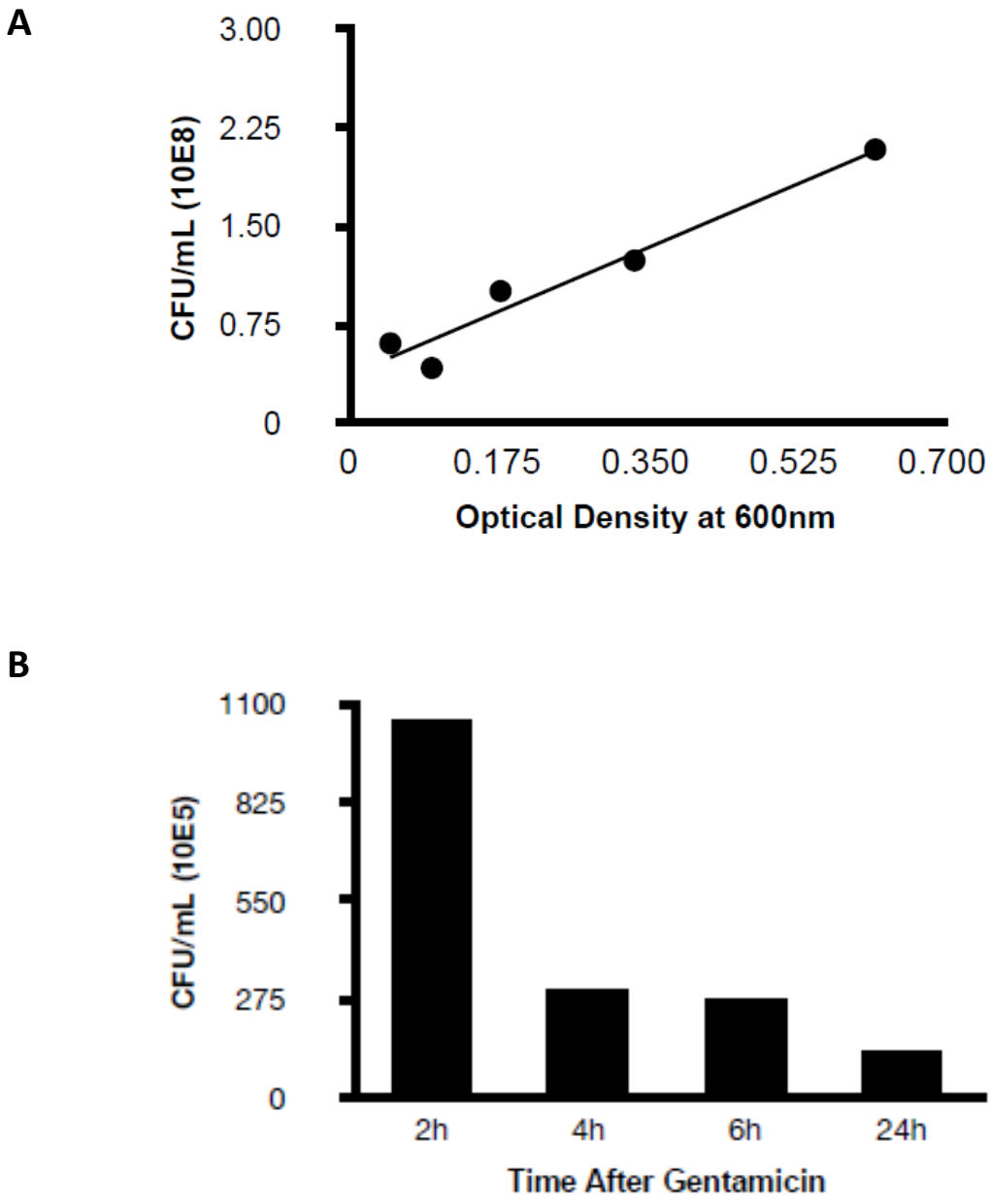
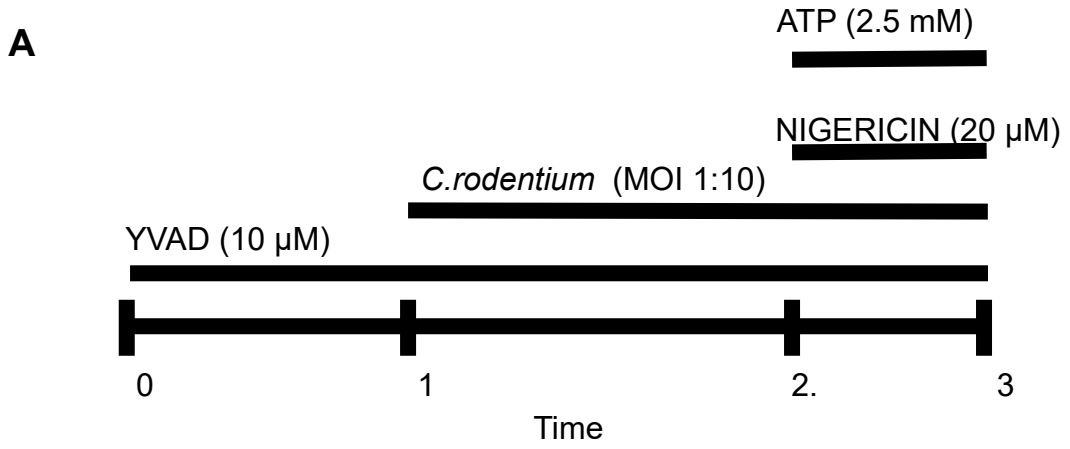


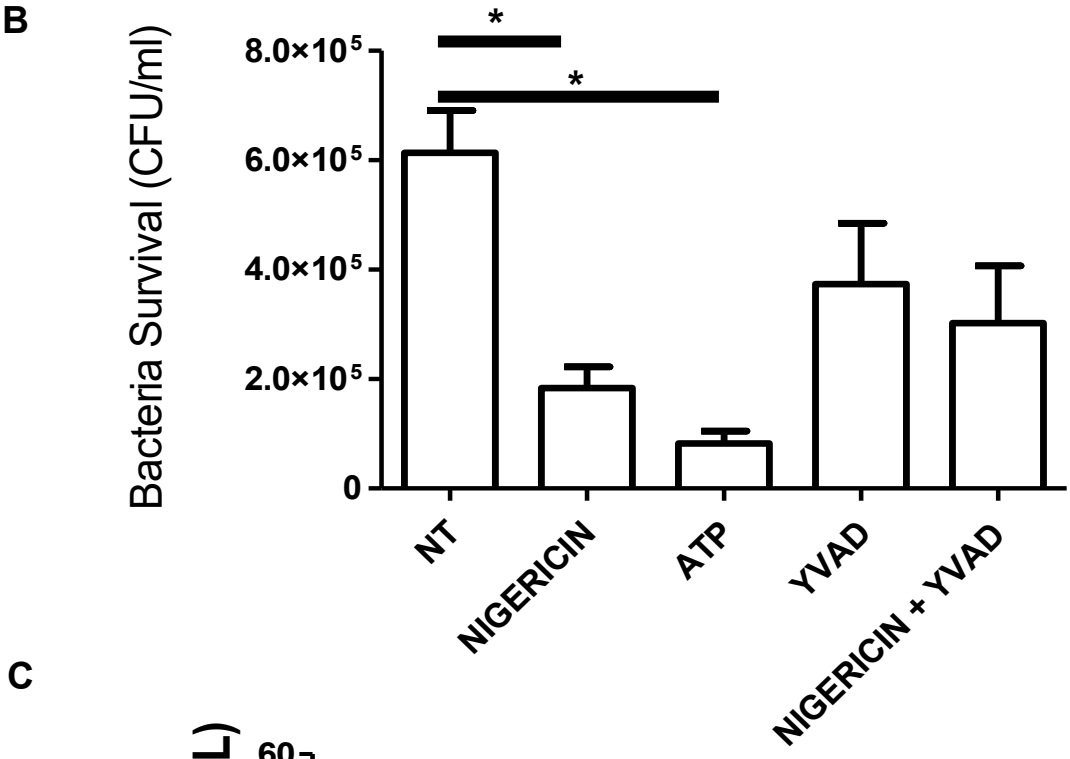
Fig 2.2. Growth rate of *C. rodentium*. (**A**) LB medium or (**B**) following gentamicin treatment for indicated time points where time 0h contained 10^8 CFU/mL. Turbidity was determined by serial dilutions of an overnight LB culture of *C. rodentium* plated and grown on LB agar overnight. Manual colony counts were performed on the serially diluted cultures grown on LB agar plates to determine CFU/mL. N=3.

Microbial killing of *C. rodentium* in Raw 264.7 is increased by nigericin

To examine the effects of nigericin on bacterial killing, Raw 264.7 cells were treated with nigericin (20 μM; 30 min), ATP (2.5 mM; 30min), or Ac-YVAD-cmk (10 μM; 3h; Sigma), in the presence of *C. rodentium* as indicated (**Fig. 2.3A**). Raw 264.7 cells were inoculated with *C. rodentium* without any treatment (NT) as a negative control, or with ATP as a positive control (**Fig. 2.3A**). Nigericin treatment of Raw 264.7 cells cultured with *C. rodentium* resulted in approximately 70% reduction in CFU/mL compared to control not treated (NT) cultures ($p<0.005$; **Fig. 2.3B**). ATP led to a similar reduction in bacterial invasion compared to NT ($p<0.001$). No significant change was found between YVAD and NT ($p >0.1$), YVAD+nigericin and NT ($p >0.1$), or nigericin and YVAD+nigericin ($p >0.1$) suggesting that addition of YVAD (caspase-1 inhibition) was able to partially counteract the bacterial killing effects of nigericin.

To test whether microbial killing occurred through activation of the NLRP3 inflammasome, Raw 264.7 cells were treated as indicated (**Fig 2.3A**) and supernatants were collected for ELISA to examine levels of secreted IL-1β, the main outcome of inflammasome activation. J774A.1 macrophage cells were used as a positive control as this cell line expresses ASC [53]. Only J774A.1 activated macrophages displayed secretion of IL-1β, indicating that microbial killing was independent of NLRP3 in Raw 264.7 macrophages (**Fig 2.3C**).


A
ATP (2.5 mM)
NIGERICIN (20 μM)
C.rodentium (MOI 1:10)
YVAD (10 μM)
0
1
2.
3
Time


B
Bacteria Survival (CFU/ml)
8.0×10⁵
6.0×10⁵
4.0×10⁵
2.0×10⁵
0
*
*
NT
NIGERICIN
ATP
YVAD
NIGERICIN + YVAD

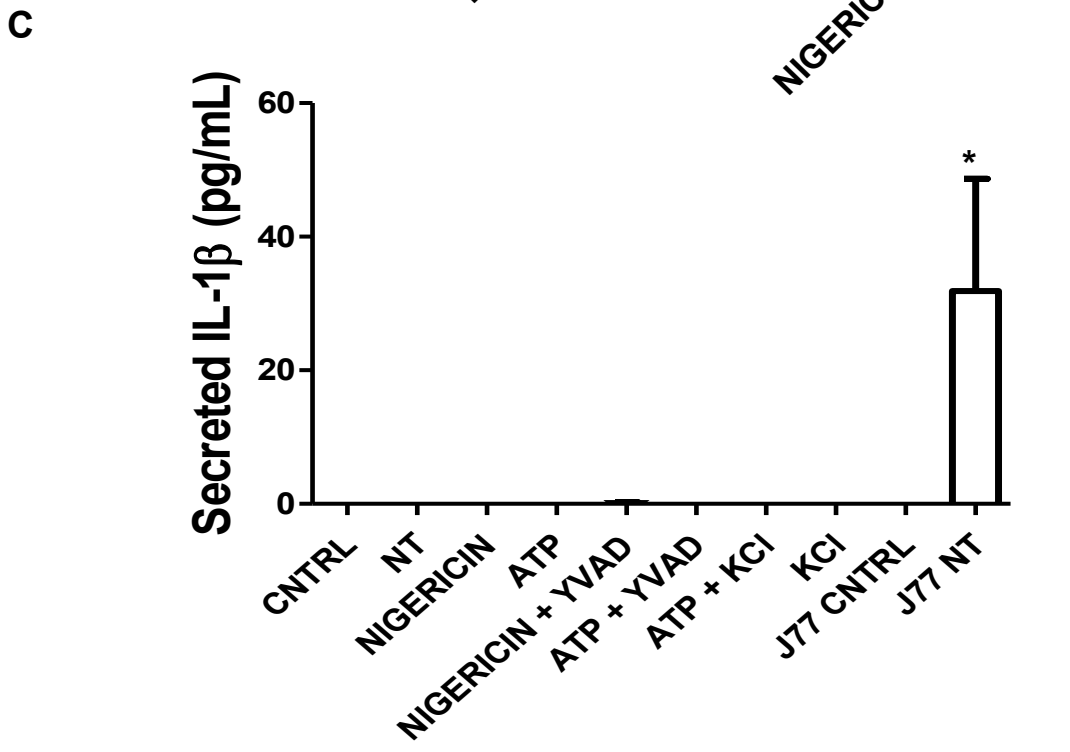
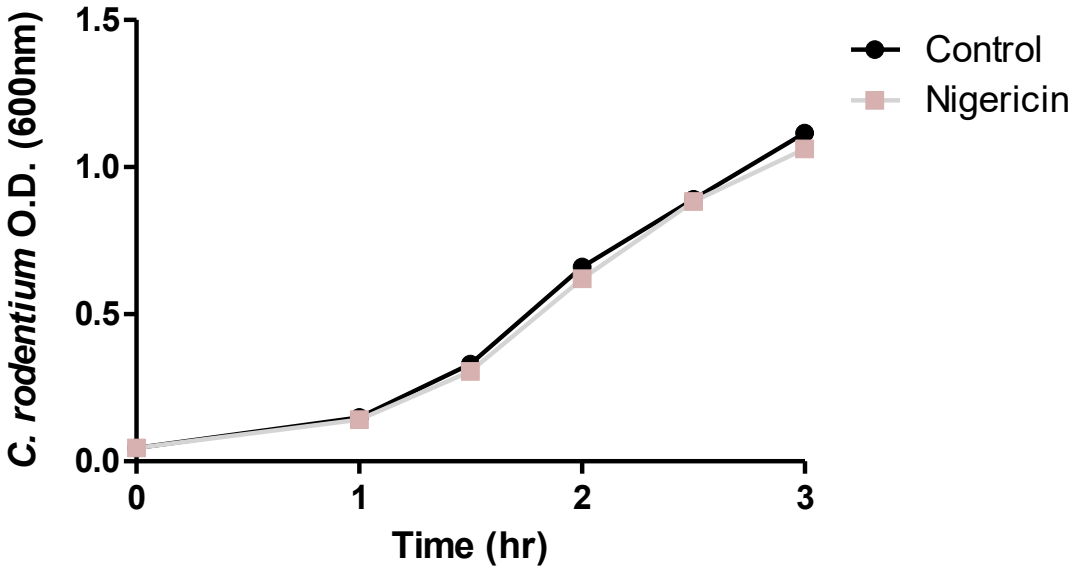

C
Secreted IL-1β (pg/mL)
60
40
20
0
*
CNTRL
NT
NIGERICIN
ATP
NIGERICIN + YVAD
ATP + YVAD
ATP + KCl
KCl
J77 CNTRL
J77 NT

Figure 2.3. Nigericin improves bacterial killing of *C. rodentium* in Raw264.7 cells independent of the NLRP3 inflammasome. (A) Raw 264.7 cells were first treated with YVAD as indicated for a total of 3 hr. Inoculation of Raw 264.7 with *C. rodentium* (MOI 1:10) occurred one hour after initial addition of YVAD. 1.5 hr after *C. rodentium* inoculation, Nigericin (20 μM) or ATP control (25 mg/mL) was added to the culture media. (B) Gentamicin (25 ug/mL) was used to eliminate non-invasive bacteria. Cell lysates were collected and grown on LB agar overnight and colonies were manually counted and expressed as CFU/mL (C) Raw 264.7 cells or control J77 cells were treated as indicated and inoculated with *C. rodentium* (MOI = 1:10). Supernatants were collected and run on ELISA for IL-1β. Samples were compared to control (CNTRL) cells which received no treatment or infection. Values represent mean±SEM between 3 independent experiments; *$p < 0.05$ using t-test. YVAD (Ac-Tyr-Val-Ala-Asp-Chloromethylketone); MOI (multiplicity of infection); CFU (colony forming units).

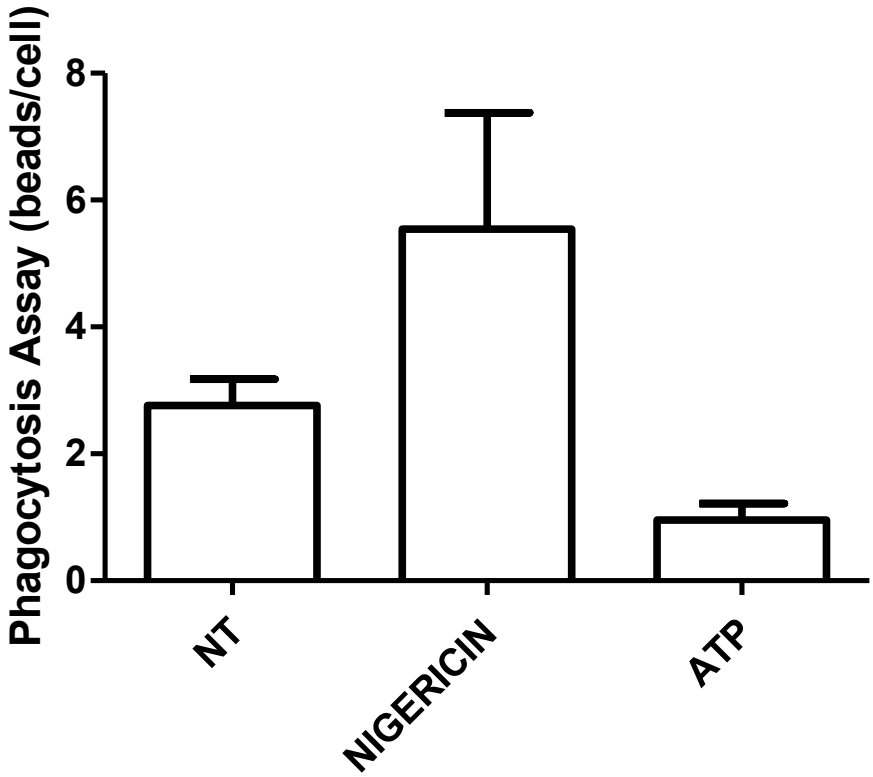
Nigericin did not directly reduce bacterial growth or alter phagocytosis in Raw 264.7 cells

The effect of nigericin on growth of *C. rodentium* was examined by OD_{600} nm spectrophotometer readings taken at indicated time points (**Fig. 2.4A**). Nigericin had no effect on the growth rate of *C. rodentium* compared to untreated control over 3h. Next, the effect of nigericin on Raw 264.7 cell phagocytosis was determined by examining uptake of fluorescein-tagged beads. There was no significant change in phagocytosis between NT, nigericin, or ATP control treated cells ($p>0.15$; **Fig. 2.4B** and **Fig 2.4C**). In all samples, it appeared that beads were engulfed by Raw 264.7 cells, suggesting that nigericin impacted bacterial killing but not phagocytosis.

A


C. rodentium O.D. (600nm)
1.5
1.0
0.5
0.0
0
1
2
3
Time (hr)
Control
Nigericin

B


Phagocytosis Assay (beads/cell)
8
6
4
2
0
NT
NIGERICIN
ATP

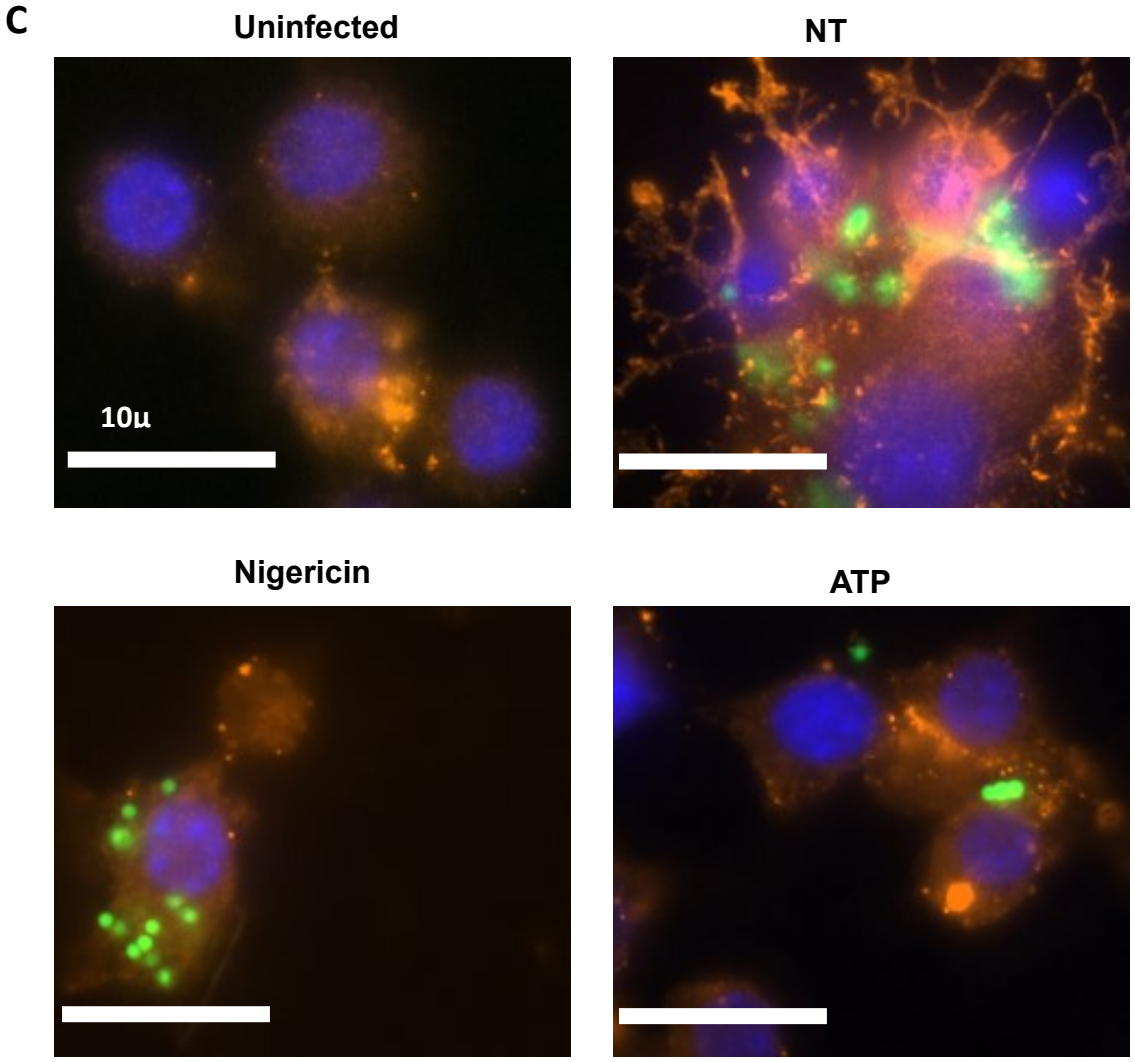

C
Uninfected
NT
10µ
Nigericin
ATP

Figure 2.4. *C. rodentium* growth and phagocytosis of are not altered with nigericin treatment. (**A**) *C. rodentium* were diluted to an initial OD600 of 0.1 and treated with nigericin (20 μM) at time 0hr. OD600 readings were taken at indicated times and compared to non-treated cultures. (**B**) Raw 264.7 cells were cultured with 2 μm conjugated beads (Polysciences Inc.) then treated with nigericin (20 μM) or ATP (25 mM) for 30 min. (**C**) Phagocytosis was measured by manual counting. Cells were fixed and stained using DAPI (cell DNA; blue), Phalloidin (F-actin; red), and Alexa Fluor 488 (*C. rodentium*; green). Cells were imaged using a Leica SP5 confocal microscope at 60X. N=3.

Nigericin does not alter the classic NLRP3 inflammasome pathway

To better determine the pathways that nigericin alters in Raw 264.7 cells, which may enhance bacterial killing, we performed an inflammasome gene expression array (**Fig. 2.5; Fig. 2.6**). This array demonstrated altered expression of a series of genes, which were the validated by qPCR (**Fig. 2.7**). Neither NLRP3 nor its downstream target IL-1β resulted in altered expression profile in *C. rodentium* infected cells treated with nigericin when compared to untreated (NT), ATP-treated, YVAD-treated, or nigericin in combination with YVAD. While IL-10 and TNFα expression were elevated slightly in response to ATP, no changes were found in response to nigericin. Nigericin treatment resulted in statistically significant increase in Cd40lg, Ciita (NLRA), IL-12, IFNγ, Mapk11 (p38), Nlrp4e, and Tab2 as well as a statistically significant decrease in Tnfsf11.

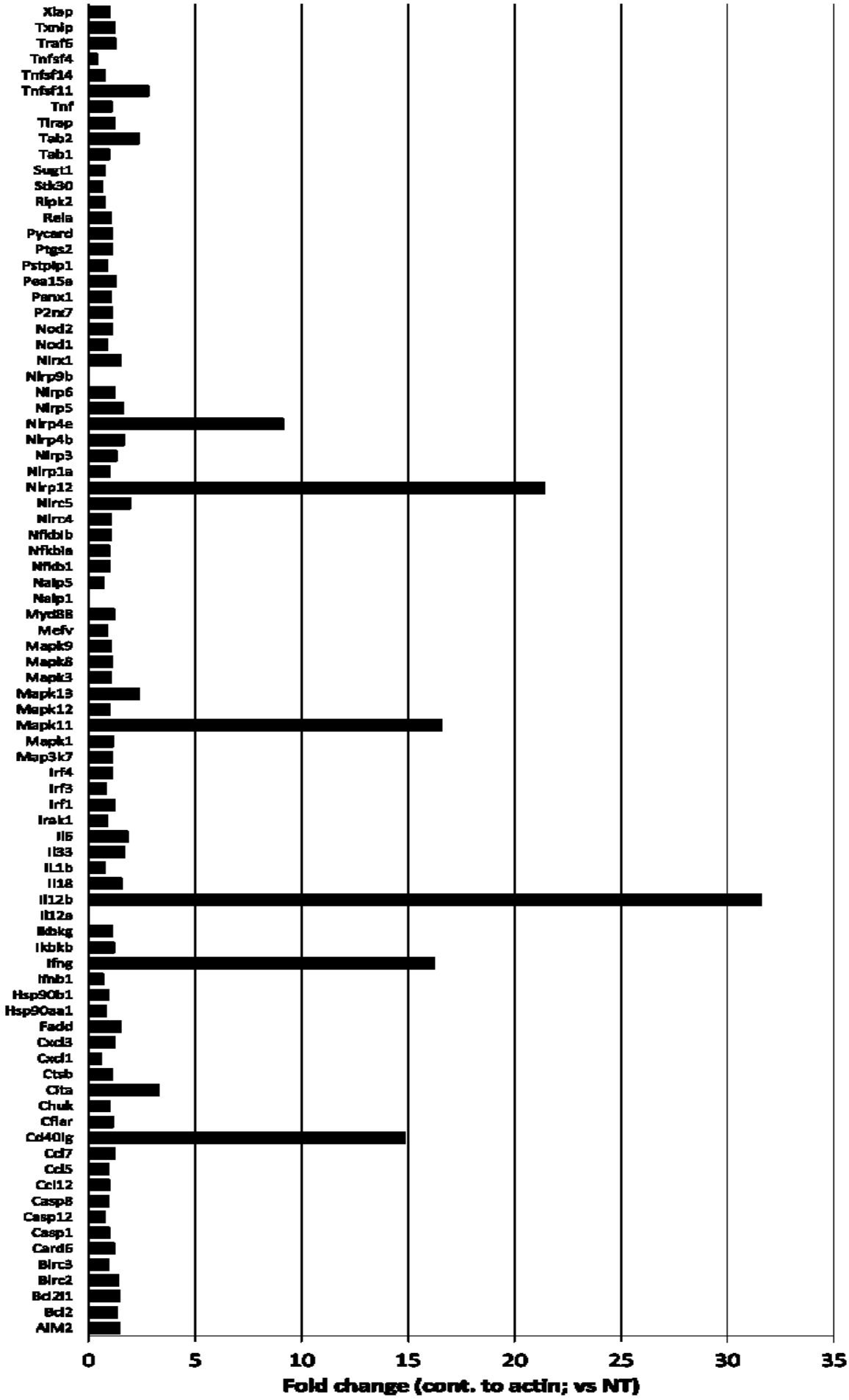
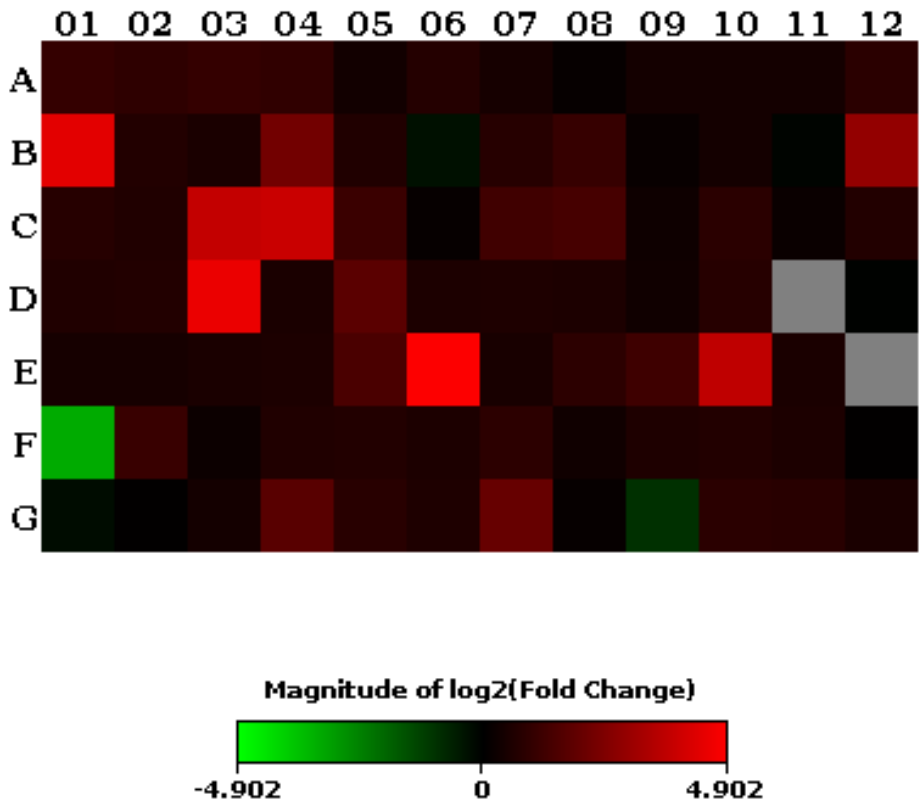

Xiap
Txnip
Traf6
Tnfsf4
Tnfsf14
Tnfsf11
Tnf
Tirap
Tab2
Tab1
Sugt1
Stk30
Ripk2
Rela
Pycard
Ptgs2
Pstpip1
Pea15a
Panx1
P2rx7
Nod2
Nod1
Nlrx1
Nlrp9b
Nlrp6
Nlrp5
Nlrp4e
Nlrp4b
Nlrp3
Nlrp1a
Nlrp12
Nlrc5
Nlrc4
Nfkbib
Nfkbia
Nfkb1
Naip5
Naip1
Myd88
Mefv
Mapk9
Mapk8
Mapk3
Mapk13
Mapk12
Mapk11
Mapk1
Map3k7
Irf4
Irf3
Irf1
Irak1
Il6
Il33
IL1b
Il18
Il12b
Il12a
Ikbkg
Ikbkb
Ifng
Ifnb1
Hsp90b1
Hsp90aa1
Fadd
Cxcl3
Cxcl1
Ctsb
Ciita
Chuk
Cflar
Cd40lg
Ccl7
Ccl5
Ccl12
Casp8
Casp12
Casp1
Card6
Birc3
Birc2
Bcl2l1
Bcl2
AIM2
0
5
10
15
20
25
30
35
Fold change (cont. to actin; vs NT)

Figure 2.5. Effects of nigericin on expression of inflammasome-associated genes. QIAGEN inflammasome gene array was performed following the manufacturer's instructions on RNA collected from Raw2 64.7 cells treated with nigericin and was compared to untreated cells, following *C. rodentium* infection. Fold changes display nigericin/untreated. N=3.

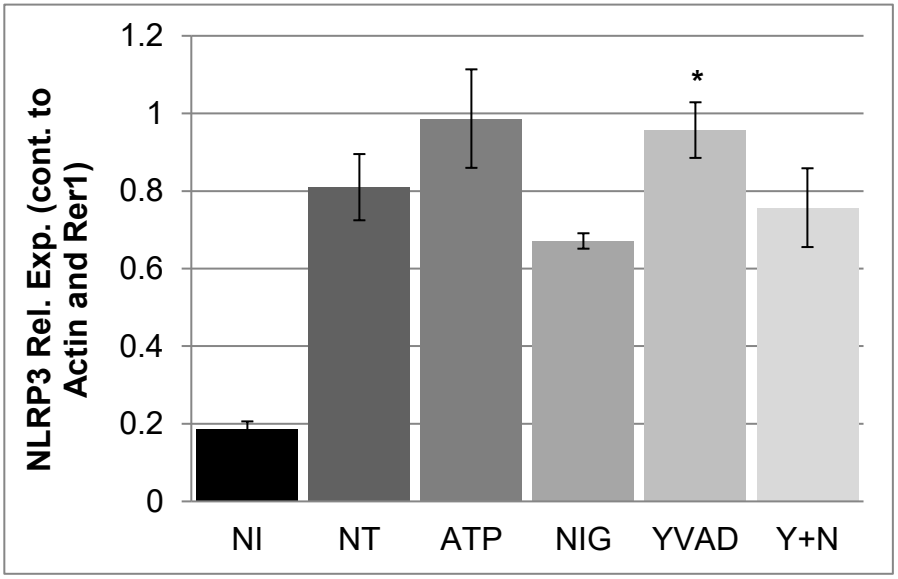
A

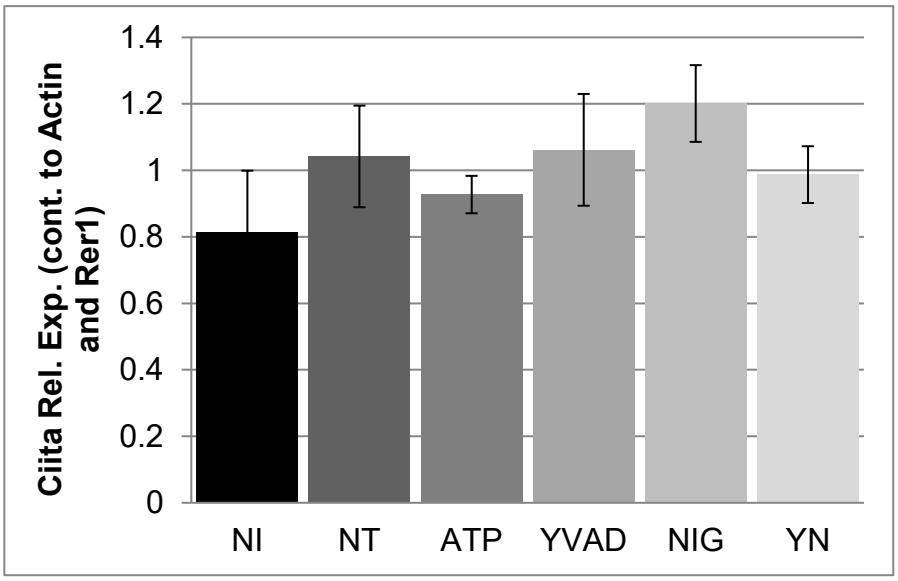


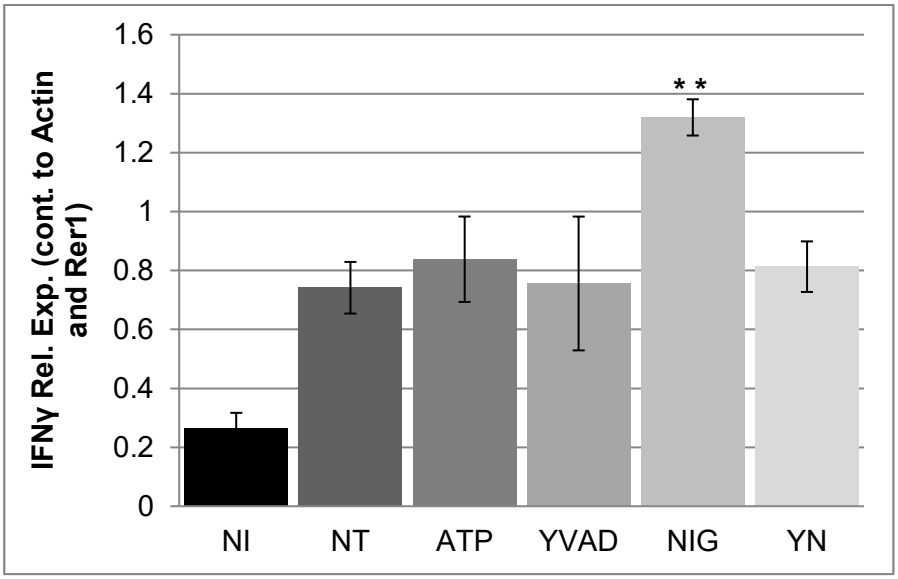
B

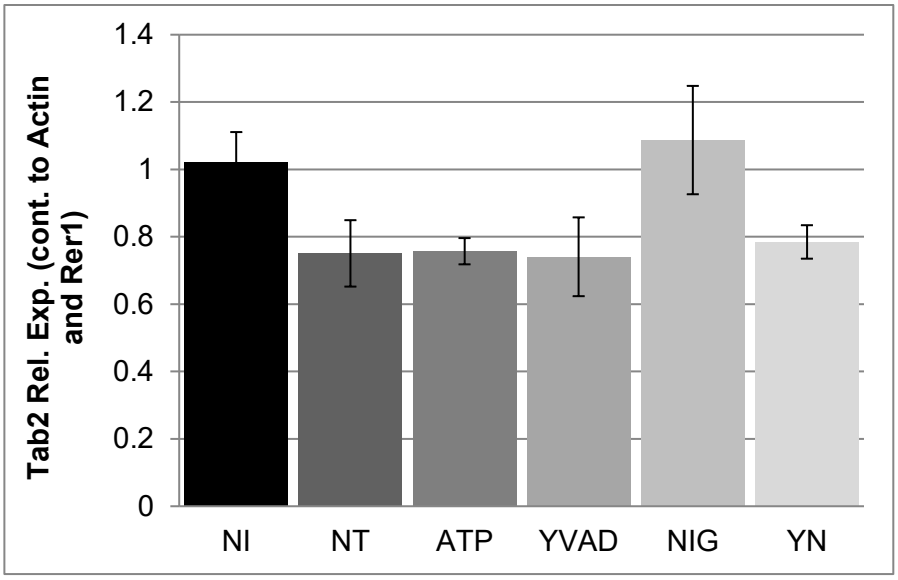
Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	Aim2 **1.99**	Bcl2 **1.83** B	Bcl2l1 **1.97**	Birc2 **1.92**	Birc3 **1.28**	Card6 **1.63**	Casp1 **1.33**	Casp12 **1.09** B	Casp8 **1.29**	Ccl12 **1.32** B	Ccl5 **1.30**	Ccl7 **1.73** B
B	Cd40lg **20.70** B	Cflar **1.59**	Chuk **1.40**	Ciita **4.57** A	Ctsb **1.52**	Cxcl1 **-1.22** B	Cxcl3 **1.66** B	Fadd **2.06**	Hsp90aa1 **1.13**	Hsp90b1 **1.30**	Ifnb1 **-1.05**	Ifng **7.17** B
C	Ikbkb **1.65**	Ikbkg **1.54** B	Il12a **13.47** B	Il12b **14.74** B	Il18 **2.18**	Il1b **1.08**	Il33 **2.32** B	Il6 **2.55** B	Irak1 **1.22**	Irf1 **1.73**	Irf3 **1.14**	Irf4 **1.57**
D	Map3k7 **1.55**	Mapk1 **1.58**	Mapk11 **23.13** A	Mapk12 **1.41** B	Mapk13 **3.32** B	Mapk3 **1.46**	Mapk8 **1.51**	Mapk9 **1.44**	Mefv **1.23** B	Myd88 **1.65**	Naip1 **-1.03** C	Naip5 **-1.02** B
E	Nfkb1 **1.36**	Nfkbia **1.33**	Nfkbib **1.42**	Nlrc4 **1.46** B	Nlrc5 **2.72** A	Nlrp12 **29.89** B	Nlrp1a **1.39**	Nlrp3 **1.79**	Nlrp4b **2.28** B	Nlrp4e **12.74** A	Nlrp5 **1.42** B	Nlrp6 **-1.03** C
F	Nlrp9b **-9.77** B	Nlrx1 **2.09** A	Nod1 **1.18** B	Nod2 **1.52** B	P2rx7 **1.57**	Panx1 **1.45**	Pea15a **1.78**	Pstpip1 **1.22**	Ptgs2 **1.51**	Pycard **1.57** B	Rela **1.45**	Ripk2 **1.04**
G	Mok **-1.17** B	Sugt1 **1.03**	Tab1 **1.32** B	Tab2 **3.23**	Tirap **1.71**	Tnf **1.49**	Tnfsf11 **3.87** B	Tnfsf14 **1.09** B	Tnfsf4 **-1.89** B	Traf6 **1.76**	Txnip **1.70**	Xiap **1.41**

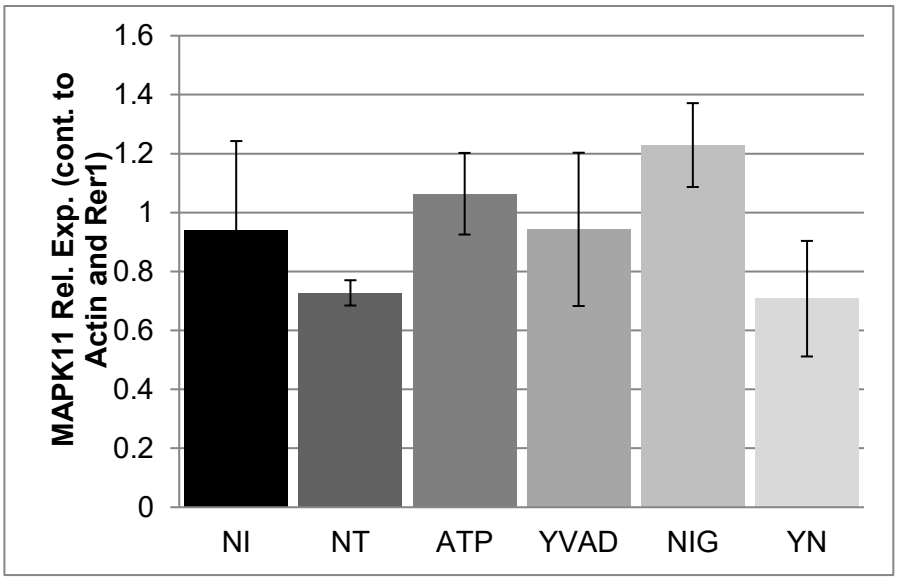
Fig 2.6. **RAW 264.7 macrophage gene array after treatment with Nigericin.** QIAGEN inflammasome gene array was performed following the manufacturer's instructions on RNA collected from Raw 264.7 cells treated with nigericin and was compared to untreated cells, following *C. rodentium* infection. (**A**) Fold change (treated/untreated) results are displayed as a heat map with (**B**) gene layout and associated fold change values. 'A' signifies a statistically significant difference between control and nigericin treatment; 'B' signifies p> 0.05; 'C' signifies the expression of this gene was undetectable. Statistical analysis was done using a student's *t*-test.

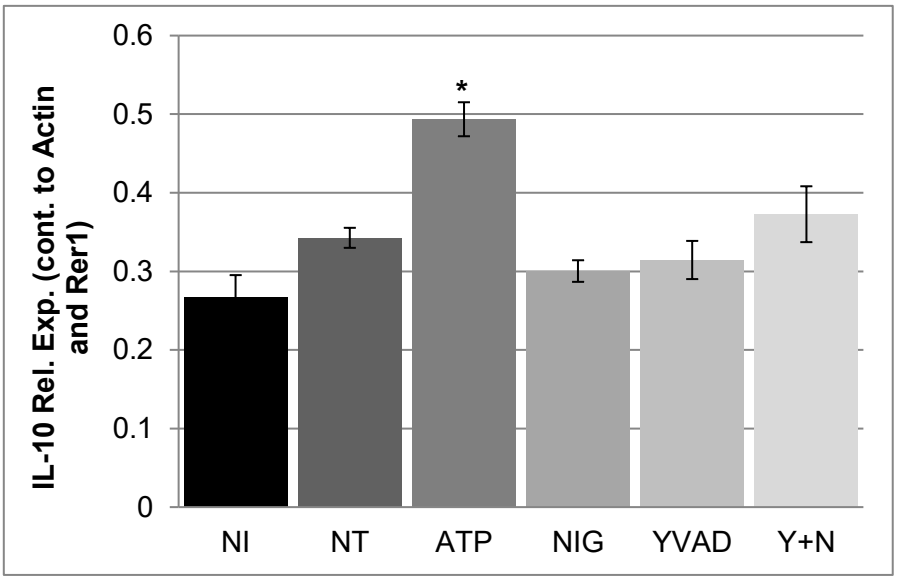

NLRP3 Rel. Exp. (cont. to Actin and Rer1)
1.2
1
0.8
0.6
0.4
0.2
0
*
NI
NT
ATP
NIG
YVAD
Y+N

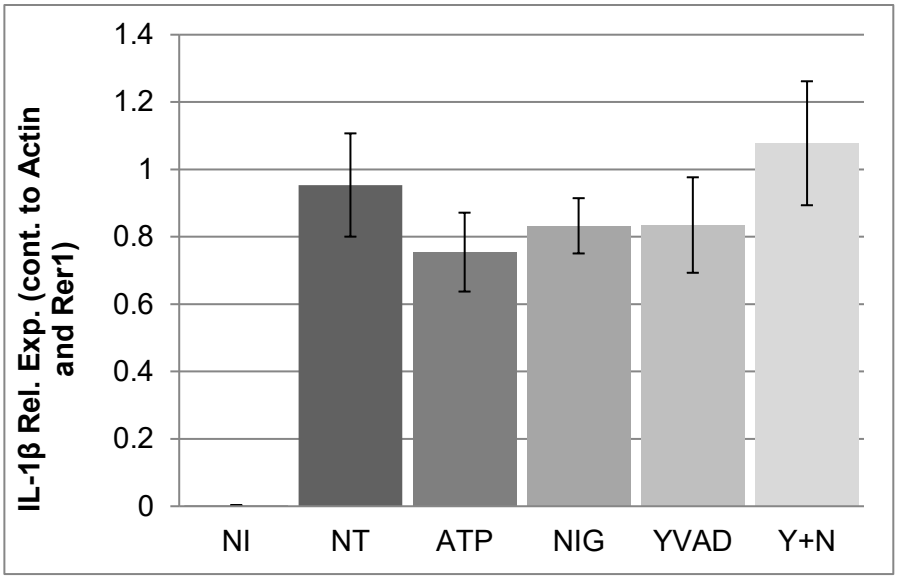

Ciita Rel. Exp. (cont. to Actin and Rer1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NI
NT
ATP
YVAD
NIG
YN

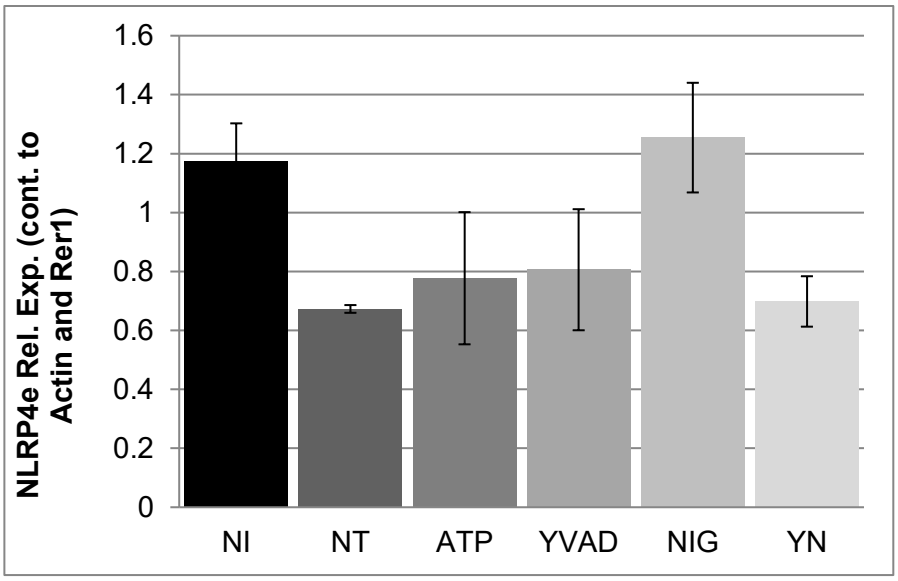

IFNγ Rel. Exp. (cont. to Actin and Rer1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
* *
NI
NT
ATP
YVAD
NIG
YN

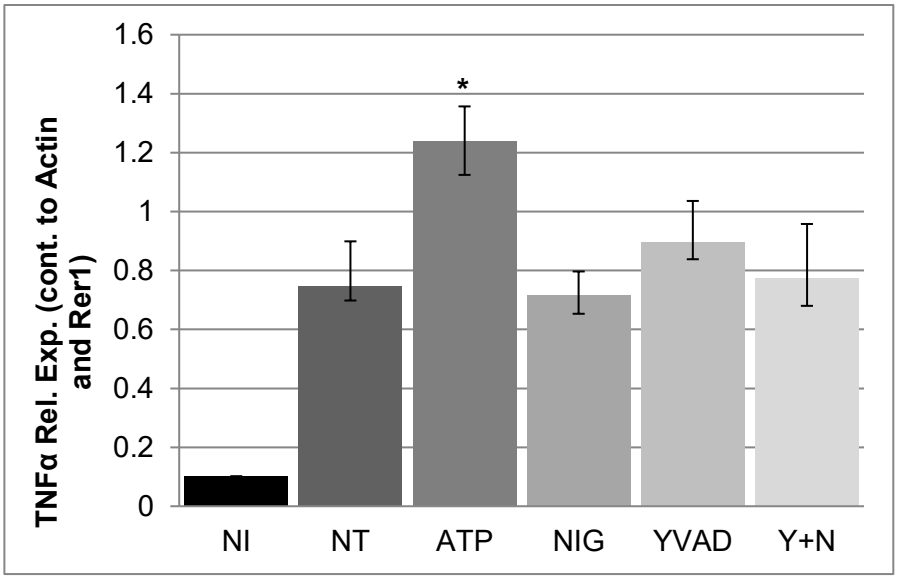

Tab2 Rel. Exp. (cont. to Actin and Rer1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NI
NT
ATP
YVAD
NIG
YN

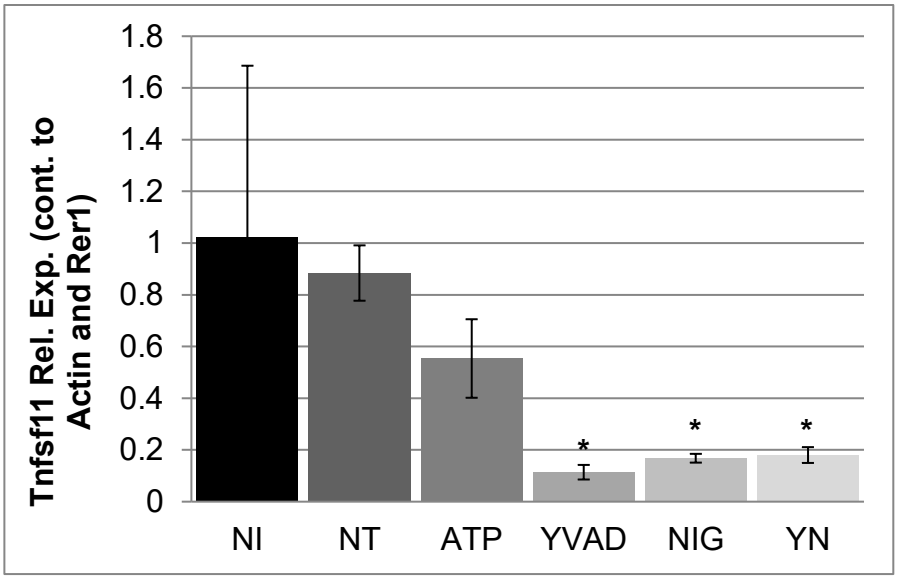

MAPK11 Rel. Exp. (cont. to Actin and Rer1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NI
NT
ATP
YVAD
NIG
YN

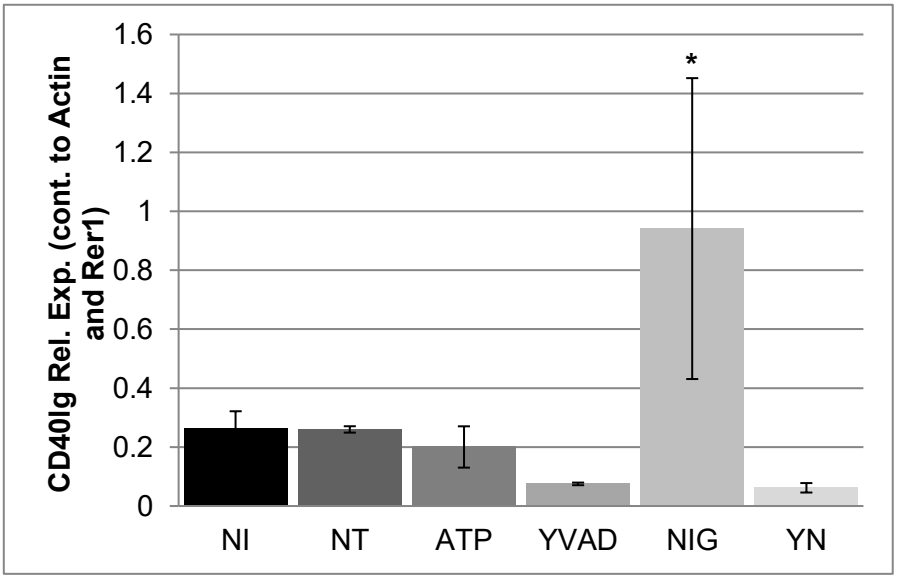

IL-10 Rel. Exp. (cont. to Actin and Rer1)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
NI
NT
ATP
NIG
YVAD
Y+N


IL-1β Rel. Exp. (cont. to Actin and Rer1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NI
NT
ATP
NIG
YVAD
Y+N


NLRP4e Rel. Exp. (cont. to Actin and Rer1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NI
NT
ATP
YVAD
NIG
YN


TNFα Rel. Exp. (cont. to Actin and Rer1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
NI
NT
ATP
NIG
YVAD
Y+N


Tnfsf11 Rel. Exp. (cont. to Actin and Rer1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
NI
NT
ATP
YVAD
NIG
YN


CD40lg Rel. Exp. (cont. to Actin and Rer1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
NI
NT
ATP
YVAD
NIG
YN

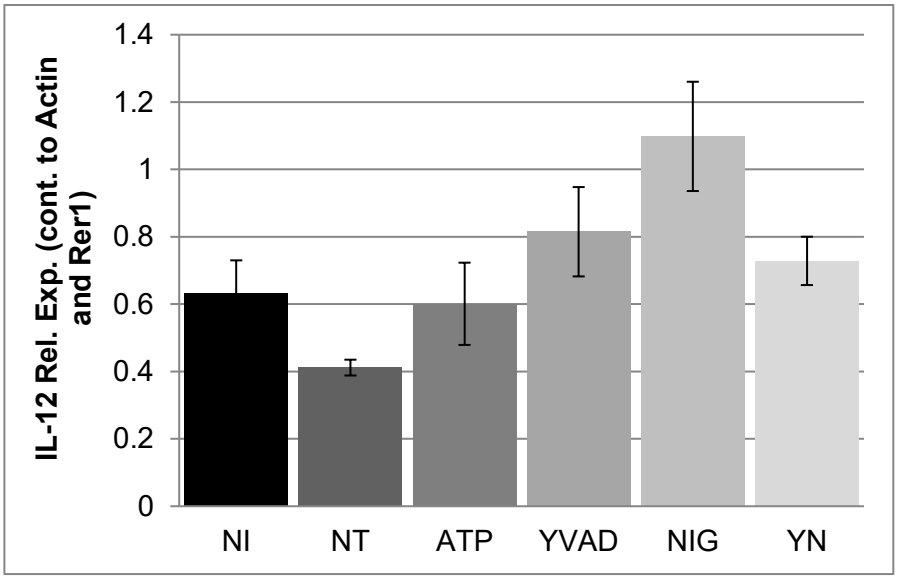

IL-12 Rel. Exp. (cont. to Actin and Rer1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NI
NT
ATP
YVAD
NIG
YN

Figure 2.7. Nigericin results in bacterial killing in Raw 264.7 cells through a mechanism unrelated to the NRLP3 inflammasome. Raw 264.7 cells were first treated with YVAD as indicated for a total of 3 hr. Inoculation of Raw 264.7 with *C. rodentium* (MOI 1:10) occurred one hour after initial addition of YVAD. 1.5 hr after *C. rodentium* inoculation, Nigericin (20 μM) or ATP control (2.5 mM) was added to the culture media. Cells were lysed with trizol and RNA extraction and cDNA processing proceeded as described in the methods. qPCR was performed to examine NLRP3, IL-1β, IL-10, Cd40lg, Ciita, IL-12, IFNγ, Mapk11, Nlrp4e, Tab2, TNFα, and Tnfsf11. Samples were controlled against β-actin and Rer1 expression. Two-tailed t-test were performed comparing treatment options against NT; $p<0.05$ *; $p<0.01$ **

2.5 DISCUSSION

While studies that aim to examine microbial killing by macrophages tend to focus on the inflammasome and its downstream effector IL-1β, here we have shown that nigericin, a potassium ionophore [104] and NLRP3 activator, remains capable of increasing bacterial killing in Raw 264.7 cells, independent of the classic NLRP3 inflammasome. Raw 264.7 do not express ASC and are therefore not capable of inflammasome activation or function [53]. Previous studies have suggested that the effects of nigericin on bacterial killing require the inflammasomes [105], while a limited number of studies have demonstrated that the inflammasome is not essential for bacterial clearance [106], although this was tested in a different setting. Interestingly, while nigericin was able to increase microbial killing in Raw 264.7, YVAD, a caspase-1 inhibitor that blocks the NLRP3 pathway, was able to partially mitigate or reverse this effect (**Fig 2.3B**).

As nigericin promotes potassium efflux, and potassium is involved in the promotion of phagocytosis [107], we sought to determine if the effects on bacterial killing were the result of increased macrophage cell death. Our results demonstrated that nigericin did not alter *C. rodentium* growth rates directly (**Fig. 2.4A**) and did not result in statistically significant changes in phagocytosis in Raw 264.7 cells (**Fig. 2.4B-C**). An inflammasome gene array of Raw 264.7 cells was used to identify key targets involved in response to nigericin and compare expression of these genes to untreated (NT) cells (**Fig. 2.5**). Validation of expression of key target genes demonstrated that nigericin resulted in increased expression of Cd40lg, Ciit (NLRPA), IL-12, and IFNγ, and a reduction in Tnfsf11 in nigericin-treated cells (**Fig. 2.7**). Based

on these results we hypothesize a possible model of nigericin-mediated bacterial killing without involvement of the inflammasome (**Fig. 2.8**). Caspase-1 has previously been shown to be modulated by nigericin via interactions with cathepsin B [108]. Interestingly, caspase-1 promotes cleavage of the pro-inflammatory cytokine pro-IL-18 to active IL-18, leading to up-regulation of Cd40lg [109], a cytokine which was increased in response to nigericin in the current study. CD40 is thought to play a key role in inflammatory diseases as hyperactivation of CD40 leads to increased production of pro-inflammatory cytokines, including IL-12 [110]. IL-12 was also found to be increased in response to nigericin. Results from the current study support previous research indicating that both IL-12 and IL-18 promote production of IFNγ. This cytokine plays a vital role in induction of the MHC class II complex, which is critical in macrophage defense against viral, protozoal, and some bacterial infections [111]. IFNγ is an upstream activator of both MAPK and Ciita, also known as NLRA. Both Mapk11 and Ciita were increased following nigericin treatment, adding further support to our hypothesized pathway. The Ciita/STAT1 pathway is important for MHCII activation and antigen presentation following phagocytosis of bacteria in macrophages [112], while IFNγ induction of MAPK11 promotes phagocyte and bacterial killing pathways via oxidative burst by increased ROS production [113, 114]. While we have not shown direct evidence to support all aspects of this model, this work should stimulate further investigation into alternative, non-inflammasome mediated pathways for immune activation and bacterial killing in macrophages.

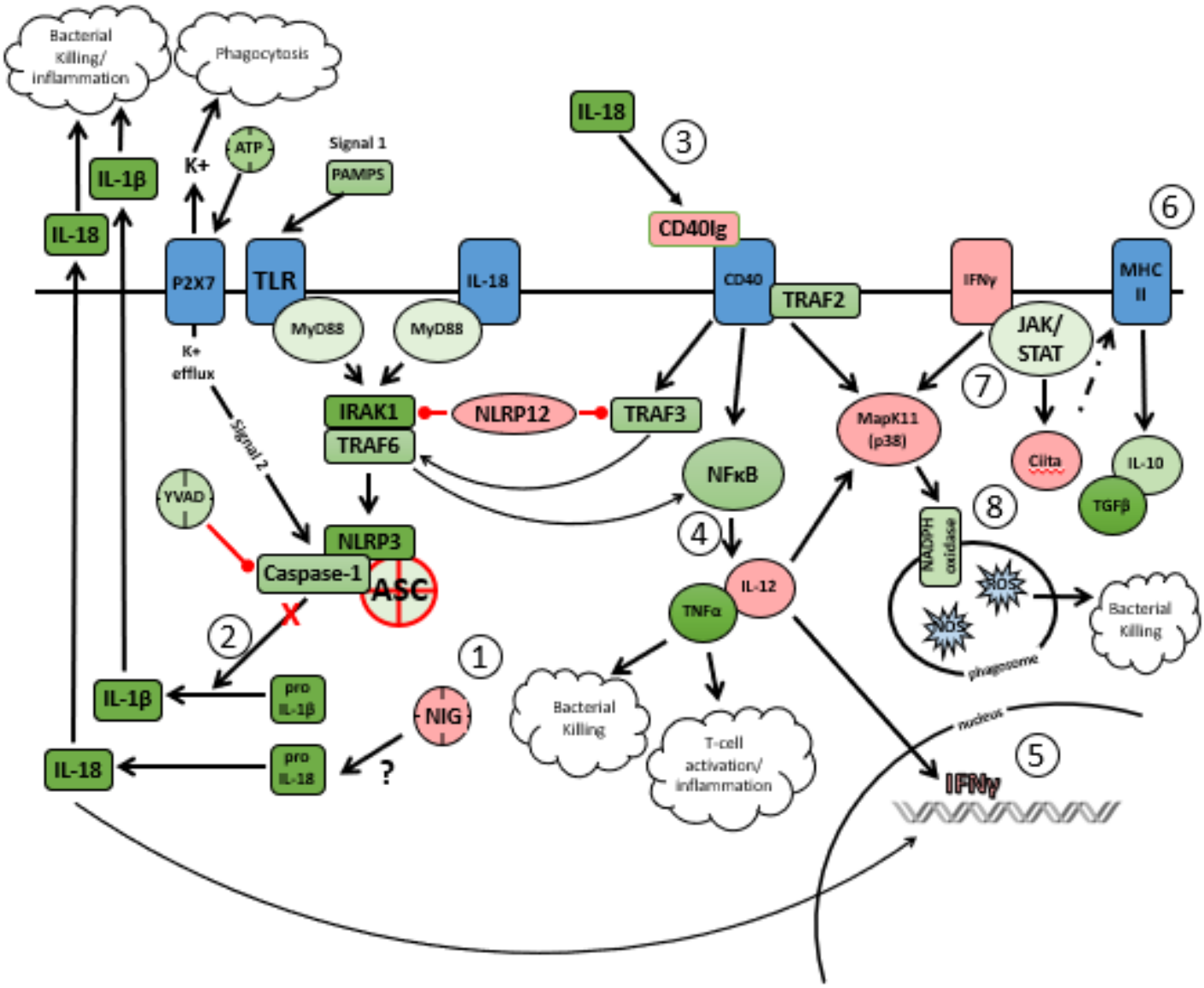

Bacterial Killing/ Inflammation
Phagocytosis
IL-18
3
ATP
K+
Signal 1
PAMPS
IL-1β
IL-18
CD40lg
6
P2X7
TLR
IL-18
CD40
TRAF2
IFNγ
MHC II
MyD88
MyD88
JAK/ STAT
K+ efflux
Signal 2
IRAK1
NLRP12
TRAF3
TRAF6
MapK11 (p38)
7
NFκB
Cilta
IL-10
TGFβ
YVAD
NLRP3
4
8
Caspase-1
ASC
NADPH oxidase
ROS
NOS
phagosome
Bacterial Killing
IL-12
TNFα
2
1
IL-1β
pro IL-1β
NIG
Bacterial Killing
T-cell activation/ inflammation
nucleus
5
IL-18
pro IL-18
?
IFNγ

Figure 2.8. Proposed model of the effects of nigericin on bacterial killing in ASC deficient macrophages. (**1**) Caspase-1 may promote cleavage of the pro-inflammatory cytokine pro-IL-18 (**2**) to active IL-18, which has been shown to induce upregulation of Cd40lg (**3**). CD40 is thought to play a key role in inflammatory diseases as hyperactivation of CD40 leads to increased production of pro-inflammatory cytokine, including IL-12 (**4**). Both IL-12 and IL-18 promote production of IFNγ (**5**). This cytokine plays a vital role in induction of the MHC class II complex (**6**), which is critical in macrophage defense against viral, protozoal, and some bacterial infections. IFNγ is an upstream activator of both MAPK and Ciita (**7**), also known as NLRA. The Ciita/STAT1 pathway is important for MHCII activation and antigen presentation following phagocytosis of bacteria in macrophages, while IFNγ and MAPK11 promote phagocyte and bacterial killing pathways via oxidative burst by increased ROS production (**8**). Key mediators that were identified by gene array as increased by nigericin are highlighted in pink.

We have demonstrated here the potential for alternate pathways of bacterial killing in macrophage cells lacking an active inflammasome by utilizing nigericin *in vitro*. This is important for a number of inflammatory diseases including cystic fibrosis, inflammatory bowel diseases, infection, and even cancer, as this could introduce innovative treatment options to mitigate disease pathogenesis by improving efficiency of bacterial clearance. Macrophages play a key role in clearance of pathogenic and pathobiont microorganisms in inflammatory disease [115]. Understanding the alternate mechanisms of bacterial clearance within a macrophage cell system will aid in our ability to identify new methods of modulating immune response during infection.

Chapter 3

Inflammasome Activation by ATP Enhances *Citrobacter rodentium* Clearance through ROS Production

CHAPTER 3. INFLAMMASOME ACTIVATION BY ATP ENHANCES CITROBACTER RODENTIUM CLEARANCE THROUGH ROS GENERATION

This article has been published in the Journal of Cellular Physiology and Biochemistry, Karger Publishing Group. Doi: 10.1159/000455988

Authors: Michael Bording-Jorgensen, Misagh ALipour, Ghazal Danesh, Eytan WIne

3.1 ABSTRACT

Background*:* Nod-like receptor family, pyrin domain containing 3 (NLRP3) is an important cytosolic sensor of cellular stress and infection. Once activated, NLRP3 forms a multiprotein complex (inflammasome) that triggers the maturation and secretion of interleukin (IL)-1β and IL-18. We aimed to define the consequences of NLRP3 induction, utilizing exogenous adenosine triphosphate (ATP) as an inflammasome activator, to determine if inflammasome activation increases macrophage killing of *Citrobacter rodentium* and define mechanisms.

Methods*:* Bacterial survival was measured using a gentamicin protection assay. Inflammasome activation or inhibition in mouse J774A.1 macrophages were assessed by measuring IL-1β; cytokines and reactive oxygen species (ROS) were measured by ELISA and DCFDA, respectively.

Results*:* Activation of the inflammasome increased bacterial killing by macrophages and its inhibition attenuated this effect with no impact on phagocytosis or cell death. Furthermore, inflammasome activation suppressed pro-inflammatory cytokines during infection, possibly due to more effective bacterial killing. While the

infection increased ROS production, this effect was reduced by inflammasome inhibitors, indicating that ROS is inflammasome-dependent. ROS inhibitors increased bacterial survival in the presence of ATP, suggesting that inflammasome-induced bacterial killing is mediated, at least in part, by ROS activity.

Conclusion*:* Improving inflammasome activity during infection may increase bacterial clearance by macrophages and reduce subsequent microbe-induced inflammation.

3.2 INTRODUCTION

The Nod-like receptor family pyrin domain containing 3 (NLRP3) protein is a cytosolic sensor of danger-associated molecular patterns (DAMPs) and microbe associated molecular patterns (MAMPs) [116]. When activated, the multiprotein complex NLRP3 inflammasome is formed, leading to the maturation and release of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 [117]. Inflammasome activation requires two signals, the first being production of pro-IL-1β/IL-18 and the second involves autocleavage of pro-caspase-1 when the inflammasome assembles. Mature caspase-1 then cleaves pro-IL-1β into the active form, which is subsequently secreted from the cell [117-119]. Inflammasomes and IL-1β have been linked to a variety of chronic immune-mediated disorders, such as gout and rheumatoid arthritis, where increased inflammasome activity appears to be detrimental [120]. In contrast, there is significant evidence that inflammasomes play an opposite role in inflammatory bowel diseases (IBD, including Crohn disease and ulcerative colitis) [121-125]. Individuals with Crohn disease have been shown to have lower expression of IL-1β and the *NLRP3* gene, suggesting a protective role for the inflammasome, which we speculate may be explained by more effective control of invasive microbes [126].

Adenosine triphosphate (ATP), a DAMP with low extracellular levels under homeostatic conditions, can be released by damaged cells, secreted by neutrophils, or directly from intestinal bacteria during infection or necrosis, eliciting an immune response [51]. The extracellular ATP sensor purinergic receptor $P2X_7$ has been

proposed to activate the NLRP3 inflammasome through reactive oxygen species (ROS) production and potassium (K^+) efflux, although the precise mechanism of activation is not known [52-54]. The inflammasome is important during bacterial infection and ATP can have a protective role through promoting the production of ROS, which then eliminates bacteria [127, 128]. $P2X_7$ recruits pannexin 1, which forms a pore in the cell membrane, allowing for efflux of K^+, another activator of the NLRP3 inflammasome [55]. The ROS scavenger *N*-acetylcysteine (NAC) and the NADPH oxidase ROS inhibitors diphenyleneiodonium (DPI) and apocynin (all of which will be referred to as ROS inhibitors) result in decreased NLRP3 expression and activation in murine macrophages [118]. Xiang *et al.* showed that ATP offered protection from peritonitis in mice infected with *Staphylococcus aureus* and *Escherichia coli* [51]. Csoka *et al.* demonstrated the ability of macrophages to increase intracellular killing of *E. coli* through purinergic receptors during sepsis, highlighting the beneficial effects of ATP through the inflammasome in an *in vivo* model [129].

Citrobacter rodentium is a mouse pathogen that colonizes the colon and causes IBD-like colitis and is used as a model for enterohemorrhagic *E. coli* and enteropathogenic *E. coli* infections. In our previous work, we showed that extraneous IL-1β improves the ability of macrophages to phagocytose *C. rodentium* in *Nlrp3*$^{-/-}$ mice and that clearance of infection is dependent on the NLRP3 inflammasome [49]. As an extension of these findings, we aimed to optimize bacterial handling and hypothesized that increasing inflammasome activation would also improve the ability of macrophages to kill these pathogens. In this study we examined the role of ATP-

induced inflammasome activation in the murine macrophage cell line J774A.1 in clearing *C. rodentium* infection. We found that inflammasome activation decreased intracellular microbial survival along with a decrease in pro-inflammatory cytokines after the clearance of the pathogen. In addition, inflammasome activation induced ROS production, which was the cause of decreased bacterial survival. This study illustrates that the protective role of ATP-induced inflammasome activation in J774A.1 macrophages is mediated by ROS production through inflammasome activation and that optimization of inflammasome function could reduce inflammation.

3.3 MATERIALS AND METHODS

Chemicals

2, 7-dichlorofluorescein diacetate (DCFDA, D6883), N-acetylcysteine (NAC, A7250), diphenyleneiodonium (DPI, D2926), apocynin (W508454), and Amicon Ultra-4 centrifugal filters (Z648035) were purchased from Sigma Aldrich. ATP (BP413-25) was purchased from Acros Organics.

Cell culture

J774A.1 murine macrophage cell line (ATCC, TIB-67) was seeded into either 24- or 96-well plates as indicated for each experiment. Cells were maintained in DMEM, supplemented with 10% heat-inactivated fetal bovine serum; medium was replaced every two days and cells were passaged at 80% confluence for a maximum of 22 passages.

C. rodentium infection

J774A.1 cells were seeded in 24-well tissue culture plates at a density of 5 x 10^5 cells per well (80% confluence) overnight to allow for adhesion. Prior to infection, the medium was changed to antibiotic- and serum-free DMEM. *C. rodentium* (DBS100, a gift from the Sherman lab, University of Toronto) was cultured overnight in lysogeny broth (LB) at 37°C. Cells were treated with the caspase 1 inhibitor AC-YVAD-CMK (25 µM, Enzo Life Sciences, ALX-260-028), or excess K^+ (45 mM) to inhibit the inflammasome, for 1 h pre-infection. *C. rodentium* was added at a multiplicity of infection (MOI) of 10:1 for 2 h of infection; then ATP (2.5 mM) with or without ROS inhibitors NAC (20 µM), DPI (10 µM), or apocynin (4 mM) were added for 30 min. The medium was changed to DMEM with gentamicin (100 µg/ml, Fisher Scientific, 15750078) for 1 h to ensure killing of extracellular bacteria (confirmed by plate growth, not shown; gentamicin does not penetrate the cell membrane so intracellular bacteria remain alive). After incubation with gentamicin, cells were washed thrice with PBS, lysed with Triton X-100 (1%, 20 min), and bacteria were serially diluted and grown in LB agar plates overnight at 37°C for quantification, expressed as CFU/ml. Results are presented as percent bacterial survival compared to infected well, not treated with inhibitors ('no treatment'). Only colony counting was used for bacterial quantification.

Immunofluorescence was used as a complementary approach for each treatment using green fluorescent protein-expressing *C. rodentium* (GFP-DBS 100, a generous gift from Dr. Bruce Vallance, University of British Columbia). Cells were grown on 13 mm coverslips, fixed with 4% PFA (15 min), and blocked in 2% Goat

Serum and 1% bovine serum albumin (15 min). Actin was stained with Alexa Fluor-594 phalloidin (1:40 dilution, 0.1% Triton, 0.2% Goat Serum, and 0.1% BSA, Fisher Scientific); 4', 6-diamidino-2-phenylindole (DAPI) (1:1000 dilution, 0.1% Triton, 0.2% Goat Serum, and 0.1% BSA) was used for nuclear staining. Slides were analyzed using Zeiss Axio Observer.Z1 microscope with ZEN Imaging software (Carl Zeiss Canada Ltd., Toronto, ON, Canada) and the illustrations were formatted for noise reduction and increased sharpness using Image J. All images were processed identically.

Phagocytosis was determined using the same method as above except with Fluoresbrite carboxylate YG one micron microsphere beads (Polysciences Inc., 18604) instead of bacteria. Beads were used to quantify phagocytosis as they are inert objects that cannot be destroyed by the maturation of the phagosomes. Quantification of intracellular beads was done using Image J and was presented as the mean number of beads per macrophage.

Cell toxicity

J774A.1 cells were seeded into 6-well dishes at 1 x 10^6 cells/well (80% confluence) overnight. Cells were either infected or left uninfected for 2 h, then ATP (2.5 mM) was added for 30 min, and then trypan blue (Life Technologies, 15250061) was added to the medium (only penetrates damaged/dying cells). Dead cells (stained by trypan blue) were blindly counted per field of view, using 20X magnification from

six random locations in each treatment. The percentage was calculated as the number of dead cells out of the total number of cells in each field of view.

Multiplex cytokine assay and IL-1β ELISA

J774A.1 cells were seeded into 6-well dishes at 1 x 10^6 cells/well (80% confluence) overnight. Infection proceeded as above, except gentamicin (100 µg/mL was left on overnight). Supernatants were collected the following day and analyzed using a multiplex Cytokine kit (Meso Scale Discovery, K15012B), measuring IL-1β, IL-6, IL-10, IL-12p70, keratinocyte chemoattractant/human growth-related oncogene (KC/GRO), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, following the manufacturer's instructions.

Intracellular pro and mature IL-1β were measured from cell lysates, along with secreted IL-1β collected from supernatants, using ELISA (R&D, DY401). In brief, cells were either inhibited for 1 h with YVAD (25 µM) or K^+ (45 mM) or left uninhibited and then infected with *C. rodentium* for 2 h. This was followed by 30 min ATP (2.5 µM) treatment with or without ROS inhibitors [NAC (20 µM), DPI (10 µM), or apocynin (4 mM)]. Cells were washed with PBS thrice and lysed with M-PER mammalian protein extraction reagent (Fisher Scientific, 78503). Protease inhibitor cocktail (0.08M EDTA, 0.08M phenylmethylsulfonyl fluoride, 0.8M pepstein) was added and the lysate was centrifuged at 18,800g for 10 min to pellet cell debris. Cell lysate supernatants were then centrifuged using 30k Amicon Ultra-4 centrifugal filters (following the

manufacturer's instructions) to separate the mature and pro forms of IL-1β, 17 kDa and 37 kDa, respectively.

ROS measurements

ROS was measured using a previously described method [130, 131]. In brief, J774A.1 macrophages were seeded overnight at 5 x 10^4 (80% confluence) cells per well in a 96-well plate. DCFDA (1 μM) was added for 30 min, then washed with PBS, and fresh DMEM was added for infection. Cells were either left uninhibited or inhibited for 1 h with YVAD (25uM) or K^+ and then infected with *C. rodentium* for 2 h, followed by 30 min ATP treatment (2.5 μM) with or without NAC (20 μM), DPI (10 μM), or apocynin (4 mM). Fluorescence was measured at Ex_{485} and Em_{535}. Fold changes in ROS production were normalized to control cells and compared to infected, untreated cell, which were considered as positive controls.

Statistical analysis

Statistical analysis was performed using Prism5 for Windows (Graph Pad, San Diego, CA, USA). Mann-Whitney two-tailed *t*-test was used for comparison between different treatments. Kruskal-Wallis ANOVA with Dunn's Multiple comparison test were used for phagocytosis determination and cell viability. Bar graphs represent the mean ± SEM and all comparisons with $P < 0.05$ were considered significant.

3.4 RESULTS

Addition of extracellular ATP enhances bacterial killing by macrophages

To determine if inflammasome activation using extracellular ATP would enhance bacterial killing, J774A.1 macrophages were stimulated with exogenous extracellular ATP after being infected with *C. rodentium*. A dose response analysis showed that both 2.5 mM and 5 mM concentrations of extracellular ATP for 30 min caused a significant reduction in intracellular survival of *C. rodentium* [($P<0.05$; **Fig.** 3.**1A&B**)]. A 2.5 mM concentration of extracellular ATP has been shown to be physiologically relevant to the gut setting and to activate the NLRP3 inflammasome and was therefore used in subsequent experiments [53, 132]. While the inflammasome inhibitors K^+ and YVAD had no effect on bacterial survival alone, YVAD and K^+ significantly increased bacterial survival ($P<0.05$; **Fig. 3.1C-E**) in the presence of ATP as compared to no treatment (NT). In addition, ATP was found to have no effect on phagocytosis as uptake of fluorescent microspheres was unaltered by the various treatments (**Fig. 3.1F&G**). Trypan blue exclusion showed that ATP-induced inflammasome activation did not result in increased cell death (**Fig.** 3.**1H**).

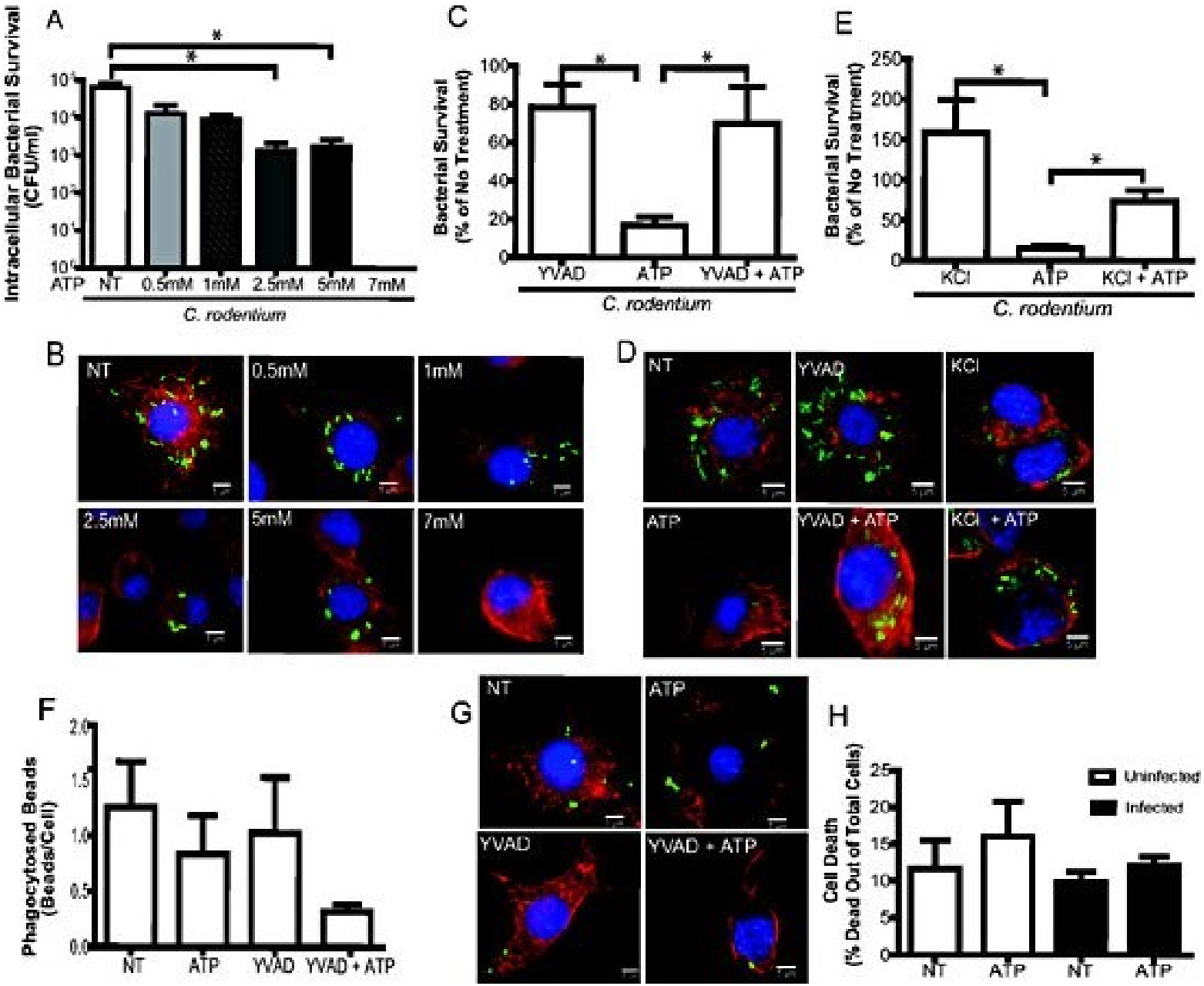

A
Intracellular Bacterial Survival (CFU/ml)
ATP NT 0.5mM 1mM 2.5mM 5mM 7mM
C. rodentium
C
Bacterial Survival (% of No Treatment)
YVAD ATP YVAD + ATP
C. rodentium
E
Bacterial Survival (% of No Treatment)
KCl ATP KCl + ATP
C. rodentium
B
NT
0.5mM
1mM
2.5mM
5mM
7mM
D
NT
YVAD
KCl
ATP
YVAD + ATP
KCl + ATP
F
Phagocytosed Beads (Beads/Cell)
NT ATP YVAD YVAD + ATP
G
NT
ATP
YVAD
YVAD + ATP
H
Cell Death (% Dead Out of Total Cells)
Uninfected
Infected
NT ATP NT ATP

Fig. 3.1. ATP induces and YVAD and K+ inhibit *C. rodentium* killing by macrophages with no effect on phagocytosis or cell death. ATP dose analysis was generated to determine optimal concentrations for bacterial killing ($P<0.05$; **A and B**). Macrophages were inhibited with YVAD (25 μM) or K+ (45mM) for 1 hr, infected with *C. rodentium* for 2 hr, and ATP (2.5 mM) was added for 30 min. Cells were then rinsed and gentamicin introduced to kill extracellular bacteria. Intracellular bacteria were quantified by plating overnight. In addition to confirming enhanced killing after addition of ATP, (**C**) YVAD significantly inhibited, while (**E**) K+ inhibition partially inhibited bacterial killing, supporting specific effects of ATP on the inflammasome. (**D**) Representative immunofluorescence images using DAPI (blue) for the nucleus, Phalloidin (red) for actin, and GFP-labelled (green) *C. rodentium*. Data in panels **C** and **E** are shown as a percent survival of *C. rodentium* as compared to infected, untreated cells (NT). Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test. N= 9-12. * $P<0.05$.(**F**) Macrophages were inhibited with YVAD (25 μM); fluorescent beads and 2.5 mM ATP were added. Numbers of intracellular beads were counted and presented as beads per macrophage. N=4. (**G**) Representative immunofluorescence images using DAPI (blue) for the nucleus, Phalloidin (red) for actin, and microsphere beads (green). (H) Macrophages were inhibited with YVAD (25 μM) or activated with 2.5 mM ATP. Cells were either infected (solid bars) or left uninfected (clear bars) for 2.5 hr; trypan blue was added and viability calculated by counting the proportion of dead cells per field of view. Statistical Analysis was done using one-way ANOVA with Tukey's multiple comparison test. N=3.

Extracellular ATP induces IL-1β maturation and reduces pro-inflammatory cytokine production

Addition of ATP did not increase secreted total IL-1β; however, both YVAD and K^+ significantly inhibited IL-1β secretion ($P<0.05$; **Fig. 3.2A**). The amount of the pro IL-1β was not changed by any of the treatments of infected cells, suggesting that, as expected, 'signal one' is independent of inflammasome manipulation (**Fig. 3.2B)**. Further supporting a specific effect on inflammasome activation, intracellular mature IL-1β was significantly increased by ATP and inhibited by K^+ and YVAD ($P<0.05$; **Fig. 3.2C**).

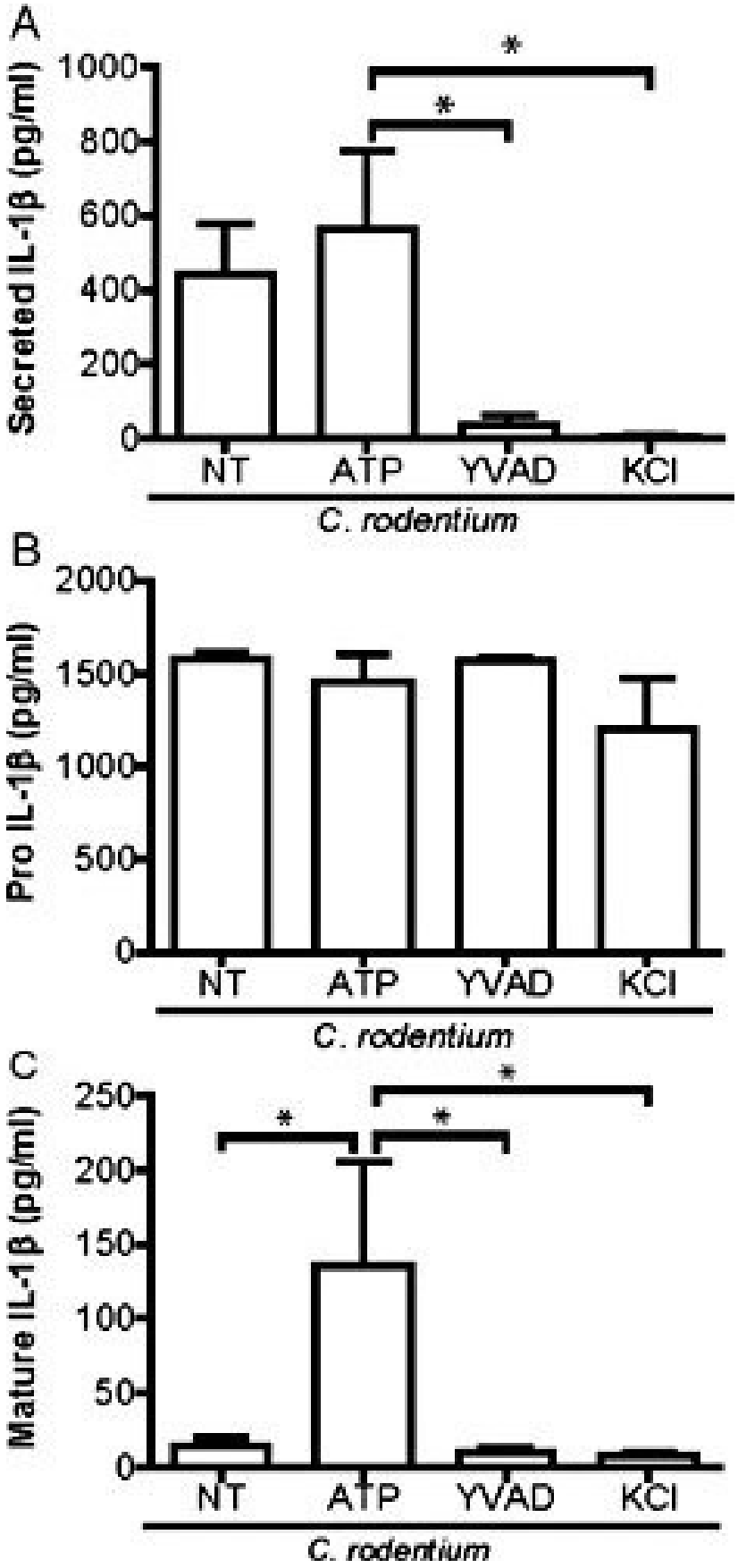
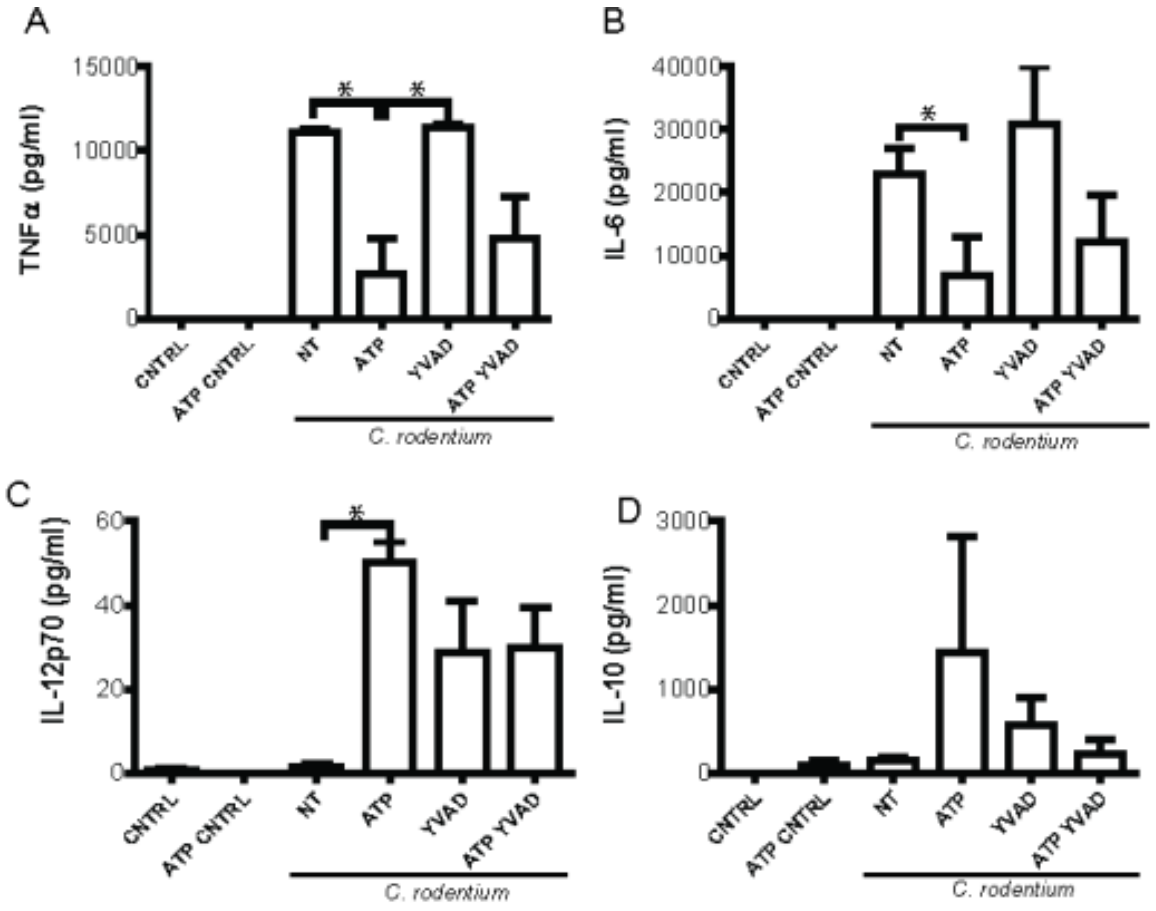

A
Secreted IL-1β (pg/ml)
1000
800
600
400
200
0
*
*
NT
ATP
YVAD
KCl
C. rodentium
B
Pro IL-1β (pg/ml)
2000
1500
1000
500
0
NT
ATP
YVAD
KCl
C. rodentium
C
Mature IL-1β (pg/ml)
250
200
150
100
50
0
*
*
NT
ATP
YVAD
KCl
C. rodentium

Fig. 3.2. ATP increases intracellular mature IL-1β but not pro IL-1β. Macrophages were inhibited with YVAD (25 μM) or K+ (45mM), infected, and 2.5 mM ATP added. (A) Secreted IL-1β, measured using ELISA, was not increased with the addition of ATP but was significantly reduced with the addition of K+ or YVAD. Cells were lysed using mammalian lysis buffer; pro (B) and mature (C) IL-1β were separated using 30 kDa centrifugal filters and measured using ELISA. ATP significantly increased intracellular mature IL-1β and K+ inhibited this increase. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test. *P<0.05. N=3 for pro IL-1β; n=8 for mature IL-1β

To determine potential effects of ATP activation of the inflammasome, beyond bacterial killing, specifically on the associated immune responses, we used a multiplex cytokine assay. TNF-α and IL-6 were decreased ($P<0.05$; **Fig. 3.3A&B**), whereas IL-12p70 was significantly increased after exposure of infected macrophages to ATP ($P<0.05$; **Fig. 3.3C**). There was no effect of ATP on KC/GRO, IFN-γ, or secreted IL-1β (data not shown). IL-10 did not show a significant change but did indicate an increasing trend with the addition of ATP (**Fig. 3.3D**). These findings not only support activation of the inflammasome by ATP but also demonstrate effects on other cytokines, specifically a reduction in the pro-inflammatory cytokines IL-6 and TNFα, related to bacterial clearance.


A
TNFα (pg/ml)
15000
10000
5000
0
*
*
CNTRL
ATP CNTRL
NT
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YVAD
ATP YVAD
C. rodentium
B
IL-6 (pg/ml)
40000
30000
20000
10000
0
*
CNTRL
ATP CNTRL
NT
ATP
YVAD
ATP YVAD
C. rodentium
C
IL-12p70 (pg/ml)
60
40
20
0
*
CNTRL
ATP CNTRL
NT
ATP
YVAD
ATP YVAD
C. rodentium
D
IL-10 (pg/ml)
3000
2000
1000
0
CNTRL
ATP CNTRL
NT
ATP
YVAD
ATP YVAD
C. rodentium

Fig. 3.3. **Pro-inflammatory cytokines are reduced by addition of ATP to infected macrophages**. Macrophages were inhibited with YVAD (25 μM), infected, and 2.5 mM ATP was added. Cells were then washed, and gentamicin introduced overnight to kill extracellular bacteria. Supernatants were collected and a multiplex cytokine array was conducted; data are shown for (**A**) TNFα, (**B**) IL-6, (**C**), IL-12, and (**D**) IL-10. ATP caused a reduction in pro-inflammatory mediators IL-6 and TNFα while showing an increase in IL-12 and a trend for the anti-inflammatory mediator IL-10. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test. N=5; *P<0.05.

Bactericidal effects of ATP are associated with an increase in ROS

To assess whether ROS is associated with, and possibly mediates the inflammasome-induced bacterial clearance, we used 2, 7-dichlorofluorescein diacetate (DCFDA) to measure ROS production. ROS production was significantly increased with the addition of ATP and this was inhibited by the inflammasome inhibitors YVAD and K^+ ($P<0.05$; **Fig. 3.4A&B**), as well as the ROS inhibitors NAC, apocynin, and DPI (used as controls; $P<0.05$; **Fig. 3.4C-E**), suggesting that ROS is associated with inflammasome-induced bacterial killing; however, it was not clear whether this was the cause (upstream) or result (downstream) of inflammasome activation.

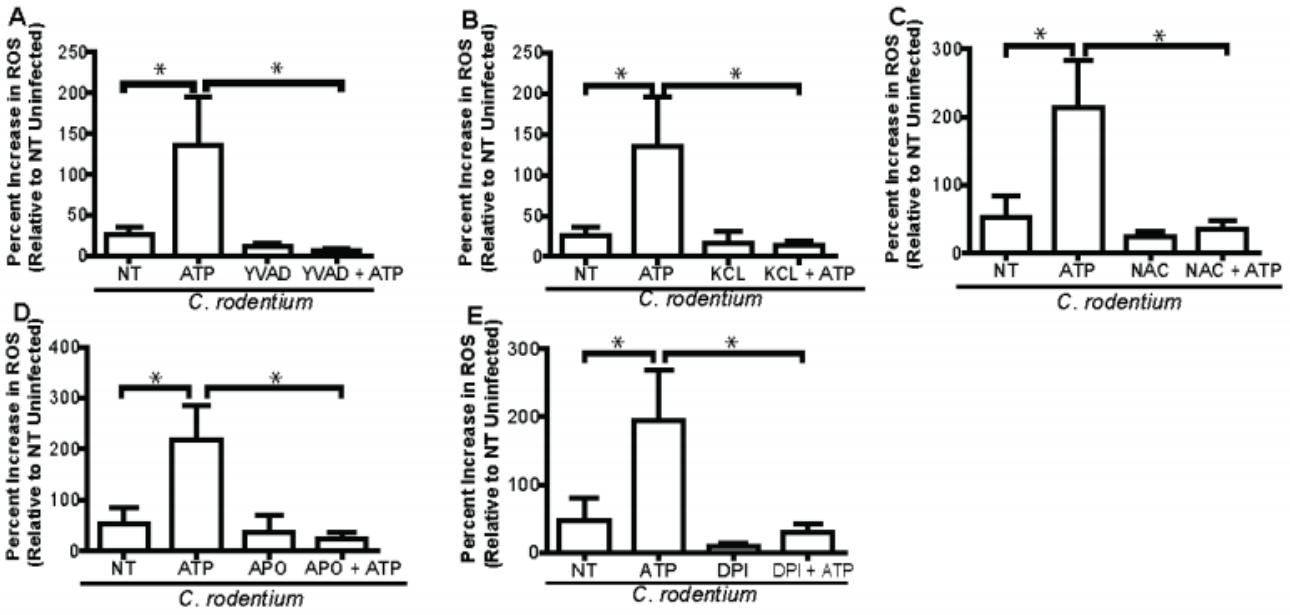


Fig. 3.4. **ATP-induced inflammasome activation increases intracellular reactive oxygen species (ROS) generation**. DCFDA was added to macrophages, which were then inhibited with YVAD (25 μM, A) or K+ (45mM, B), infected, and treated with 2.5 mM ATP with and without ROS inhibitors N-acetyl cysteine (NAC) (20 μM, C), apocynin (APO) (4 mM, D), or diphenyleneiodonium (DPI) (10 μM, E) followed by ROS measurement. Changes in ROS production were normalized to uninfected, untreated cells. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test. * $P<0.05$. N=3.

To address the causal relationship between ROS, the inflammasome, and bacterial killing by macrophages we used ROS inhibitors and assessed their impact on IL-1β production and bacterial survival with inflammasome activation. Inhibition of ROS did not reduce IL-1β release, indicating that inflammasome activation is not dependent on ROS (**data not shown**); however, NAC and apocynin significantly increased bacterial survival in the presence of ATP ($P<0.05$; **Fig. 3.5A&B**; the effect of DPI did not reach significance; **Fig.** 3.**5C**). This suggests that ATP-induced inflammasome activation and bacterial killing by macrophages are at least partially mediated by and dependent on ROS activity.

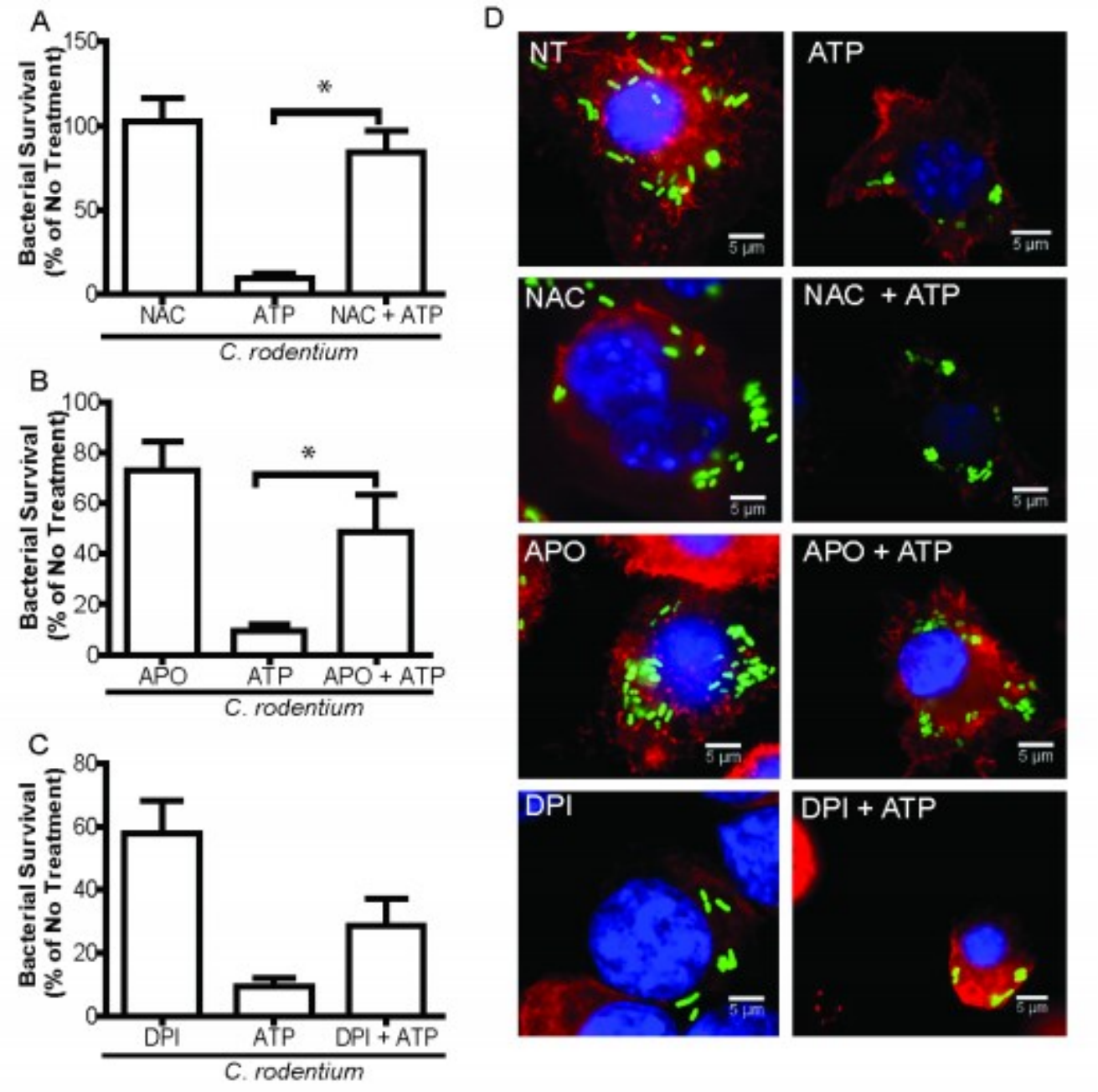

A
Bacterial Survival (% of No Treatment)
150
100
50
0
*
NAC
ATP
NAC + ATP
C. rodentium
B
Bacterial Survival (% of No Treatment)
100
80
60
40
20
0
*
APO
ATP
APO + ATP
C. rodentium
C
Bacterial Survival (% of No Treatment)
80
60
40
20
0
DPI
ATP
DPI + ATP
C. rodentium
D
NT
ATP
NAC
NAC + ATP
APO
APO + ATP
DPI
DPI + ATP
5 µm

Fig. 3.5. **Inhibition of ATP-driven intracellular reactive oxygen species (ROS) generation increases intracellular *C. rodentium* survival**. Macrophages were infected for 2 hr, and then inhibited with N-acetyl cysteine (20 μM), diphenyleneiodonium (10 μM), or apocynin (4 mM) with or without 2.5 mM ATP added. Cells were then washed and gentamicin introduced to kill extracellular bacteria. Intracellular bacteria were quantified by plating overnight and serial dilutions. (**A**) N-acetyl cysteine (NAC) and (**B**) apocynin (APO) significantly inhibited bacterial killing while (**C**) diphenyleneiodonium (DPI) did not.

(**D**) Representative immunofluorescence images using DAPI (blue) for the nucleus, Phalloidin (red) for actin, and GFP-labelled (green) *C. rodentium*. Data are shown as a percent survival of *C. rodentium* as compared to infected, no treatment (NT). Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test. N= 8-10. * $P<0.05$.

3.5 DISCUSSION

The complex relationship between bacteria and the immune response they induce, especially in the gut, is thought to contribute to immune-mediated conditions, such as IBD [133]. NOD2 is an innate immune cytosolic microbial sensor, which detects the muramyl dipeptide component of peptidoglycan; a frameshift mutation causing a NOD2 deficiency has been linked to Crohn disease susceptibility [20, 134]. The NLRP3 inflammasome is also an innate sensor of bacteria, and closely related to NOD2, that appears to have conflicting roles in different inflammatory conditions – detrimental in extraintestinal conditions and protective in IBD [135, 136]. We hypothesized that this discrepancy could be explained by the role of the NLRP3 inflammasome in bacterial killing by macrophages in the microbe-rich gut (in contrast to relatively sterile extraintestinal sites).

Using ATP to activate the NLRP3 inflammasome, we demonstrated an increased capacity of inflammasome-activated macrophages to kill intracellular bacteria, which is retarded when the inflammasome is suppressed using inhibitors, ROS scavengers, and ROS inhibitors, supporting specificity of our model. Interestingly, we found that more effective bacterial killing through inflammasome activation also reduced pro-inflammatory responses, with reductions in TNFα and IL-6; this may be relevant to IBD, where the inflammasome appears to be protective, as improved bacterial clearance by inflammasome-activated macrophages may reduce the uncontrolled immune activation, observed when bacteria are not effectively controlled. In contrast, in other chronic immune-mediated conditions, such as familial

Mediterranean fever, gout, and rheumatoid arthritis, inflammasomes are recognized as major contributors to pathogenesis, possibly since bacteria are not central to these conditions and failure of bacterial killing will not lead to more inflammation but secretion of excess IL-1β by the activated inflammasome might [120]. The common theme between these extraintestinal conditions is the overabundance of IL-1β and, in fact, anti-IL-1β drugs are being considered as potential treatments for these conditions [120]. However in Crohn disease an opposite (and protective) role for the inflammasome is suggested as reduced production of IL-1β and decreased expression of the *NLRP3* gene have been observed, which, given our findings, may reflect a deficiency in killing bacteria, leading to inappropriate immune activation [126].

Bacterial-induced inflammasome activation requires live bacteria, as ATP has been shown to protect mice against *E. coli* and *S. aureus* infections through neutrophil recruitment and inflammasome activation [51, 137]. We showed that, at physiologic concentrations, ATP reduced the survival of intracellular *C. rodentium* with inhibition by YVAD and partial inhibition by K^+. This suggests that ATP-induced inflammasome activation plays an important role in intracellular bacterial killing, supporting the findings of Xiang *et al.* with *E. coli* and *S. aureus* [51].

In order to differentiate between uptake by macrophages (phagocytosis), replication within macrophages, and bacterial killing by macrophages, we incubated macrophages with inert fluorescent beads and assessed effects of inflammasome manipulation. Neither inflammasome activation nor inhibition affected the rate of bead phagocytosis, suggesting that the inflammasome is not directly involved in

phagocytosis but rather in elimination of bacteria (or inhibiting bacterial replication), likely through the phagosome maturation process. Inflammasome activation can lead to pyroptosis, a form of programmed cell death, which could result in a loss of membrane integrity and gentamicin leakage, causing microbial cell death. Macrophage cell death was not increased by either ATP addition or *C. rodentium* infection, suggesting that the duration of infection and ATP exposure used were not causing late stages of cell death.

Intracellular pro-IL-1β was not different between any of the inflammasome manipulation treatments, which is expected as this requires 'signal one' that is provided by *C. rodentium.* As anticipated, intracellular mature IL-1β was significantly increased by ATP and inhibited by K^+ and YVAD. Another interesting observation was the change in cytokine profiles with the addition of ATP. This may suggest a switch from a Th_1/Th_{17} proinflammatory response to a more regulatory IL-10-mediated response due to activation of NLRP3 by ATP. IL-6 is required for differentiation of naïve T cells into Th_{17} cells and TNF-α is a major product of Th_1 cells; therefore, this observation is important in understanding the role of macrophages and bacterial killing during chronic infections. [138] As Crohn disease is known to be a Th_{17}-mediated inflammatory condition, the dysregulation (or dysfunction) of the inflammasome could lead to increased concentration of IL-6 and TNF-α, resulting in infiltration of Th_{17} cells into the lamina propria and more inflammation. This could be the consequence of impaired bacterial killing, as ATP appeared to reduce pro-inflammatory cytokines in our study [139].

There is some debate in the literature whether ROS activates the inflammasome or the reverse [140]. Our findings suggest that ATP activates the inflammasome, which then increases ROS production, as shown by ROS reduction with inflammasome inhibitors without a change in IL-1β production with ROS inhibitors. NAC is a ROS scavenger that is not specific to any organelle or complex and therefore does not contribute to our understanding of the mechanism for inflammasome-activated ROS production. DPI, a NADPH oxidase inhibitor, did not show any bactericidal inhibition, suggesting involvement of a different ROS producer outside the phagolysosome. Apocynin, another inhibitor of the NADPH oxidase complex and hydrogen peroxide scavenger, showed an increase in bacterial survival, suggesting that the ROS species responsible for microbial death is hydrogen peroxide and that this is not mediated by the NADPH oxidase complex [141]. In contrast to our findings, alveolar macrophages have been shown to have ROS-induced inflammasome activation in an LPS-ATP model [142]. Another study showed that inhibition of ROS results in decreased LPS and ATP-induced inflammasome activation in J774A.1 cells [118]. However, these studies illustrate the relationship between ROS production and the inflammasome either in a sterile inflammatory state or where there are no live bacteria present. In addition, patients with chronic granulomatous disease, lacking NADPH oxidase activity, are still capable of secreting normal levels of IL-1β [143]. This is in accordance with our findings that inflammasome activation was not affected when the three ROS inhibitors (NAC, DPI, or Apocynin) were added along with ATP to the macrophages during the infection. Nevertheless, it is possible that both ROS and the inflammasome are affected by

LPS, or other bacterial factors, through NOX activation, for example. While our study did not directly address this possibility, we did show that at least in part, inhibiting the inflammasome reduces ROS and that ROS is required to eliminate bacteria. Future studies will focus on delineating the mechanism of inflammasome-induced ROS production as there is some evidence suggesting that mitochondria, which can be affected by $P2X_7$ activation, interact with the NLRP3 inflammasome [144, 145].

In Summary, our *in vitro* study reinforces the importance of the inflammasome in regulating and clearing enteric pathogens and possibly explains the unique role of inflammasomes in the gut, in the context of IBD, and why this is different from other settings. Bacterial killing by the inflammasome is mediated by ROS production, as shown by use of ROS inhibitors. We also show that ATP (through inflammasome activation) changes the immune milieu in a way that could reduce the vicious inflammatory cycle in the gut. With these findings, we propose a model (**Fig. 3.6**) for ATP-induced inflammasome activation and ROS production during *C. rodentium* phagocytosis in J774A.1 macrophages. Understanding the mechanisms used by macrophages to kill bacteria, and therefore affect inflammatory responses, could lead to valuable insight into chronic inflammatory conditions, such as IBD, and potential new treatment approaches for these chronic conditions. Appropriate activation of the inflammasome may, therefore, improve control of intestinal bacteria and reduce the exaggerated compensatory immune response seen in IBD.

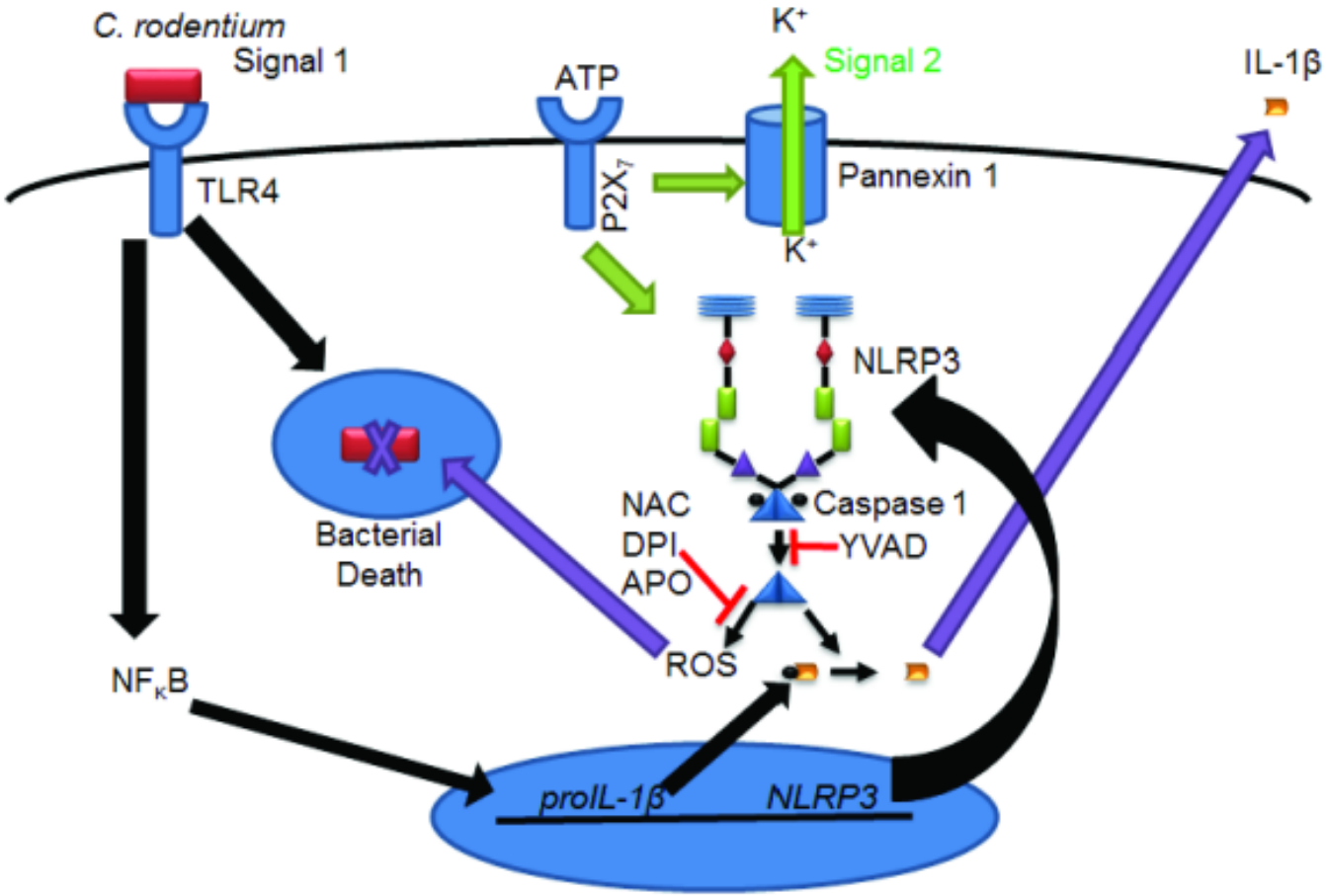

C. rodentium
Signal 1
TLR4
ATP
P2X7
K+
Signal 2
Pannexin 1
K+
IL-1β
NLRP3
Bacterial
Death
NAC
DPI
APO
Caspase 1
YVAD
ROS
NFκB
proIL-1β
NLRP3

Fig 3.6. Suggested model of ATP-induced inflammasome activation and ROS production. Black arrows: Bacterial components from *C. rodentium* act as 'signal 1', through Toll-like receptor (TLR) 4, which leads to the activation of nuclear factor-κB (NF-κB) and phagocytosis. NFκB signalling induces the transcription and translation of pro-IL-1β and NLRP3 proteins.

Green arrows: Extracellular ATP then provides 'signal 2' through the purinergic receptor P2X7, causing pannexin 1 activation and K+ efflux, leading to inflammasome activation. Activation of the inflammasome causes autocleavage of caspase-1 (inhibited by YVAD), which can then cleave pro IL-1β into its mature form that is secreted from the cell.

Purple arrows: Inflammasome activation also induces ROS production that can eliminate the phagocytosed bacterium and prevent inappropriate proinflammatory responses.

Chapter 4

Inflammasome Activation by ATP Induces ROS Generation through Mitochondrial Damage

CHAPTER 4. INFLAMMASOME ACTIVATION BY ATP INDUCES ROS GENERATION THROUGH MITOCHONDRIAL DAMAGE

This manuscript in preparation for submission

Authors: Michael Bording-Jorgensen, Heather Armstrong, Jeremy Jerasi, Dawson Lafleur, Eytan Wine

4.1 *ABSTRACT*

Background: The NLRP3 inflammasome is required for the maturation and secretion of the proinflammatory cytokine IL-1β. This complex has been shown to be important for macrophages to clear infections such as *Citrobacter rodentium*. The inflammasome requires two signals for activation, originating from many different stimuli. Extracellular ATP is a well characterized inflammasome activator acting as the second signal through the purinergic receptor P2X7. We have previously shown this activation can increase the ability of J774A.1 macrophages to clear *C. rodentium* in an *in vitro* environment through the generation of reactive oxygen species (ROS). In addition, we showed that ATP can induce the macrophages to change their cytokine profile. Our hypothesis was that extracellular ATP was inducing mitochondrial stress, causing the production of ROS, leading to microbial death.

Methods: We used the murine macrophage cell line J774A.1 and pathogen *C. rodentium*. Lysotracker red and MitoSOX were used to determine cellular location of bacteria and quantify mitochondrial ROS induction, respectively. Conditioned media

was used to determine if the effects could be transferred to newly infected macrophages. Cytokines were measured using ELISA and a proteome profiler, ROS was measured using DCFDA, Gasdermin D and Caspase 11 activities were observed by Western Blot.

Results: Lysotracker red staining shows *C. rodentium* is isolated within phagolysosomes and not in the cytoplasm. We observed the activation of mitochondrial ROS with the addition of ATP, independent of infection. sICAM-1, MIP-1α, and MCP-2 were all increased by ATP but not inhibited by YVAD. IP-10, TNFα, MIP-1β, M-CSF, and MIP-2 were increased by infection and not influenced by ATP or YVAD. Conditioned media from ATP-activated macrophages increased secretion of IL-1β, increased ROS production, and decreased intracellular survival of *C. rodentium*. Cleavage of Gasdermin D was increased with the addition of ATP but not inhibited by YVAD whereas Caspase 11 was unchanged between treatments.

Conclusion: Mitochondrial ROS production by extracellular ATP may be involved in the decrease of bacterial survival. Activation of the purinergic receptor induces the secretion of cytokines, chemokines, and other factors that affect newly infected macrophages independent of ATP. Gasdermin D cleavage independent of caspase 11 suggests that a different, yet uncharacterized pathway is activated; this may explain the lack of pyroptotic cells in our study. Understanding how the NLRP3 inflammasome is activated and the downstream pathways may lead to potential therapy for many conditions.

4.2 INTRODUCTION

The nod-like receptor protein complex 3 (NLRP3) is an immune sensor that is activated by a plethora of stimuli [146]. This complex requires two signals for activation. The first activates nuclear factor kappa B for transcription and translation of NLRP3, IL-1β, and IL-18. The second signal leads to the formation of the NLRP3 complex, which recruits the ASC domain; this induces autocleavage of the caspase 1 dimer to the active form, which can then cleave the pro IL-18 and pro IL-1β into their mature forms [48]. Neither of these proteins have a signal peptide sequence; therefore, they are either released from damaged cells or secreted via an unknown process. Recent evidence indicated that the inflammasome can activate caspase 11 in certain conditions, which cleaves gasdermin D. Gasdermin D then forms a pore in the cell membrane, initiating the inflammasome activated cell death process, termed pyroptosis [35, 147].

The NLRP3 inflammasome has been linked to many diseases such as rheumatoid arthritis, gout, diabetes, and inflammatory bowel diseases (IBD). This proinflammatory pathway is usually associated with an increased expression and secretion of IL-1β; conversely, polymorphisms in the *NLRP3* gene in some individuals with Crohn disease (CD) show increased expression but with a decreased secretion of IL-1β [126]. Individuals with IBD have a disrupted microbiome leading to dysbiosis; this dysbiosis coupled to a dysregulated immune system may lead to an inability to control potential pathogens or result in a loss of tolerance to commensals.

Extracellular ATP is a well characterized damage-associated molecular pattern (DAMP) that is released from necrotic cells. Macrophages can recognize extracellular ATP via the purinergic receptor P2X7, recruiting pannexin 1, which forms a transmembrane pore. Pannexin 1 causes K^+ efflux from the cell, down the concentration gradient through a poorly understood mechanism of activation; however, evidence shows K^+ efflux to be a crucial component [148]. It has been assumed that the P2X7 receptor induces ROS production and this activates the inflammasome; however, we have previously shown in certain conditions that the inflammasome can cause ROS generation. In addition, we have shown that ROS produced with the addition of extracellular ATP also reduces the survival of intracellular *C. rodentium*. This reduction of intracellular survival also leads to a change in the macrophage cytokine profile once the infection has been cleared to an IL-10 mediated response [82].

In this study we sought to understand the mechanism by which extracellular ATP induces ROS production. In addition, we also determined whether activated macrophages secrete factors, which will induce the same effect in newly infected macrophages without additional activation. Our hypothesis was that extracellular ATP causes mitochondrial stress, which produces ROS that then decreases intracellular bacterial survival.

4.3 MATERIALS AND *METHODS*

Cell culture

J774A.1 murine macrophage cell line (ATCC, TIB-67) was seeded as indicated for each experiment. Cells were maintained in DMEM, supplemented with 10% heat-inactivated fetal bovine serum; medium was replaced every two days and cells were passaged at 80% confluence for a maximum of 22 passages from thaw.

Immunofluorescence and live cell imaging

For live cell imaging, J774A.1 cells were seeded in 35mm tissue culture plates (Sarstedt) at a density of 5 x 10^5 cells (80% confluence) overnight to allow for adhesion. The medium was changed to antibiotic- and serum-free DMEM without phenol red and MitoSox (to visualize mitochondrial ROS, Life Technologies), or Lysotracker Red (binds to lysosomes, Life Technologies) were added 3 hours before imaging. At the time of imaging Hoescht (Immunochemostry) was added as a DNA label. Fluorescein labelled beads (2 μm, PolySciences Inc.) were added and at 15 min; ATP (2.5 mM) was added to the dish. Videos were taken using a spinning disk confocal microscope for the indicated time lapse.

Immunofluorescence images were taken by seeding J774A.1 macrophages on 12mm round coverslips at a density of 5 x 10^5 cells (80% confluency) overnight for adhesion. Experiment proceeded as for live cell imaging except that macrophages were fixed using 4% PFA and DAPI was used as the nuclear stain. Images were taken using a

spinning disk confocal microscope and processed using Image J (National Institute of Health, Maryland, USA).

Conditioned Media infection

J774A.1 cells were seeded in 24-well tissue culture plates at a density of 5 x 10^5 cells per well (80% confluence) overnight to allow for adhesion. Prior to infection, the medium was changed to antibiotic- and serum-free DMEM. *C. rodentium* (DBS100, a gift from the Sherman lab, University of Toronto) was cultured overnight in lysogeny broth (LB) at 37°C. *C. rodentium* was added at a multiplicity of infection (MOI) of 10:1 for 2 h of infection; then ATP (2.5 mM, Acros) was added for 30 min. Supernatants were centrifuged at 18,800 g for 10 min to pellet cell debris and syringe filtered (2 µm) to remove live cells. Protease inhibitor cocktail (0.08M EDTA, 0.08M phenylmethylsulfonyl fluoride, 0.8M pepstein, Sigma) was added and the samples stored at -20°C. Subsequent infection was done by adding the conditioned media (CDM,10X dilution) or conditioned media from macrophages previously exposed to ATP (CDMA, 10X dilution) at the time of infection. Bacterial survival was determined by lysing the macrophages with 1% Triton X-100 (Fisher Scientific) and serial dilutions and plating the *C. rodentium* on LB agar overnight (**Fig 4.1**).

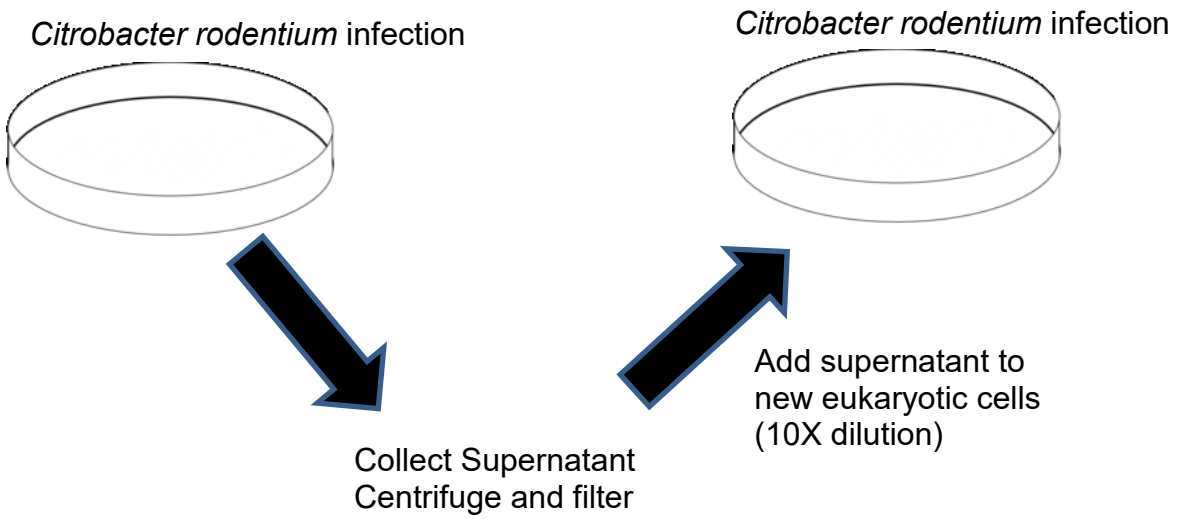


Fig 4.1. Generation, collection, and use of conditioned media from J774A.1 macrophages. J774A.1 macrophages were seeded onto plates overnight to allow for adhesion. Prior to infection, the medium was changed to antibiotic- and serum-free DMEM. *C. rodentium* was added at a multiplicity of infection (MOI) of 10:1 for 2 h of infection, then ATP (2.5 mM) was added for 30 min. Supernatants were centrifuged at 18,800 g for 10 min to pellet cell debris and syringe filtered (2 µm). Protease inhibitor cocktail was added, and the samples stored at -20°C. Subsequent infection was done by adding the conditioned media (10X dilution) at the time of infection.

ROS production quantification

ROS was measured using a previously described method [21, 22]. In brief, J774A.1 macrophages were seeded overnight at 5 x 10^4 (80% confluence) cells per well in a 96-well plate. DCFDA (1 μM, Sigma) was added for 30 min, then washed with PBS and fresh DMEM was added for infection. Cells were infected with *C. rodentium* for 2 h, followed by 30 min ATP treatment (2.5 μM) or Rotenone (40 μM, Sigma) with or without mitoTEMPO (100 μM, Sigma). Fluorescence was measured at ex485 nm and em535 nm using a spectrophotometer. Fold changes in ROS production were normalized to control cells and compared to infected, untreated cell, which were considered as positive controls.

Cytokine measurements

Secreted IL-1β was collected from supernatants, measured using ELISA (R&D, DY401). Cells were infected with live *C. rodentium*, heat killed (65°C for 15 min) *C. rodentium* (confirmed by plate growth), paraformaldehyde (4%) fixed *C. rodentium*, *E. coli* lipopolysaccharide (LPS; 10 ng/ml, Sigma), conditioned media (10X dilution), or LB broth supernatant from overnight growth of *C. rodentium* (10X diluted) for 2 hr followed by 30 min ATP (2.5 μM) treatment. Supernatants were centrifuged at 18,800g for 10 min to pellet cell debris. Protease inhibitor cocktail (as above) was added and the samples stored at -20°C.

Cytokine Profile was done using a proteome profiler following manufacturers protocol (R&D Systems). In brief, cells were seeded into 6-well dishes at 1 x 10^6 cells/well (80% confluence) overnight. Cells were then treated with the caspase 1 inhibitor AC-

YVAD-CMK (25 μM, Enzo Life Sciences) to inhibit the inflammasome for 1 h pre-infection. *C. rodentium* was added at an MOI of 10:1 for 2 h of infection; then ATP (2.5 mM) was added for 30 min. Supernatants were centrifuged at 18,800g for 10 min to pellet cell debris. Protease inhibitor cocktail was added and pooled together. Total protein content was measured using a Bradford assay and a total of 50 μg of protein was added to the kit. Cytokines were measured using fluorescence (Odyssey) and fold change was calculated based on intensity values compared to the kit controls.

Western Blot

J774A.1 cells were seeded in 100 cm^2 culture plates and allowed to grow until 80% confluence. Prior to infection, the medium was changed to antibiotic- and serum-free DMEM. *C. rodentium* was cultured overnight in LB at 37°C. Cells were treated with the caspase 1 inhibitor AC-YVAD-CMK (25 μM, Enzo Life Sciences, ALX-260-028) to inhibit the inflammasome for 1 h preinfection.

C. rodentium was added at a MOI of 10:1 for 2 h of infection; then ATP (2.5 mM) was added for 30 min. Cells were lysed using RIPA Buffer containing protease inhibitor cocktail (Sigma), then centrifuged at 18,800 g for 10 min to pellet cell debris. SDS and 2-mercaptoethanol was added to the supernatants and lysates, boiled for 5 min at 95°C and 25 μg was run on a 12% SDS-PAGE precast gel (Biorad). Proteins were transferred to a PVDF membrane, membranes were blocked (Aquablock), and then probed with their respective primary antibodies rabbit anti-caspase 11 (1:200, Abcam), rabbit anti-gasdermin D (1:200, Abcam) or mouse anti-actin (1:5000, Abcam) overnight. Membranes were washed with 0.05% PBST and the secondary

antibodies added goat anti-rabbit 680 (1:1000, IRDYE 680) and goat anti-mouse 800 (1:1000, IRDYE800). Images were captured using a LICOR odyssey and processed with FIJI.

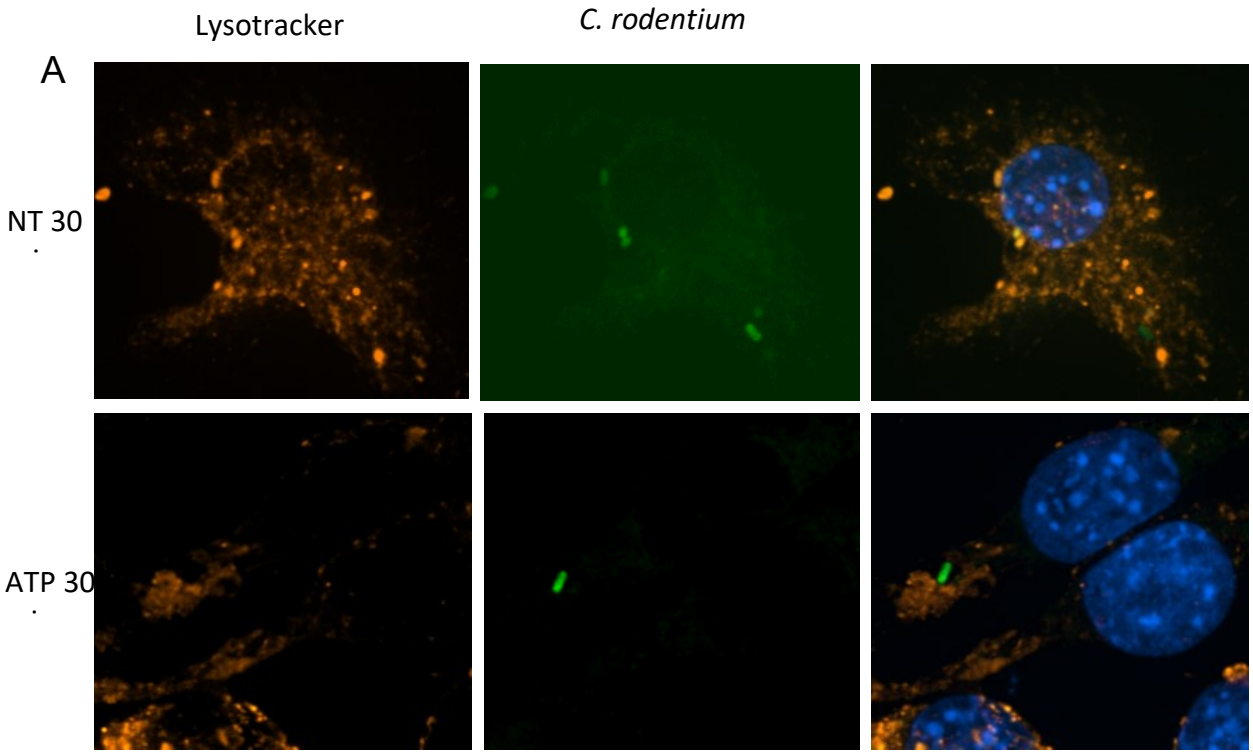
Statistical Analysis

Statistical analysis was performed using Prism5 for Windows (Graph Pad, San Diego, CA, USA). Kruskal-Wallis ANOVA with Dunn's Multiple comparison test were used for comparison. Bar graphs represent the mean $\pm$ SEM and all comparisons with $P < 0.05$ were considered significant

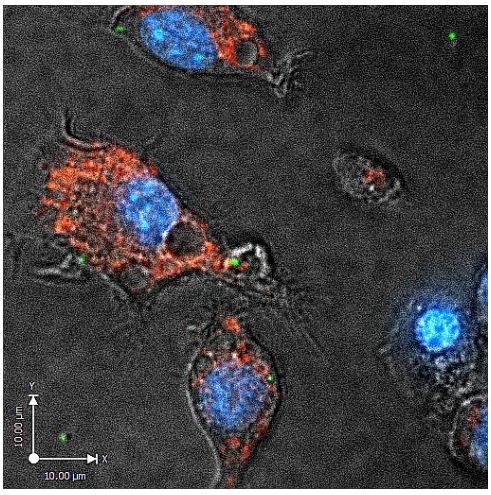
4.4 *RESULTS*

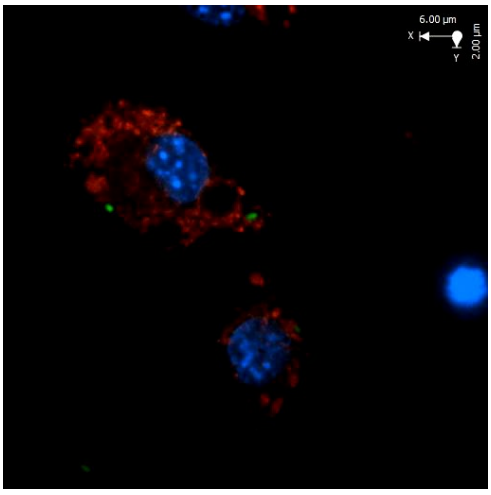
Citrobacter rodentium **clearance in macrophages is mediated through mitochondrial ROS production**

Lysotracker red was used to determine whether *C. rodentium* was found within lysosomes in macrophage upon phagocytosis. After 30 min, *C. rodentium* was found within lysosomes throughout the macrophage and this was not affected with the addition of extracellular ATP, as demonstrated using confocal or live cell imaging (**Fig 4.2 A-C**). Next, Mitosox Red showed using fixed and live cell staining that extracellular ATP induced mitochondrial ROS production without the presence of bacteria (**Fig 4.2 D-E**). Rotenone was used as a positive control as it disrupts complex I of the electron transport chain and MitoTempo is a mitochondrial ROS inhibitor, which significantly ($p<0.05$) reduced ROS production when ATP was added during *C. rodentium* infection (**Fig 4.2 F**).



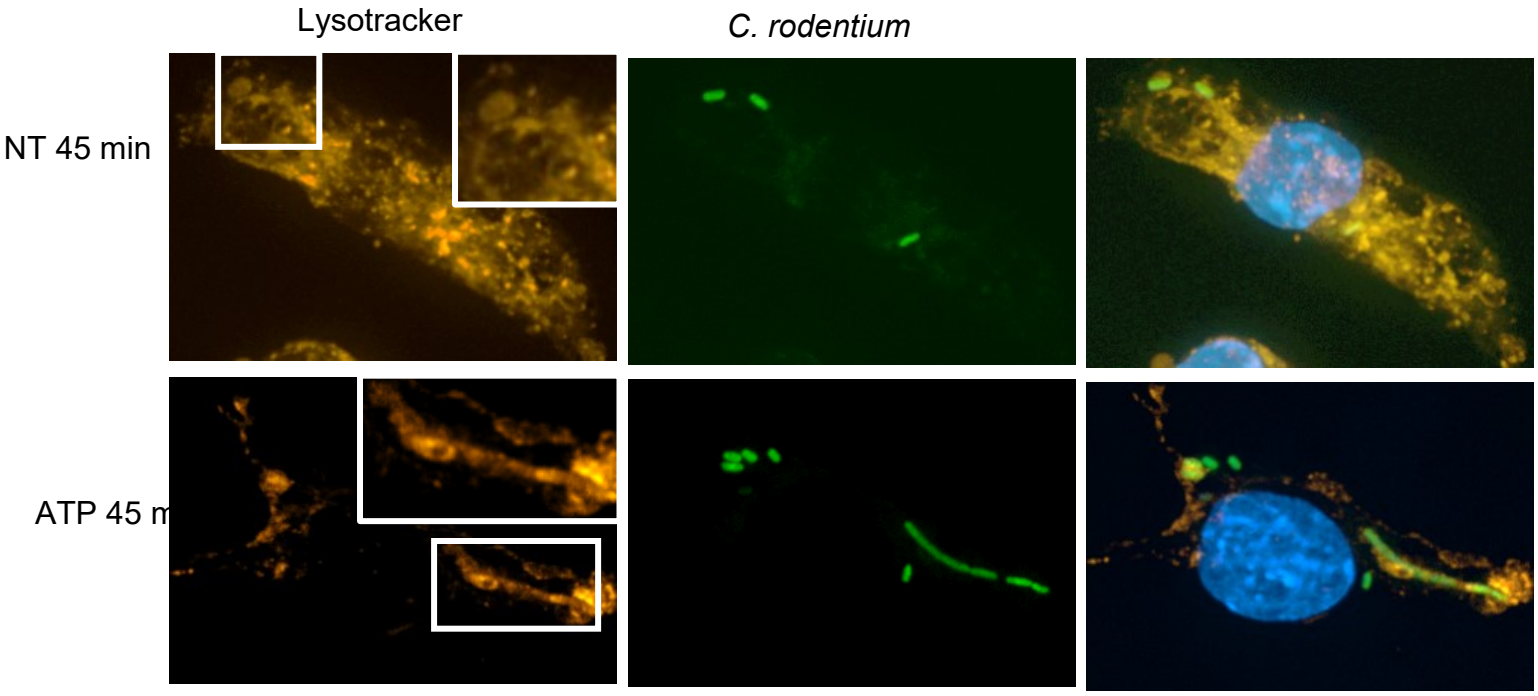
B



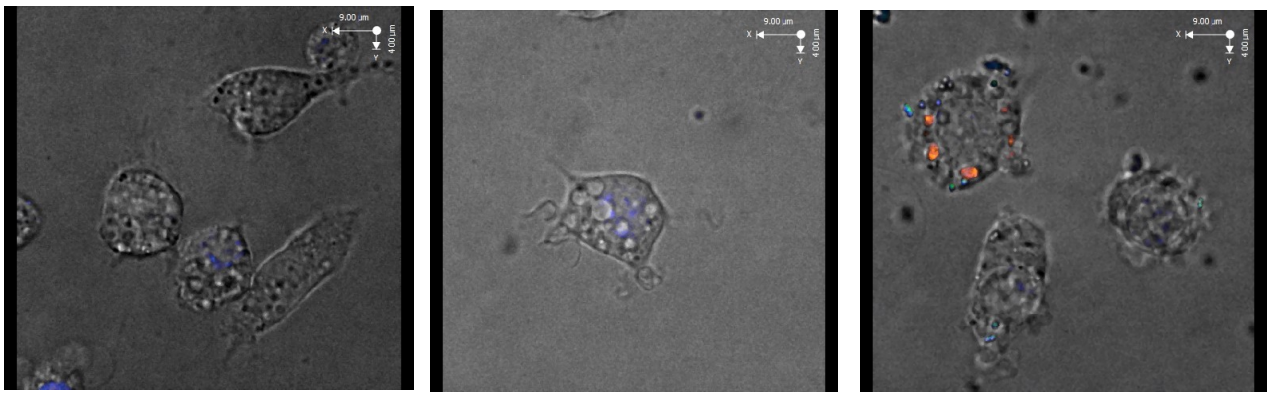


Lysotracker
Hoescht
C. rodentium

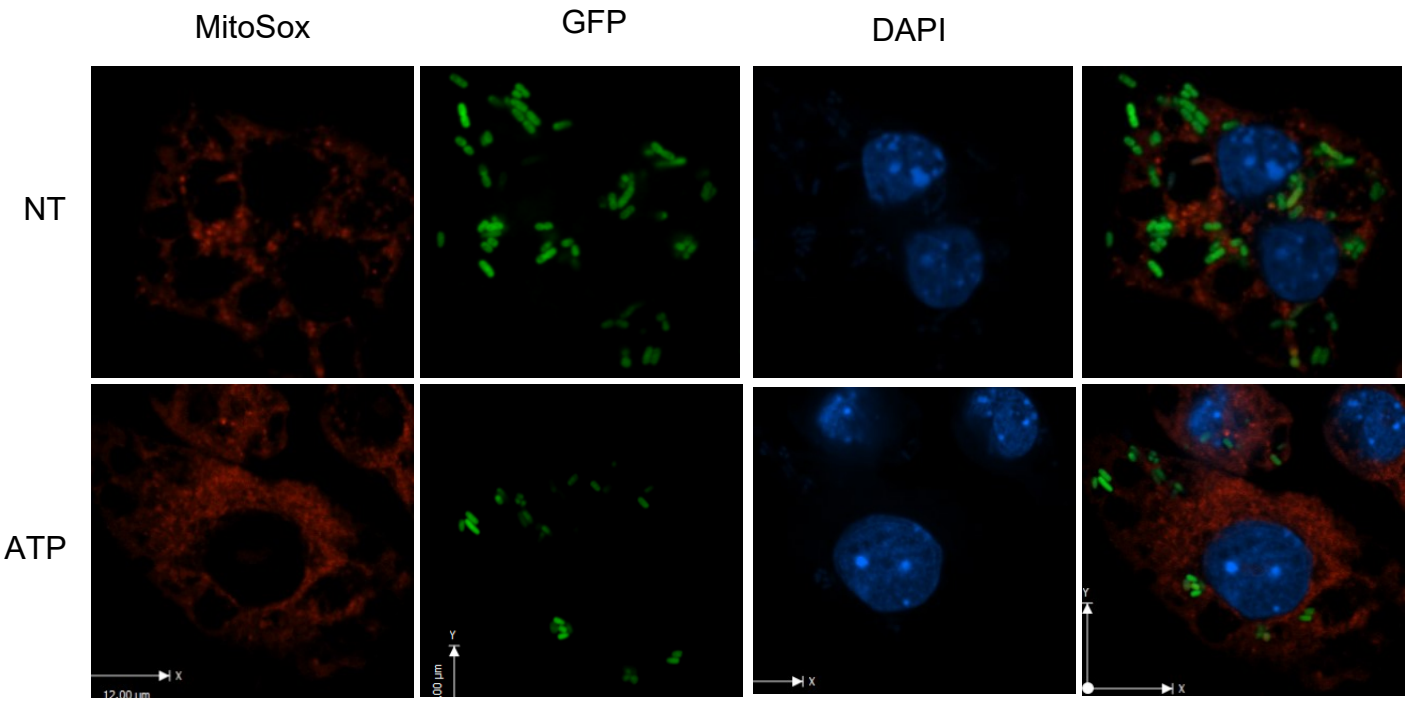
C


Lysotracker
C. rodentium
NT 45 min
ATP 45 m

D


Control
Beads
Beads + ATP

E


MitoSox
GFP
DAPI
NT
ATP

F

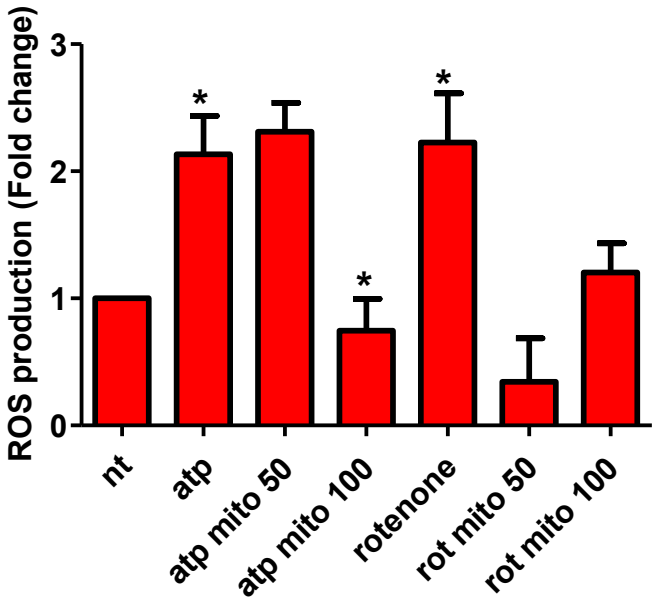
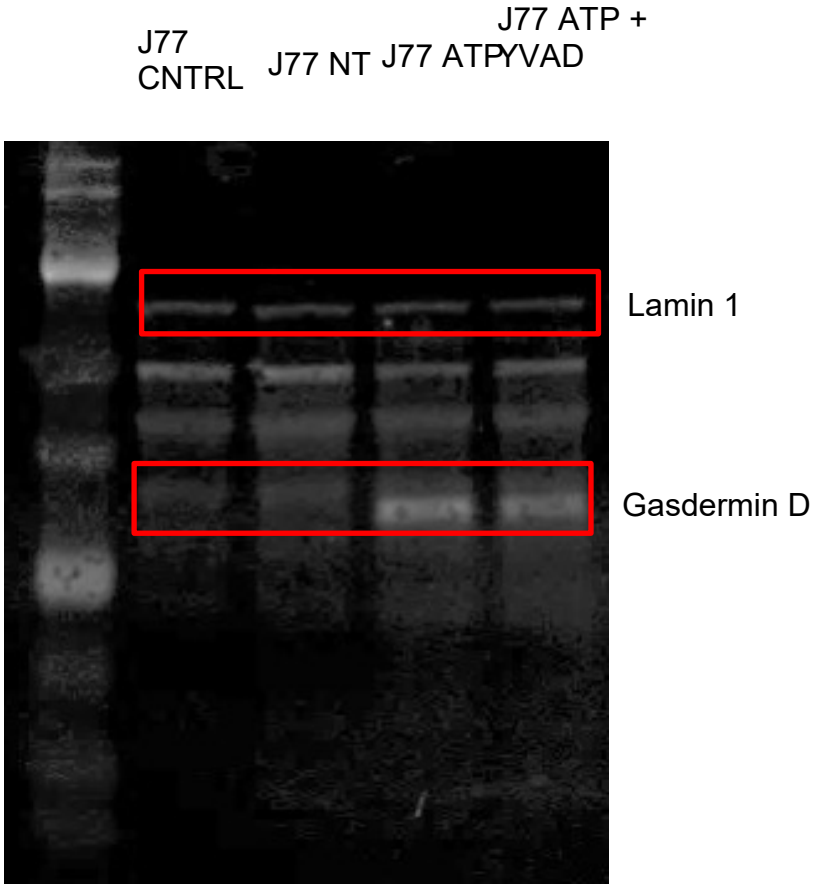

ROS production (Fold change)
0
1
2
3
*
*
*
nt
atp
atp mito 50
atp mito 100
rotenone
rot mito 50
rot mito 100

Fig 4.2. Extracellular ATP induces mitochondrial ROS production in J774A.1 macrophages. (**A-C**) J774A.1 macrophages were infected with GFP- *C. rodentium*; ATP (2.5 mM) was added for 30 min and dyed with lysotracker red, a lysosomal marker (insert indicates *C. rodenitum* within a phagolysosome. (**D**) J774A.1 macrophages were infected with GFP- *C. rodentium*, ATP (2.5 mM) was added for 30 min and dyed with MitoSox, a marker for mitochondrial ROS generation. (**E**) J774A.1 macrophages were infected with fluorescein labelled beads; ATP (2.5 mM) was added for 30 min and dyed with MitoSox, a marker for mitochondrial ROS generation and imaged using live cell imaging. (**F**) DCFDA was added to macrophages, which were then inhibited with mitoTEMPO (50 or 100 μM) or K+ (45mM) and treated with 2.5 mM ATP or rotenone (40 μM) followed by ROS measurement. Changes in ROS production were normalized to untreated cells. Images were analyzed using spinning disk confocal microscopy. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test. * $P<0.05$. N=3.

Gasdermin D is activated, independent of caspase 11, with the addition of extracellular ATP during *C. rodentium* infected.

Western Blot analysis showed an increased cleavage of Gasdermin D with the addition of ATP and this was not inhibited with the addition of YVAD (**Fig 4.3A**). Caspase 11 activity was unchanged by either infection or the addition of ATP and YVAD (**Fig 4.3B**).

A


J77 CNTRL
J77 NT
J77 ATP
J77 ATP + YVAD
50 kDa
25 kDa
Lamin 1
Gasdermin D

B

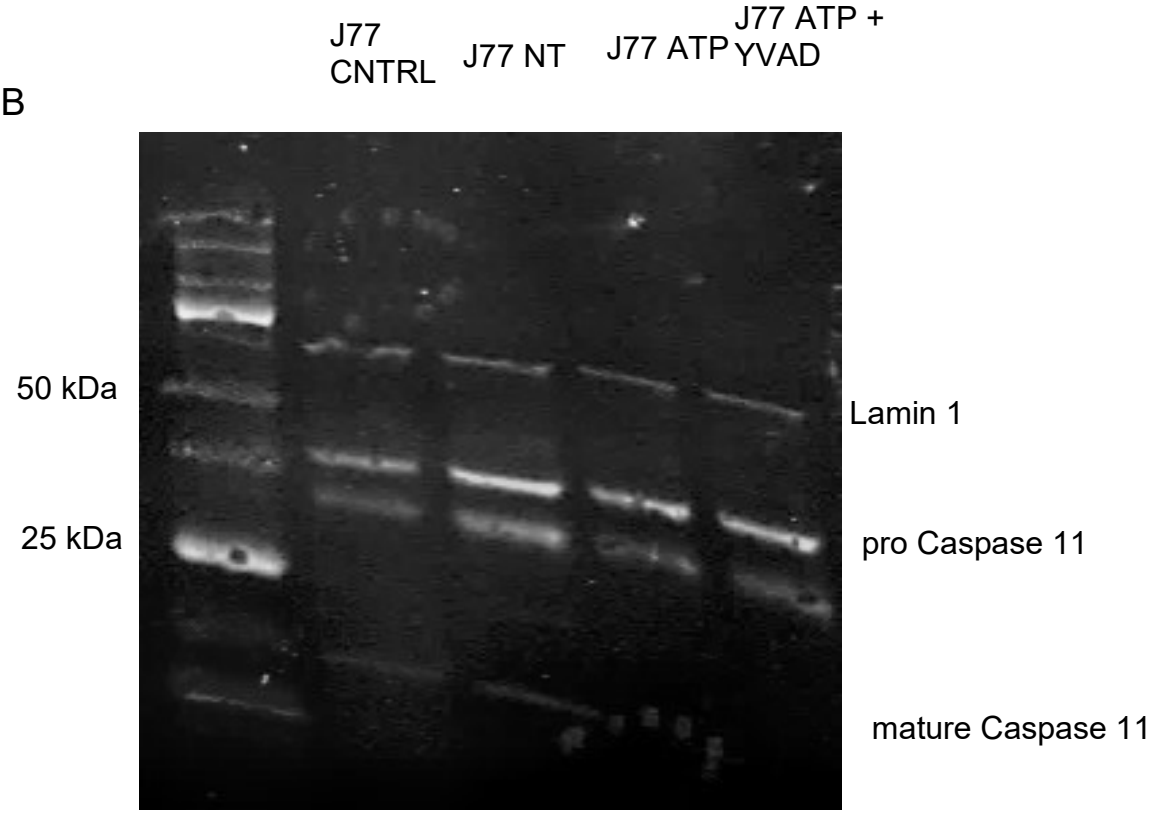
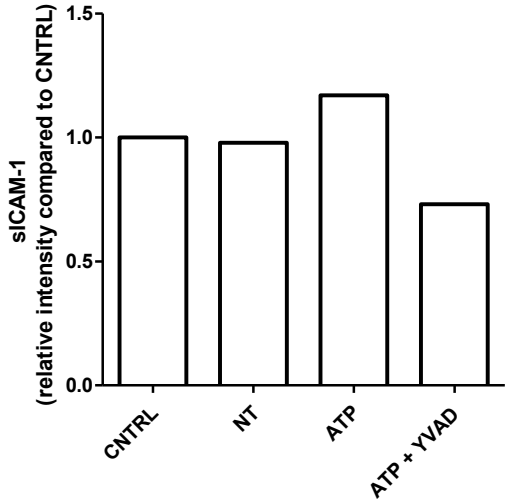

J77 CNTRL
J77 NT
J77 ATP
J77 ATP + YVAD
50 kDa
25 kDa
Lamin 1
pro Caspase 11
mature Caspase 11

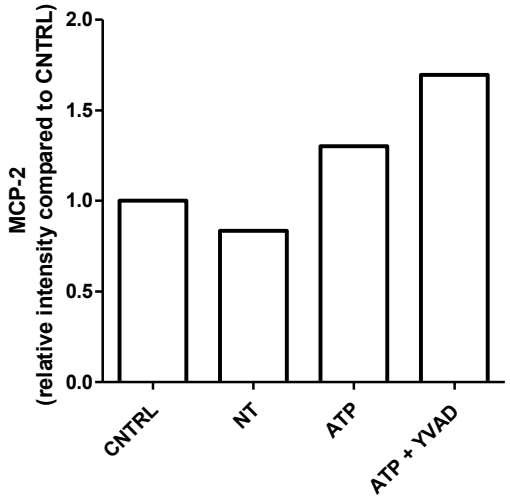
Fig 4.3. Caspase 11 is not required for Gasdermin D processing during *C. rodentium* infection. Western blot analysis of J774A.1 macrophage lysates after infection with *C. rodentium*, activated with ATP (2.5mM) and inhibited with YVAD (25 μM). Lysates were analyzed for (**A**) gasdermin D or (**B**) caspase 11 with Lamin 1 as the loading control. Images were taken using an Odyssey.

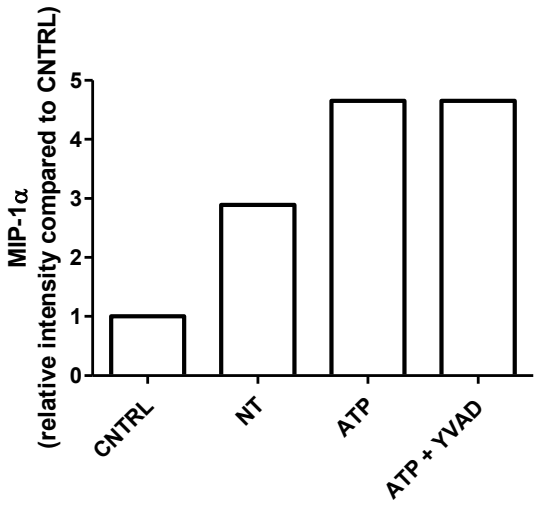
Extracellular ATP induces the secretion of cytokines independent of inflammasome activation

A proteome profiler was used to determine if the addition of ATP and subsequent mitochondrial ROS production, changed the inflammatory response during *C. rodentium* infection. Soluble inter-cellular adhesion molecule-1 (sICAM-1), MIP-1α, and MCP-2 were all increased with ATP with YVAD inhibiting sICAM-1 (**Fig 4.4A**). IL-1ra, TREM, and RANTES were not affected by any of the treatments (**Fig 4.4B**). IP-10, TNFα, M-CSF, MIP-1β, and MIP-2 were increased by infection but neither ATP nor YVAD had any additional effect (**Fig 4.4C**). MIP-1α was then assessed using ELISA but was not significantly increased with the addition of ATP (**Fig 4.4D**).

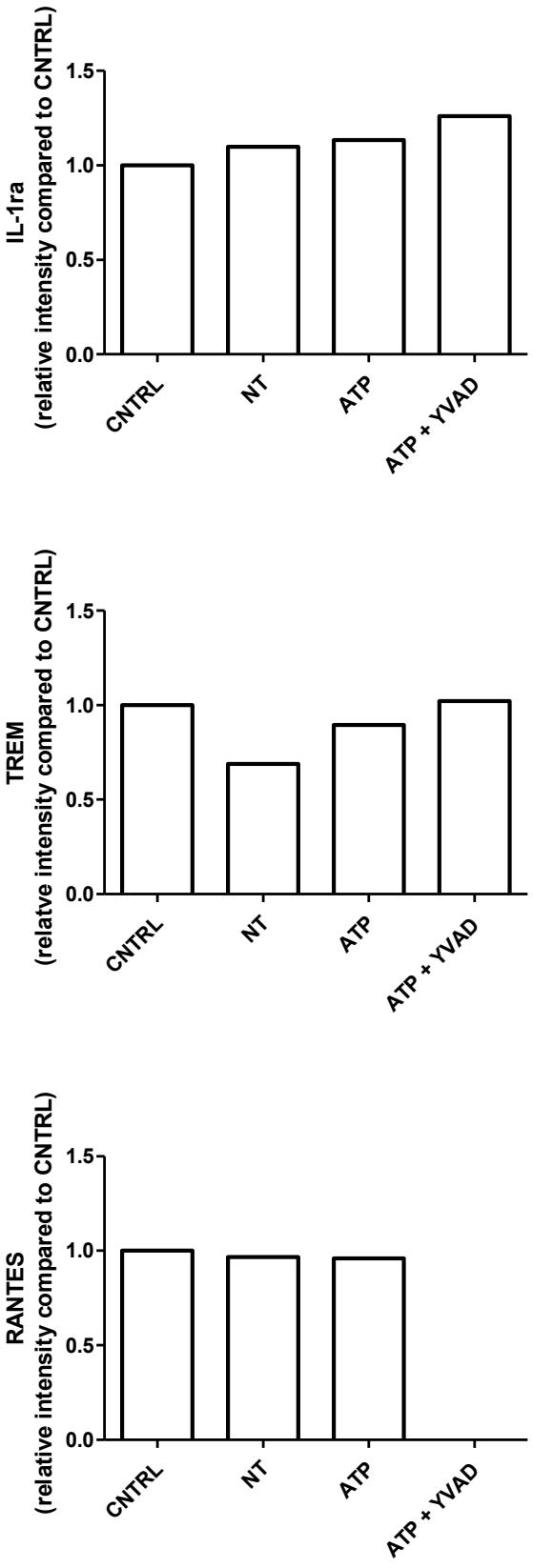
A


sICAM-1
(relative intensity compared to CNTRL)
1.5
1.0
0.5
0.0
CNTRL
NT
ATP
ATP + YVAD

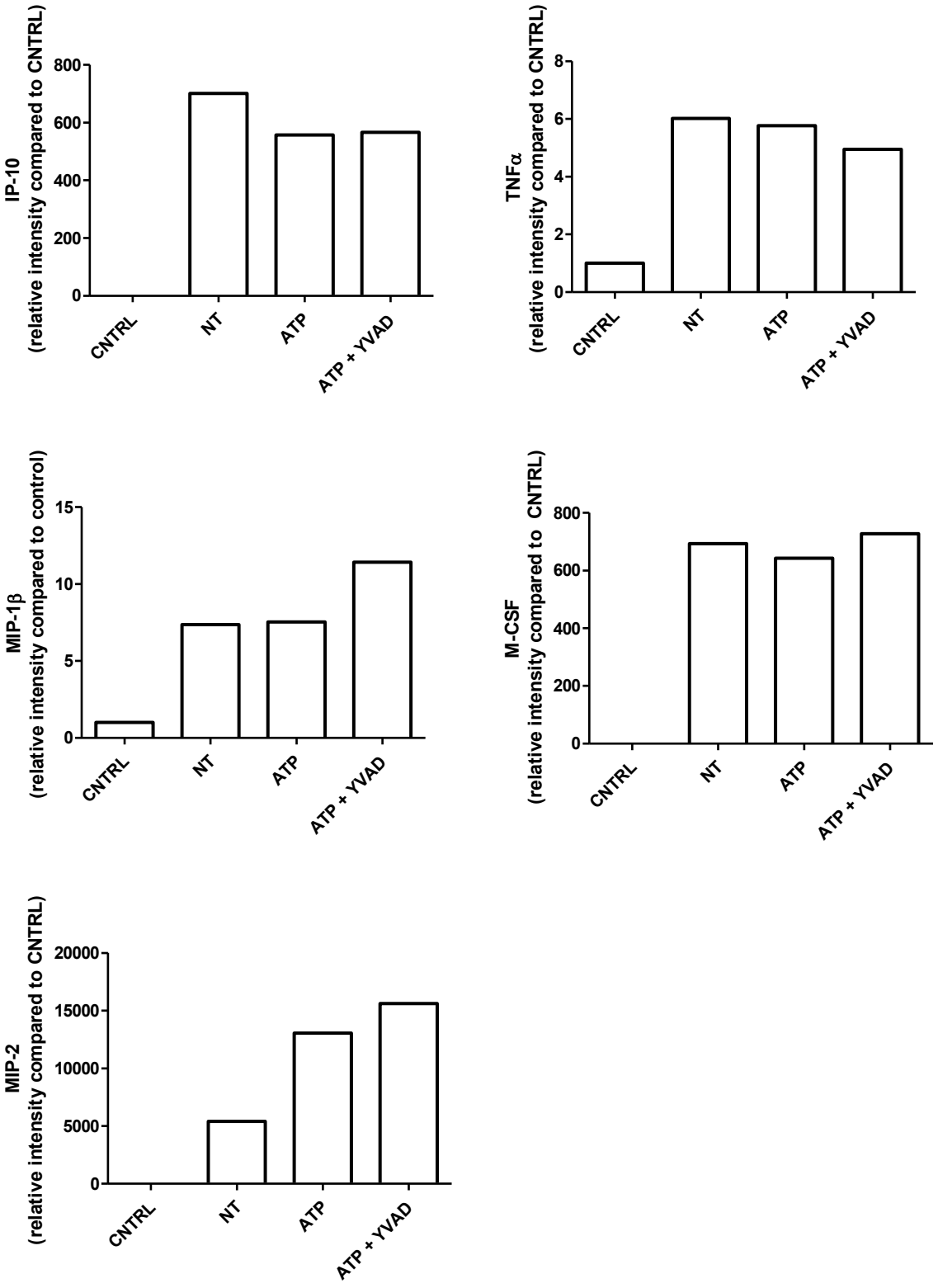

MCP-2
(relative intensity compared to CNTRL)
2.0
1.5
1.0
0.5
0.0
CNTRL
NT
ATP
ATP + YVAD


MIP-1α
(relative intensity compared to CNTRL)
5
4
3
2
1
0
CNTRL
NT
ATP
ATP + YVAD

B


IL-1ra
(relative intensity compared to CNTRL)
1.5
1.0
0.5
0.0
CNTRL
NT
ATP
ATP + YVAD
TREM
(relatve intensity compared to CNTRL)
1.5
1.0
0.5
0.0
CNTRL
NT
ATP
ATP + YVAD
RANTES
(relative intensity compared to CNTRL)
1.5
1.0
0.5
0.0
CNTRL
NT
ATP
ATP + YVAD

C


IP-10
(relative intensity compared to CNTRL)
800
600
400
200
0
CNTRL
NT
ATP
ATP + YVAD
TNFα
(relative intensity compared to CNTRL)
8
6
4
2
0
CNTRL
NT
ATP
ATP + YVAD
MIP-1β
(relative intensity compared to control)
15
10
5
0
CNTRL
NT
ATP
ATP + YVAD
M-CSF
(relative intensity compared to CNTRL)
800
600
400
200
0
CNTRL
NT
ATP
ATP + YVAD
MIP-2
(relative intensity compared to CNTRL)
20000
15000
10000
5000
0
CNTRL
NT
ATP
ATP + YVAD

D

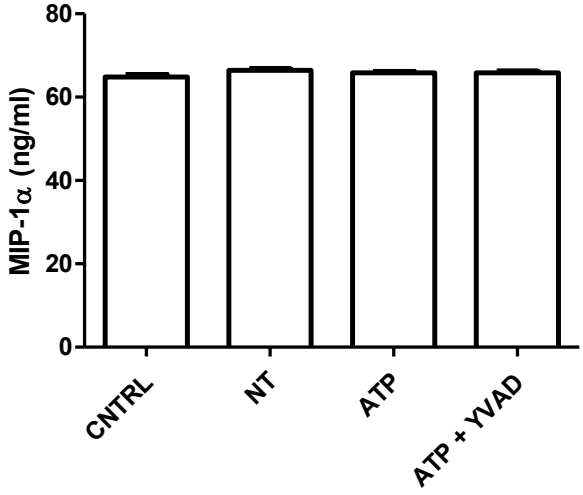
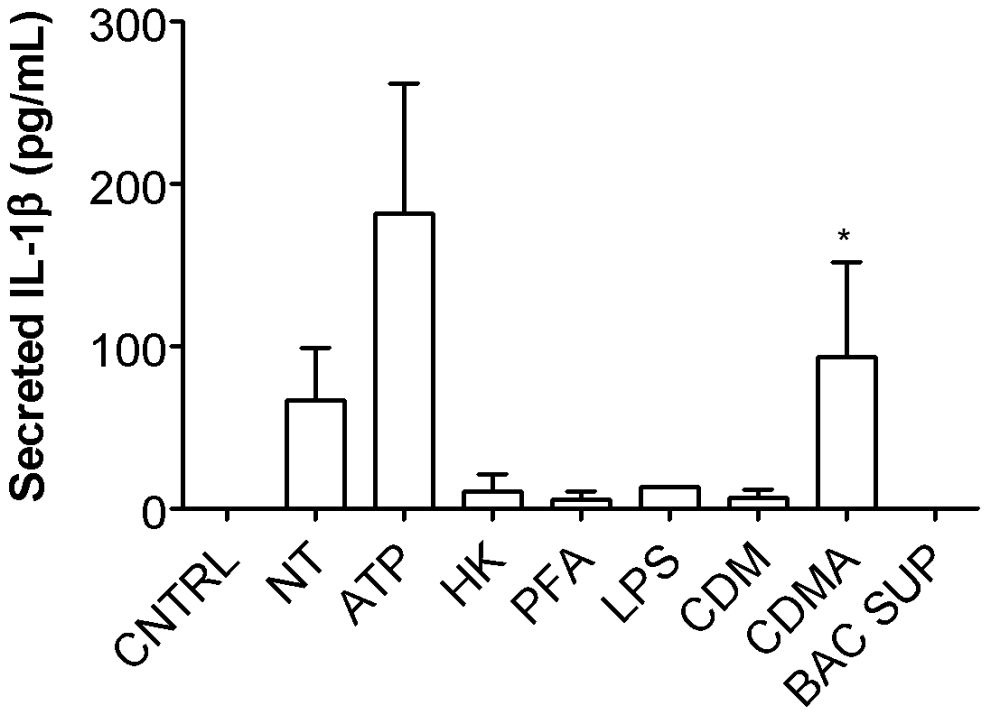


Fig 4.4. Extracellular ATP and *C. rodentium* infection induce different cytokine profiles from J774A.1 macrophages. (**A-C**) J774A.1 cells were treated with the caspase 1 inhibitor AC-YVAD-CMK (25 μM) to inhibit the inflammasome for 1 h pre-infection. *C. rodentium* was added at a multiplicity of infection of 10for 2 h of infection; then ATP (2.5 mM) was added for 30 min. Protease inhibitor cocktail was added and samples (N = 5) were pooled together. Total protein content was measured using a Bradford assay and a total of 50 μg of protein was added to the assay. Cytokines were measured using fluorescence (Odyssey) and fold change was calculated based on intensity values compared to the kit controls. Cytokine analysis are separated into those where (**A**) ATP showed an increase, (**B**) those that had no difference in secretion, and (**C**) those that were affected by infection alone. (**D**) MIP-1α was increased in the proteome profiler with the addition of ATP, so it was further analyzed using an ELISA for quantitation. N = 10.

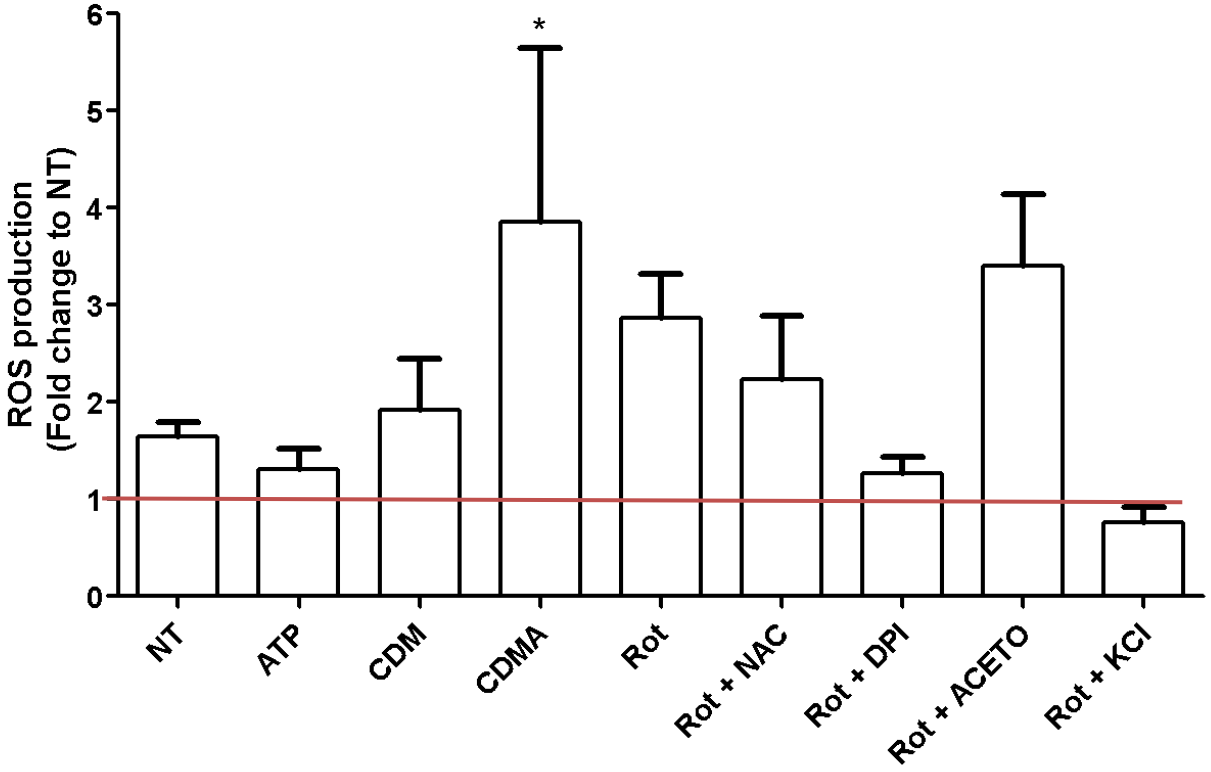
Secreted factors from ATP-induced macrophages induce ROS and activate the inflammasome during subsequent infection.

Previous results show extracellular ATP influences mitochondrial ROS production and results in cytokine secretion independent of the inflammasome. Next, we assessed whether the secretions from these cells can influence naïve macrophages. Conditioned media from ATP-induced macrophages significantly ($p < 0.05$) increased both IL-1β secretion and ROS production in non-noninfected macrophages (**Fig 4.5A-B**). Conditioned media significantly ($p < 0.05$) reduced the intracellular survival of newly infected macrophages with *C. rodentium* independent of ATP (**Fig 4.5C**).

A


Secreted IL-1β (pg/mL)
300
200
100
0
*
CNTRL
NT
ATP
HK
PFA
LPS
CDM
CDMA
BAC SUP

B


ROS production
(Fold change to NT)
6
5
4
3
2
1
0
*
NT
ATP
CDM
CDMA
Rot
Rot + NAC
Rot + DPI
Rot + ACETO
Rot + KCl

C

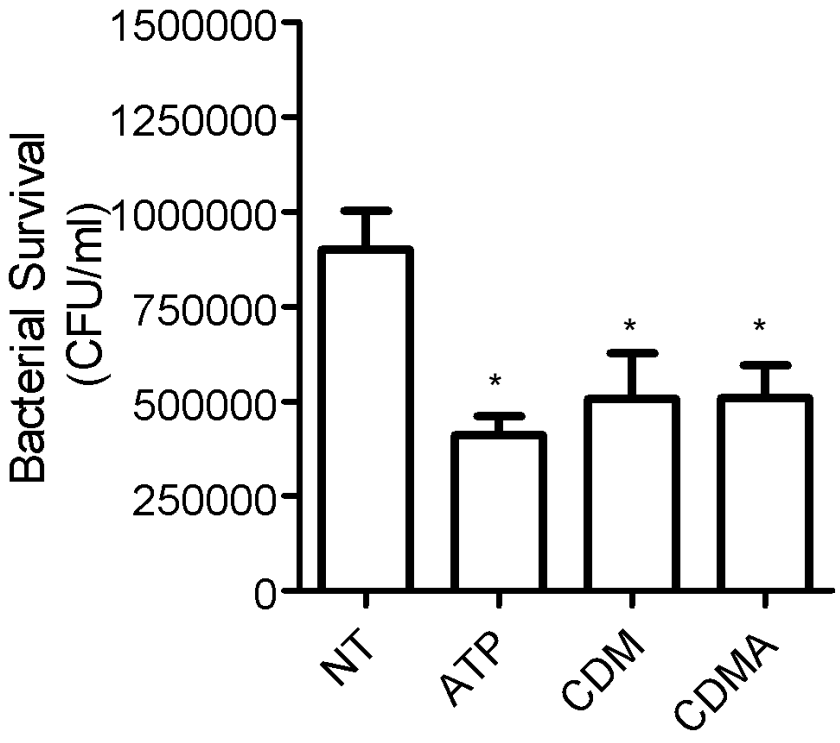

Bacterial Survival
(CFU/ml)
1500000
1250000
1000000
750000
500000
250000
0
*
*
*
NT
ATP
CDM
CDMA

Fig 4.5. Naïve J774A.1 macrophages are activated by supernatants from ATP-induced macrophages. (**A**) J774A.1 macrophages were infected with *C. rodentium*, *C. rodentium* + ATP (2.5 mM), LPS (10 ng/ml), PFA fixed *C. rodentium*, heat killed (HK) *C. rodentium*, supernatant from bacteria grown in LB, or conditioned media for 2 hrs. Supernatant was then collected and analyzed for IL-1β secretion by ELISA. N=6, *p < 0.05. (**B**) ROS was measured using DCFDA on J774A.1 macrophages treated with ATP (2.5 mM), rotenone (40 μM) or conditioned media (diluted 10X). ROS was inhibited using KCl (45 mM,) N-acetyl cysteine (NAC) (20 μM), apocynin (ACETO) (4 mM), or diphenyleneiodonium (DPI) (10 μM) followed by ROS measurement. Changes in ROS production were normalized to untreated cells. (C) Conditioned media was added to macrophages infected with *C. rodentium* for 2 hrs. The media was then changed to DMEM with gentamicin (100 μg/m) for 1 hr. Bacterial survival was determined by lysing the cells with Triton X-100 and plating the lysates on LB agar overnight at 37 °C. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test. * P<0.05 using ANOVA. N=3.

4.5 *DISCUSSION*

The NLRP3 inflammasome is a multiprotein complex responsible for the maturation and secretion of the proinflammatory cytokine IL-1β. The over production of this cytokine has been linked to various conditions such as gout, rheumatoid arthritis, and Familial Mediterranean fever [124, 149, 150]. In contrast to these sterile inflammatory conditions, there is a reduced IL-1β secretion found in Crohn disease patients [126, 151, 152]. The microbiome plays a critical role in the development of this disease and the dysregulated inflammasome may explain the aberrant immune response. Our previous work has shown that a balanced IL-1β response is critical for the clearance of the murine pathogen *C. rodentium* mediated by macrophages [49]. Although there is no evidence that IBD is caused by a specific pathogen, the overactive immune response to the recognition of the microbiome through inflammasomes may further fuel the proinflammatory microenvironment seen in IBD.

The addition of extracellular ATP has been shown to aid in the clearance of *C. rodentium* as well as other pathogens such as *Staphylococcus aureus* [51, 82]. The mechanism for bacterial clearance is not known; however, we have previously shown that extracellular ATP induces ROS production through inflammasome activation, reducing intracellular survival of *C. rodentium* [82]. Here, we sought to identify the pathways involved in the production of the ROS and delineate the autocrine and paracrine effects induced by extracellular ATP. First, we identified that *C. rodentium* was localized within lysosomes by 30 min of infection. This suggests the ROS being produced requires entrance into the lysosomes to facilitate killing the bacterium, as opposed to being in the cytosol. We have already shown that the ROS generated is

not due to NADPH oxidase activation; therefore, we looked at mitochondrial ROS as a likely source [82]. It has been shown that mitochondrial stimulation is linked to inflammasome activation through extracellular ATP [57]. Using the dye MitoSOX and inert polystyrene beads, we showed using live cell microscopy that extracellular ATP induces mitochondrial ROS independent of infection. As we did not prove that the inflammasome is not activated, we cannot confirm that this was completely independent of inflammasome activation. However, inert beads alone should not be able to activate as there is no second signal, but they still require phagocytosis which could initiate the first signal. We chose 2 μm beads as this is similar in size to *C. rodentium* and we also did not want to initiate inflammasome activation through frustrated phagocytosis; this is when the cell is trying to ingest an object that is too large. Not all of the cells were positive for MitoSOX and this could be because of inflammasome activation or due to the uptake of the dye itself.

These findings do not explain how mitochondrial ROS could affect intracellular bacterial survival although there may be a link to NADPH oxidase [153]. There is sufficient evidence that activation of mitochondrial potassium channels by the mitochondrial ATP levels increases ROS production [154]. Therefore, in our model, extracellular ATP induces potassium efflux activating the inflammasome which can then cause mitochondrial stress through the potassium channels and generate ROS. NADPH oxidases have also been linked to mitochondrial ROS production due to superoxide and hydrogen peroxide generation, which would also be activated from phagocytosis [155].

Pyroptosis is a type of programmed cell death similar to necrosis, but mediated by caspase 11 or caspase 1 through inflammasome activation [35, 147, 156]. Caspase 1 is involved in canonical inflammasome activation whereas caspase 11 is non- canonical. Activated caspases (1 or 11) can cleave gasdermin D, which binds to phosphatidylinositol phosphates and phosphatidylserine in the inner leaflet of the cell membrane generating pore formation and progression to pyroptosis [157]. This is a leading hypothesis for how IL-1β and IL-18 are secreted from the cell as they do not have a signal peptide. We found that extracellular ATP mediated cleavage of gasdermin D is independent of caspase 11 cleavage, suggesting a canonical inflammasome pathway. In our previous study, we showed that there was no increase in cell death with the addition of ATP, suggesting that gasdermin D is not causing pyroptosis. In addition to binding to the inner leaflet of eukaryotic cells, it can also bind to cardiolipin of bacterial membranes and thus it may be responsible for some of the bacterial clearance. Next, we analyzed the supernatants of infected macrophages to determine if extracellular ATP changed the cytokine and chemokine profile in macrophages. We found that sICAM-1, MIP-1α, and MCP-2 were all increased with ATP and that YVAD inhibited only sICAM-1. MIP-1α was analyzed using ELISA but was not significantly different between any of the treatments. These results suggest a change in some cytokines due to ATP, but that this is independent of the inflammasome. These are all generally increased in infections as part of TLR signaling, which is why they are increased in the non-treatment group as well [158]. MCP-1 and sICAM-1 are involved in leukocyte recruitment and cell adhesion suggesting extracellular ATP may be involved in this process.

Lastly, we collected supernatant from infected cells (conditioned media; include factors secreted by cells in response to stimuli) and gave it to naïve macrophages with and without infection. Conditioned media from cells treated with ATP induced ROS production and IL-1β secretion in naïve cells, suggesting that a positive feedback pathway is involved. This is important for both sterile conditions and infection-induced inflammation because if this pathway is altered it may lead to chronic inflammation such as that observed in IBD [159]. Although the secretion of IL-1β in IBD is a hypoproductive state, this dysregulation in the inflammasome and downstream secreted factors will ultimately affect other cells such as T cells [22]. IBD is a Th1/Th17 mediated response which is directly related to macrophage activity within the GI tract [139]. Interestingly, ATP from conditioned media had the same effect as non-ATP conditioned media on intracellular bacterial survival, suggesting that the secreted factors are not involved in the microbicidal pathway but more likely on macrophage activation. The secretion mechanism of IL-1β is unknown, however there are other secretion pathways available to similar proteins without a signal peptide. Exosomes are extracellular vesicles derived from endocytosis which may contain lipids, proteins or RNA involved in cellular crosstalk. Recently, it has been shown that macrophages infected with *Salmonella enterica* serovar Typhimurium secrete exosomes that can activate naïve macrophages [158]. It may be possible that *C. rodentium* and ATP are inducing the same pathways involved. In addition, it may be possible that IL-1β secretion is facilitated through exosome secretion.

In conclusion, to better define pathways for bacterial killing by macrophages, we provide a location of ROS production by extracellular ATP that may be

responsible for the clearance of intracellular *C. rodentium*. In addition, we observed that the effect could be transferred to newly infected macrophages, illustrating a paracrine effect between activated and naïve macrophages. This study demonstrates the complex nature of the NLRP3 inflammasome in macrophages during bacterial infection. Better understanding of this complex may lead to more effective treatments in conditions such as IBD where the immune system is dysregulated leading to chronic inflammation. Current treatment for IBD is focusing on immune modulation, the results from this work illustrate a new approach may be taken to focus on improving bacterial control by macrophages.

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CHAPTER 5

NLRP3 Activation is Protective and Aids Recovery of Intestinal Epithelial Cells after Infection with *Citrobacter rodentium*

CHAPTER 5. NLRP3 ACTIVATION IS PROTECTIVE AND AIDS RECOVERY OF INTESTINAL EPITHELIAL CELLS AFTER INFECTION WITH *CITROBACTER RODENTIUM*

This manuscript is in preparation for submission

Authors: Michael Bording-Jorgensen, Heather Armstrong, Emma Zwaigenbaum Jeremy Jerasi, Dawson Lafleur, Eytan Wine

5.1 ABSTRACT

Introduction: Crohn disease and ulcerative colitis are the two major subtypes of Inflammatory Bowel Diseases (IBD). One of the polyporphisims associated with IBD is in the nod-like receptor protein 3, which results in a hypoproduction of the proinflammatory cytokine IL-1β. NLRP3 has been linked to pathogen control by macrophages and neutrophils but its role in epithelial cells is not well understood. Our hypothesis was that activation of the NLRP3 inflammasome in colonic epithelial cells would promote recovery after infection with *Citrobacter rodentium*.

Methods: Mouse colonic epithelial cell line CMT-93 was seeded in on transwells until confluence as measured using transepithelial electrical resistance (TEER). The apical membrane was infected with *C. rodentium* while macrophage cell line J774A.1, control or inflammasome activated, was added to the basolateral membrane and TEER was measured over 24 hours. Inflammasome activation was achieved using LPS and ATP with inhibition using YVAD. Apical and basolateral media were collected for ELISA and bacterial attachment was quantified through serial dilutions from the transwells. NLRP3 and IL-1β gene expression was measured

using qPCR. Immunofluorescence was done on transwells after 24 hours using spinning disk confocal microscopy.

Results: ATP-activated macrophages were recruited to the apical membrane of epithelial cells, by claudin-1 expression, where they promoted epithelial barrier recovery and decreased *C. rodentium* adhesion. *NLRP3* expression was increased in epithelial cells; however, neither IL-18 or IL-1β secretion changed in infected epithelial cells. Epithelial cell supernatant increased macrophage ability to clear infection in an inflammasome-independent mechanism; however, ATP significantly improved epithelial barrier recovery.

Conclusion: The role of the inflammasome in epithelial cells appears to be independent of IL-18 and IL-1β secretion during *C. rodentium* infection but may have a role in promoting barrier function. Inflammasome activation in macrophages plays a dual role of pathogen clearance and improving epithelial barrier integrity. Upregulation of the inflammasome in IBD may be a potential therapy for individuals with mutations in *NLRP3*.

5.2 *INTRODUCTION*

Inflammatory Bowel Diseases (IBD) are a group of chronic conditions with the two major groups being Crohn Disease (CD) and Ulcerative Colitis (UC). The etiology remains unknown but there is evidence suggesting a link between genetics, the environment, and microbes, leading to an uncontrolled proinflammatory response [21, 62, 160-162]. Although there have been over 200 loci identified as making an individual susceptible to IBD, no single gene carries a high enough weight to determine the exact cause. The *NLRP3* gene is linked to IBD and has a major role in bacterial recognition and immune responses. It has been suggested that this gene may have a protective role in IBD by preventing colitis, contrasting sterile inflammatory conditions such as gout or rheumatoid arthritis [163]. *Citrobacter rodentium* is a Gram-negative bacterium that cause acute colitis in mice and has been used extensively as an infection model for inflammation, similar in some ways to that seen in IBD. We have previously shown that *Nlrp3*$^{-/-}$ mice infected with *C. rodentium* have higher colonization rates and that this can be alleviated by injecting exogenous interleukin-1β (IL-1β) [49].

The NLRP3 inflammasome is a multiprotein complex that forms in the cytosol, which when assembles activates caspase-1 to cleave the proinflammatory cytokine IL-1β [146]. Inflammasome activation requires two signals: first the transcription and translation of NLRP3 and pro IL-1β with the second being formation of the NLRP3 multiprotein complex and cleavage of caspase-1, leading to the maturation and secretion of mature IL-1β. In this study we used *C. rodentium* and extracellular ATP; the bacterium can act as both signals whereas extracellular ATP is signal two through

activation of the purinergic receptor P_2X7. The purinergic receptor P_2X7 is ubiquitously expressed throughout the human body with having specialized pathways in each cell [164]. We have previously shown that macrophages exposed to extracellular ATP during infection with *C. rodentium* have increased bacterial killing [82].

The intestinal epithelial barrier is a physical barrier between the microbial rich environment of the lumen and the mucosal immune system [165]. Homeostasis of this environment is maintained by the integrity of the intestinal epithelial cell layer and is critical for gastrointestinal health. As epithelial cells have innate immunity functions they can both detect potential pathogens through innate recognition and can provide defense through secretion of antimicrobial peptides (AMP) into the gastrointestinal tract [31]. Inflammasomes are not well characterized in intestinal epithelial cells; however, it is known that NLRP6 is required for mucous secretion from goblet cells and this is depleted in IBD [62]. Yet, there is limited knowledge on the function of NLRP3 in intestinal epithelial cells as this has been mostly characterized in macrophages. A recent publication has shown that during *Toxoplasma gondii* infection, small intestinal expression of *NLRP3* through P_2X7 and IL-1β secretion has a protective effect [166]. *C. rodentium* infects the colonocytes using a type 3 secretion system much the same as enterohemorrhagic *Escherichia coli*. It is an attaching effacing (A/E) pathogen that disrupts the epithelial barrier, causing inflammation and acute colitis [167-169]. Therefore, activation of the inflammasome in these epithelial cells may aid in the clearing of this infection.

We aimed to study the role of the inflammasome in murine intestinal epithelial cells during infection with *C. rodentium* in the absence and presence of macrophages. This complex relationship is critical to innate immunity within the gastrointestinal tract during infection and maintenance of the host microbiome and *in vitro* coculture model allowed us to analyze the role of the inflammasome in this complex relationship. Our hypothesis was NLRP3 inflammasome activation by ATP would improve intestinal recovery after infection with *C. rodentium*. Interestingly, we found that ATP-activation of the inflammasome in intestinal epithelial cells was not important for recovery but secreted factors from epithelial cells decreased bacterial survival inside macrophages (likely through improved bacterial clearance). We also found that ATP-activated macrophages aided in recovery of epithelial cells after infection, that macrophages are recruited to the apical membrane, and that macrophages express the tight junction protein claudin-1. Although inflammasome activation may be focused in macrophages, epithelial cells still play an important role in innate immunity.

5.3 MATERIALS AND METHODS

Chemicals

ATP (BP413-25) was purchased from Acros Organics, YVAD (SML0429) from Enzo Life Sciences and Glyburide (G2539) from Sigma Aldrich.

Cell culture

J774A.1 murine macrophage cell line (ATCC, TIB-67) and CMT-93 murine colonic cell line (ATCC, CCL-223) was seeded as indicated for each experiment. Cells were

maintained in DMEM, supplemented with 10% heat-inactivated fetal bovine serum; medium was replaced every two days and cells were passaged at 80% confluence for a maximum of 22 passages from thaw.

Transepithelial electrical resistance (TEER)

To best recreate the gut epithelial-macrophage-microbial interface in an *in vitro* setting, the following model was developed. The underside of transwells (3 µm pore size, Corning) were coated with rat tail collagen (0.33 mg/ml, Gibco) and seeded with 1 x 10^5 cells/ml of CMT-93 murine colonic cell line until confluence measured using TEER, shown as a plateau in resistance. Before measurement, the chopsticks were sterilized using ethanol and then a 1X PBS wash. After confluence, the Transwell inserts were flipped into a 24 well petri dish with 5 ml of 1X PBS and 50 µl of *C. rodentium* from overnight broth (approximately 5 x 10^6 CFU/ml) resuspended in DMEM was added to the apical membrane for 3 hours. After the initial infection, the inserts were washed once with 1X PBS to remove any not adhered bacteria and added to a new 24 well plate. ATP (2.5mM), YVAD (25 µM), or both were added to the basolateral membrane where indicated. Treated and non-treated J774A.1 macrophages were added to the basolateral membrane and Gentamicin added to the apical membrane (10 µg/ml, Sigma). The no treatment and ATP supernatants of infected J774A.1 macrophages were filtered (0.2 µm pore) and added to the basolateral membrane where indicated (10X dilution). TEER was measured every 2 hours over a 24-hour period with the percent recovery calculated relative to the initial ohmic resistance measurement before infection. The ohmic resistance of a blank

insert without cells was measured in parallel and subtracted from the sample resistance to reflect the natural resistance of the transwell and fluid (**Fig 5.1**).

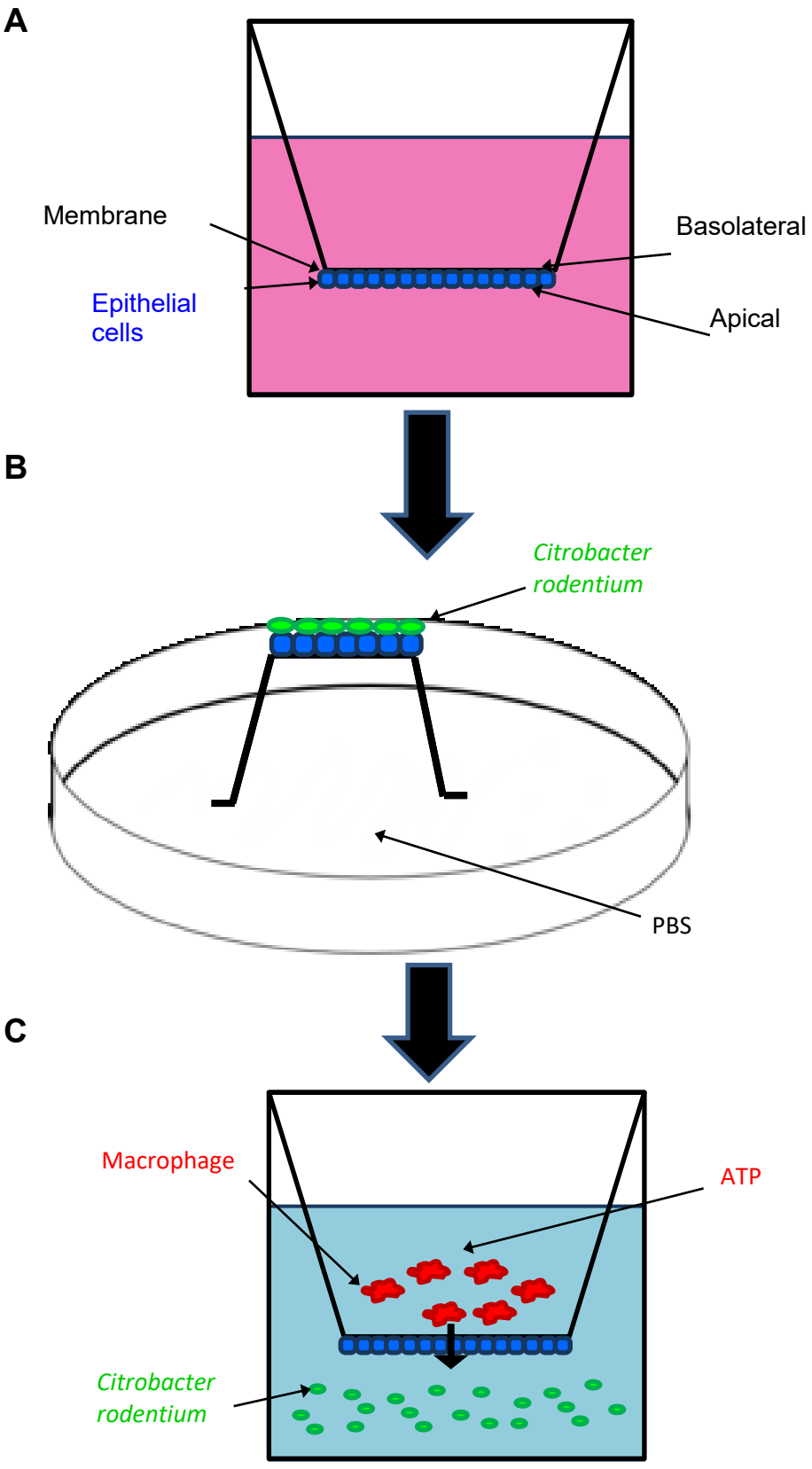


Fig 5.1. Experimental design for coculture of CMT-93 epithelial cells, J774A.1 macrophages and *C. rodentium.* (**A**) Transwells were coated with collagen to allow for attachment of epithelial cells. CMT-93 cells were seeded onto the bottom of transwells until tight junction formation, measured using trans epithelial electrical resistance (TEER) on a voltometer. (**B**) Transwells were then flipped into a petri dish and 50 µl of overnight culture *C. rodentium* (5 x 10^6 CFU/ml) was added for 4 hrs. (**C**) Transwells were washed with PBS to remove non-adhered bacteria and placed back into a 24 well plate, macrophages or ATP were added to the basolateral membrane and TEER measured for 24 hrs.

Immunofluorescence

Transwells were fixed with either methanol (for ZO-1 staining) or 4% PFA, and then blocked in 2% Goat Serum and 1% bovine serum albumin (15 min). Actin was stained with Alexa Fluor-594 phalloidin (1:40 dilution, 0.1% Triton, 0.2% Goat Serum, and 0.1% BSA, Fisher Scientific); 4', 6-diamidino-2-phenylindole (DAPI) (1:1000 dilution, 0.1% Triton, 0.2% Goat Serum, and 0.1% BSA, Thermo Fisher) was used for nuclear staining, ZO-1 (1:200 dilution, 0.1% Triton, 0.2% Goat Serum, and 0.1% BSA), F4/80, (1:200, Thermo Fisher). Transwells were cut out and slides were analyzed using Zeiss Axio Observer.Z1 microscope with ZEN Imaging software (Carl Zeiss Canada Ltd., Toronto, ON, Canada) and the illustrations were formatted for noise reduction and increased sharpness using Image J. All images were processed identically.

qPCR

J774A.1 and CMT-93 cells were cultured overnight prior to treatment as indicated. RNA was isolated using 1mL trizol as previously described [103]. RT-qPCR was performed as previously described [103] to validate findings using primers highlighted in **Table 5.1**. Both biological and technical replicates were performed on all reactions using Rer1, Rpl 21, and Rpl 27 as housekeeping genes. Data were analysed using CFX Manager Software Version 3.0 (Bio-Rad Laboratories, Inc.). Statistical significance was evaluated by the Student's unpaired *t*-test with Welch's correction using GraphPad Prism 4.0.

Table 5.1 Primer sequence for qPCR of CMT-93 murine epithelial cells

PRIMER	Forward	Reverse
Claudin 1	AGG AAA GGC CCT TCA GCA GAG CAA	GTG CCC CCT CTT GAC TCA TGC AAC
Occludin	ATG TCC GGC CGA TGC TCT C	CTT TGG CTG CTC TTG GGT CTG TAT
ZO-1	AAA TGG CCG GGC AGA GAC TTG TGT A	ACC CGA AAC TGA TGC TGT GGA TAG
NLRP3	TGC TCT TCA CTG CTA TCA AGC CCT	ACA AGC CTT TGC TCC AGA CCC TAT
Rer1	GCC TTG GGA ATT TAC CAC CT	CTT CGA ATG AAG GGA CGA AA
Rpl 21	GCC ATG AGA GCG AAG TGG	CTC CTG CAG GCG TCG TAG
Rpl 27	AAG CCG TCA TCG TGA AGA ACA	CTT GAT CTT GGA TCG CTT GGC

Citrobacter rodentium Infection

CMT-93 or J774A.1 cells were seeded in 24-well tissue culture plates at a density of 5 x 10^5 cells per well (80% confluence) overnight to allow for adhesion. Prior to infection, the medium was changed to antibiotic- and serum-free DMEM. C. rodentium (DBS100, a gift from the Sherman lab, University of Toronto) was cultured overnight in lysogeny broth (LB) at 37°C. Cells were treated with the caspase 1 inhibitor AC-YVAD-CMK (25 μM, Enzo Life Sciences, ALX-260-028) or glyburide (40 μM) to inhibit the inflammasome for 1 h preinfection.

C. rodentium was added at a multiplicity of infection (MOI) of 10:1 for 4 h of infection; then ATP (2.5 mM) was added for 30 min. Cells were washed thrice with 1XPBS, lysed with Triton X-100 and plated on LB agar overnight at 37°C. Bacterial adherence to transwells was done by adding Triton X-100 after the 24 hour infection and plating on LB agar overnight, results are shown as a percentage as compared to control without the presence of macrophages.

Supernatant from infected epithelial cells added to macrophages was collected after 24 hour infection, centrifuged (7500 rpm, 5min) and then syringe filtered (2 μm). Supernatants were 10X diluted during the subsequent infection with the macrophages.

Western blot

J774A.1 cells were seeded in 10 cm tissue culture plates until confluent. Prior to infection, the medium was changed to antibiotic- and serum-free DMEM. *C. rodentium* (DBS100, a gift from the Sherman lab, University of Toronto) was cultured

overnight in lysogeny broth (LB) at 37°C. Cells were treated with the caspase 1 inhibitor AC-YVAD-CMK (25 μM, Enzo Life Sciences, ALX-260-028) to inhibit the inflammasome for 1 h preinfection.

C. rodentium was added at a multiplicity of infection (MOI) of 10:1 for 2 h of infection; then ATP (2.5 mM) was added for 30 min. Cells were lysed using RIPA Buffer containing protease inhibitor cocktail (Sigma), then centrifuged at 18,800 g for 10 min to pellet cell debris. SDS and 2-mercaptoethanol was added to the supernatants and lysates, boiled for 5 min at 95°C and run on a 10% SDS-PAGE gel. Proteins were transferred to a PVDF membrane, membranes were blocked overnight (Aquablock) then probed with their respective primary antibodies rabbit anti-ZO-1 (1:250, Invitrogen) rabbit anti-Claudin-1(1:250, Invitrogen), mouse anti-Lamin1 (1:10,000, Proteintech). Membranes were washed with 0.05% PBST and the secondary antibodies added goat anti-rabbit 680 (IRDYE 680) and goat anti-mouse 800 (IRDYE800). Images were captured using a LICOR odyssey and processed with FIJI.

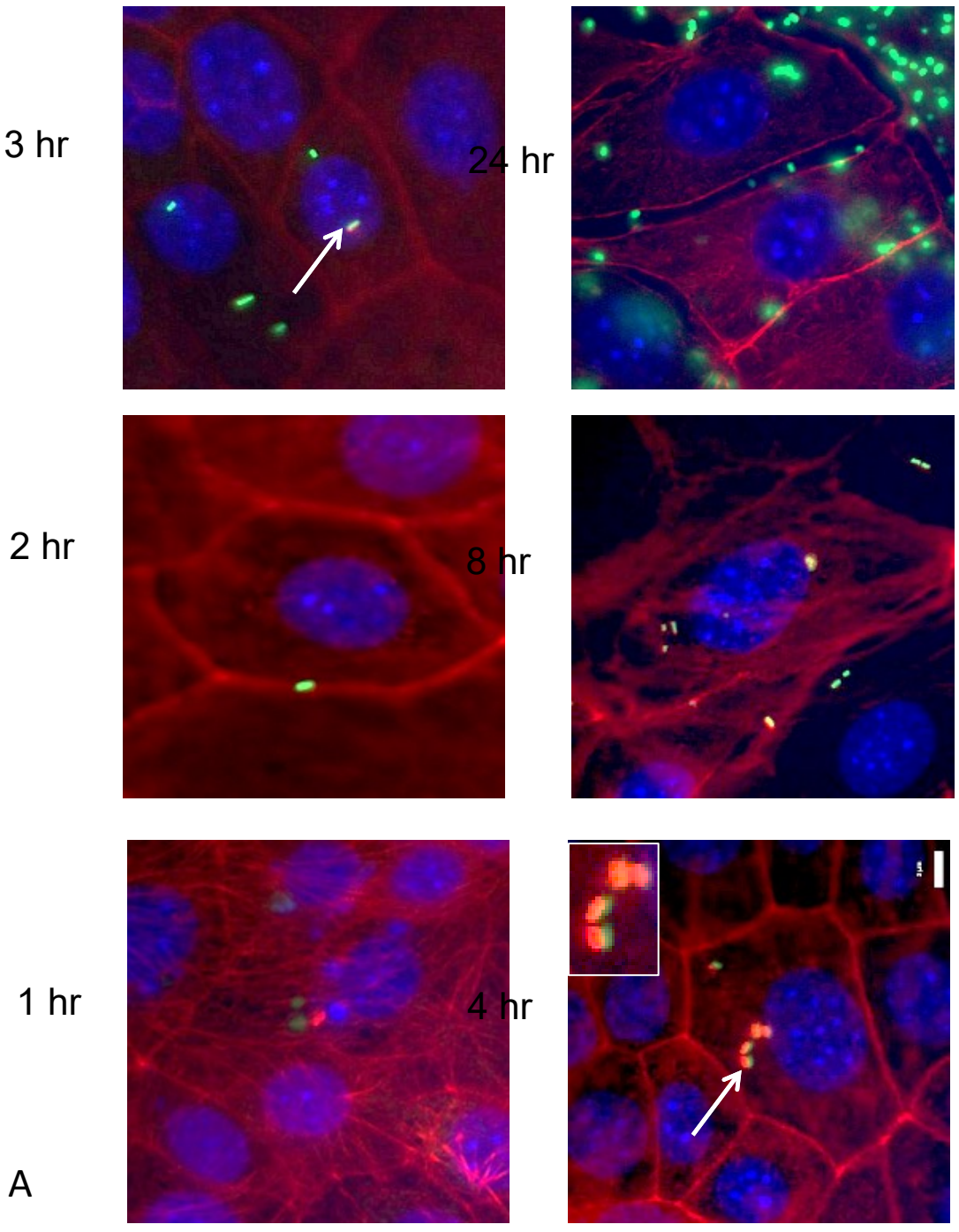
Statistical analysis

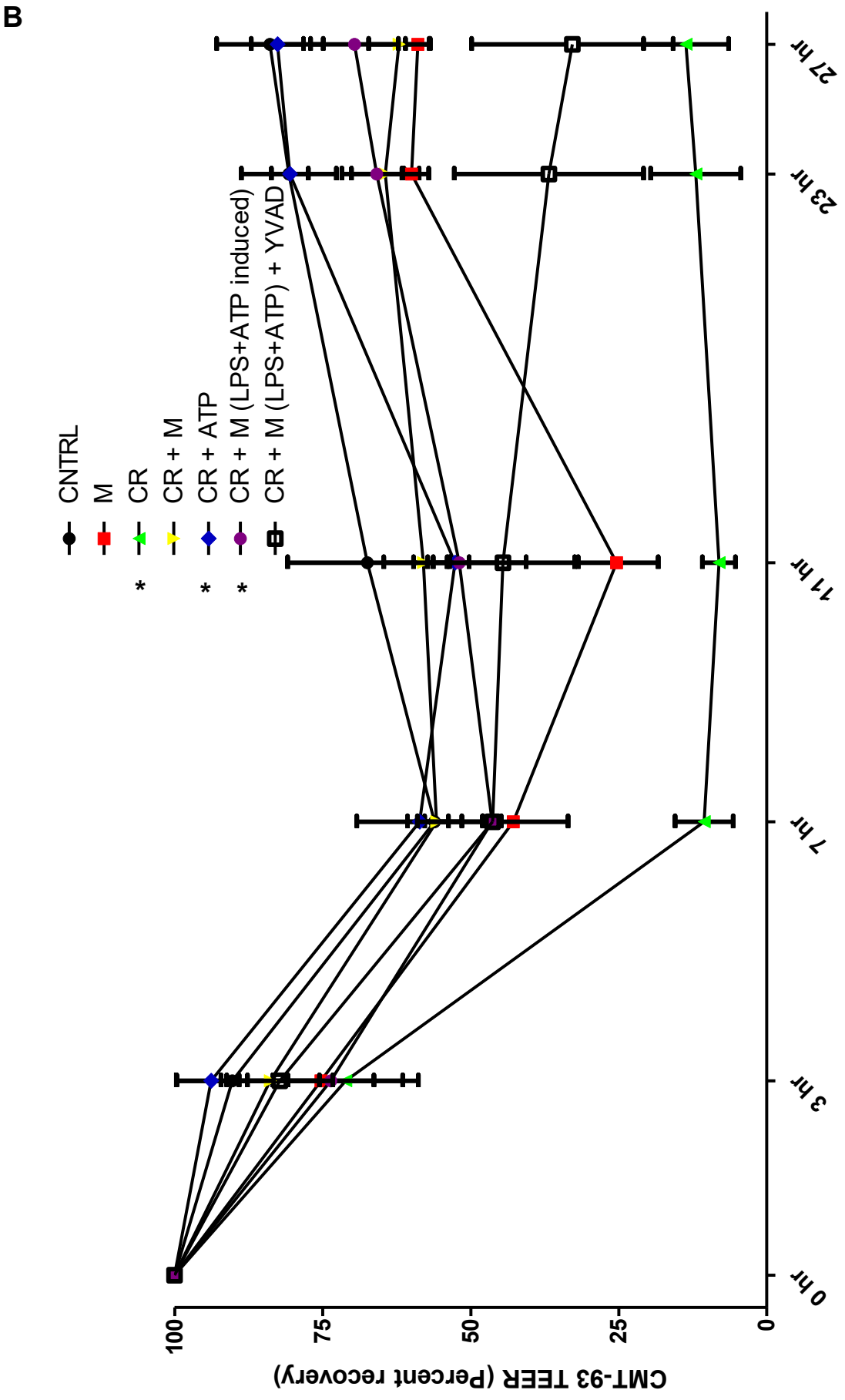
Statistical analysis was performed using Prism5 for Windows (Graph Pad, San Diego, CA, USA). Mann-Whitney two-tailed *t*-test was used for comparison between different treatments. Kruskal-Wallis ANOVA with Dunn's Multiple comparison test were used for phagocytosis determination and cell viability. Bar graphs represent the mean $\pm$ SEM and all comparisons with $P < 0.05$ were considered significant.

5.4 *RESULTS*

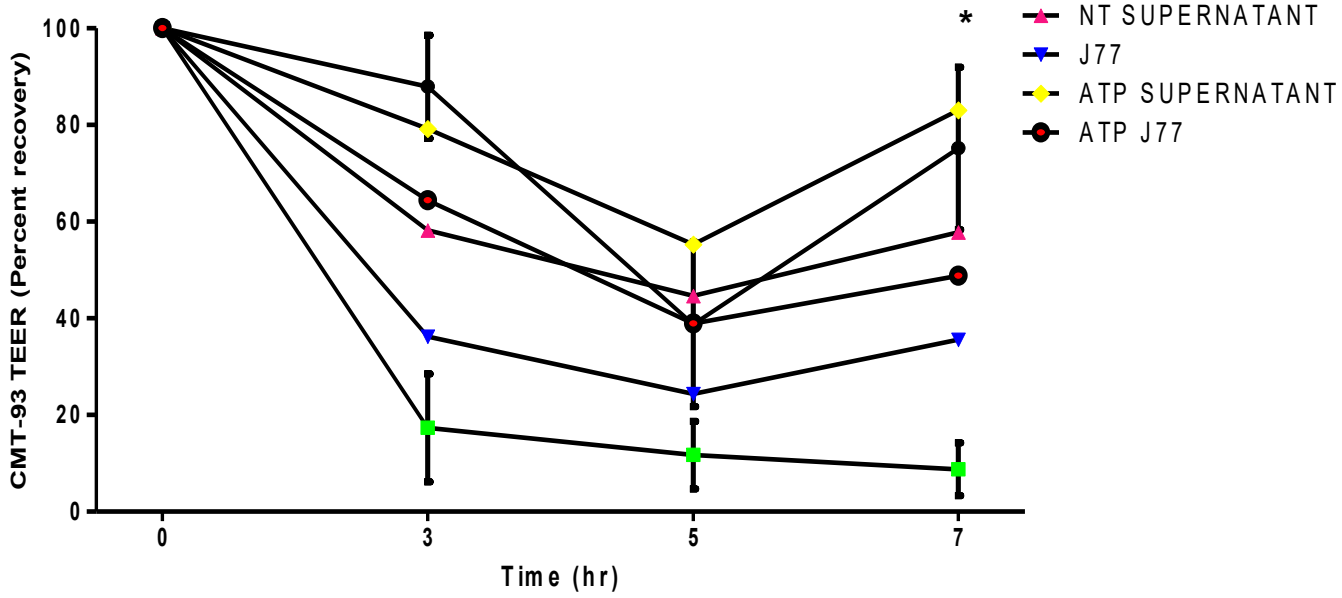
Inflammasome activated macrophages increase epithelial monolayer recovery during *Citrobacter rodentium* infection

To determine the length of infection required for A/E lesion formation, a time course infection of the colonic epithelial cell line CMT-93 with *C. rodentium* was done. Immunofluorescence showed that A/E lesions are formed after 3 hours of infection (**Fig 5.2A**). Epithelial cell barrier recovery after 24 hours (3 hours for A/E lesion formation) of infection was improved with the addition of extracellular ATP (2.5 mM), inflammasome-activated (LPS + ATP) macrophages, or the supernatants from previously infected macrophages; this effect was inhibited by YVAD (**Fig 5.2B and C**). Immunofluorescence showed that during infection, macrophages are recruited to the apical membrane and the addition of inflammasome-induced macrophages significantly reduces *C. rodentium* adherence to CMT-93 epithelial cells (**Fig 5.2D and E**). *C. rodentium* induced apical secretion of IL-1β in CMT-93 cells independent of J774A.1 macrophages (**Fig 5.2F**).

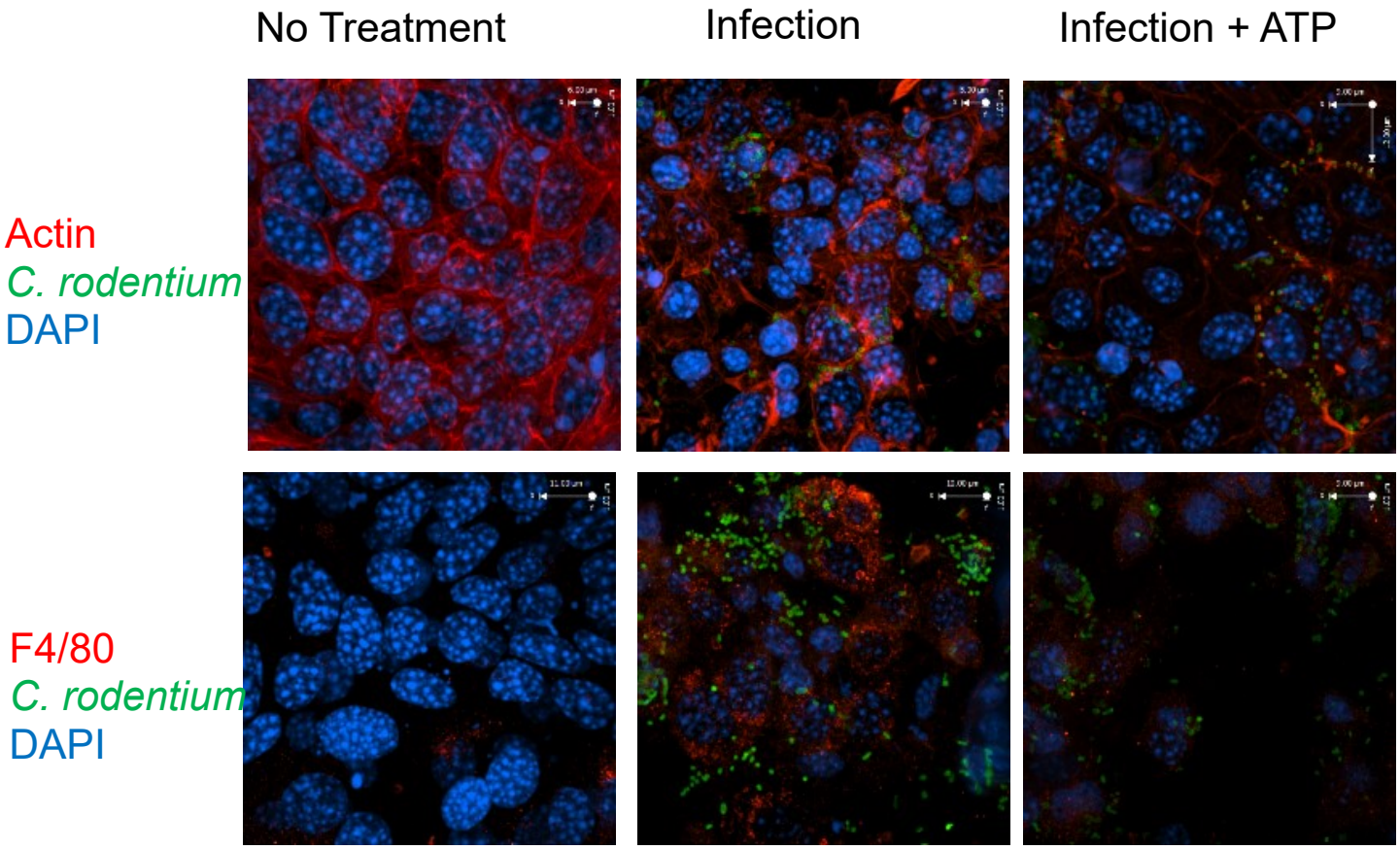

3 hr
24 hr
2 hr
8 hr
1 hr
4 hr
A


B
CMT-93 TEER (Percent recovery)
100
75
50
25
0
0 hr
3 hr
7 hr
14 hr
23 hr
27 hr
CNTRL
M
CR
CR + M
CR + ATP
CR + M (LPS+ATP induced)
CR + M (LPS+ATP) + YVAD
*
*
*

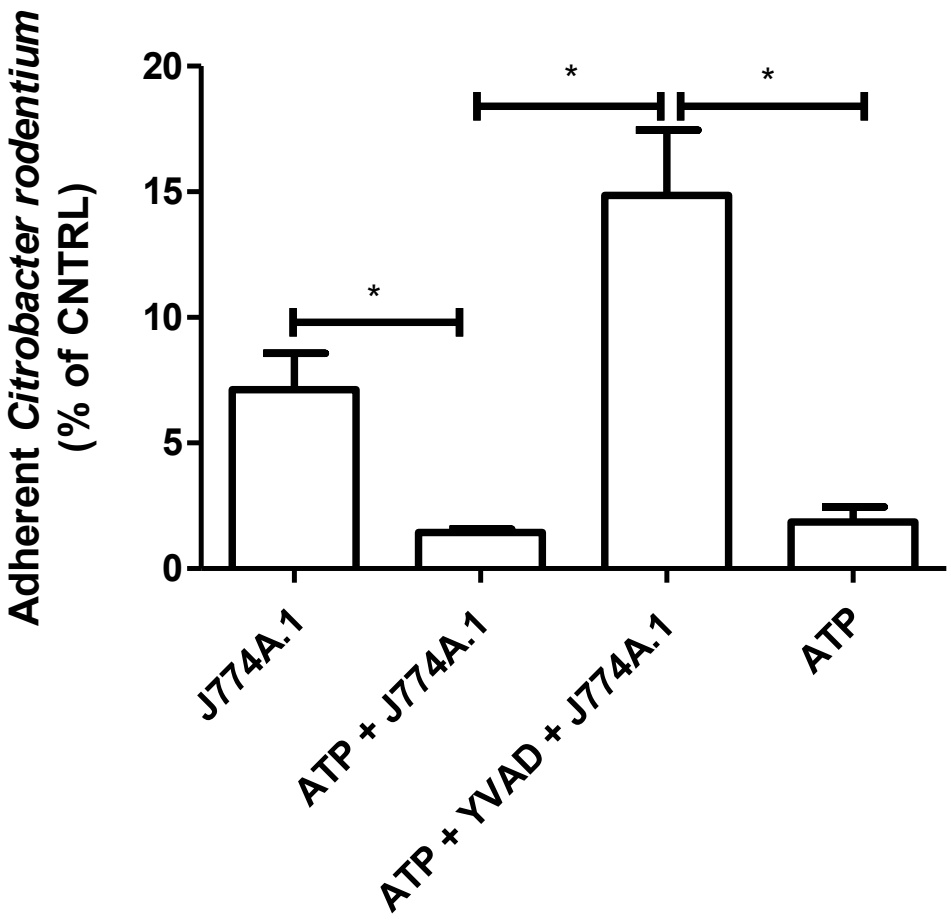
C


CMT-93 TEER (Percent recovery)
100
80
60
40
20
0
0
3
5
7
Time (hr)
*
*
CNTRL
CR
NT SUPERNATANT
J77
ATP SUPERNATANT
ATP J77

D


No Treatment
Infection
Infection + ATP
Actin
C. rodentium
DAPI
F4/80
C. rodentium
DAPI

E


Adherent Citrobacter rodentium
(% of CNTRL)
20
15
10
5
0
*
*
*
J774A.1
ATP + J774A.1
ATP + YVAD + J774A.1
ATP

F

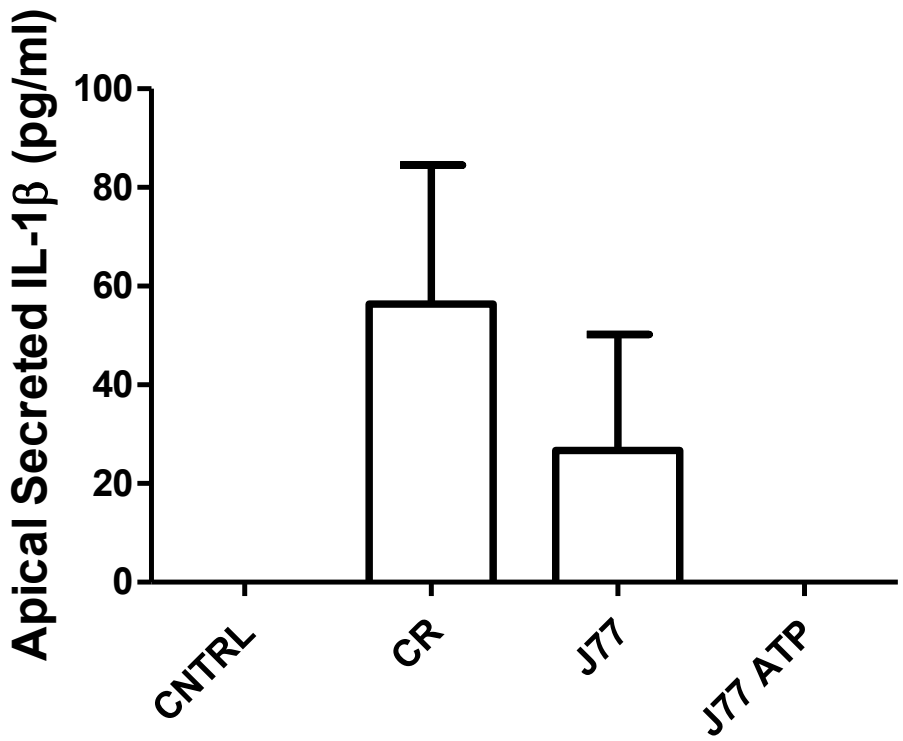
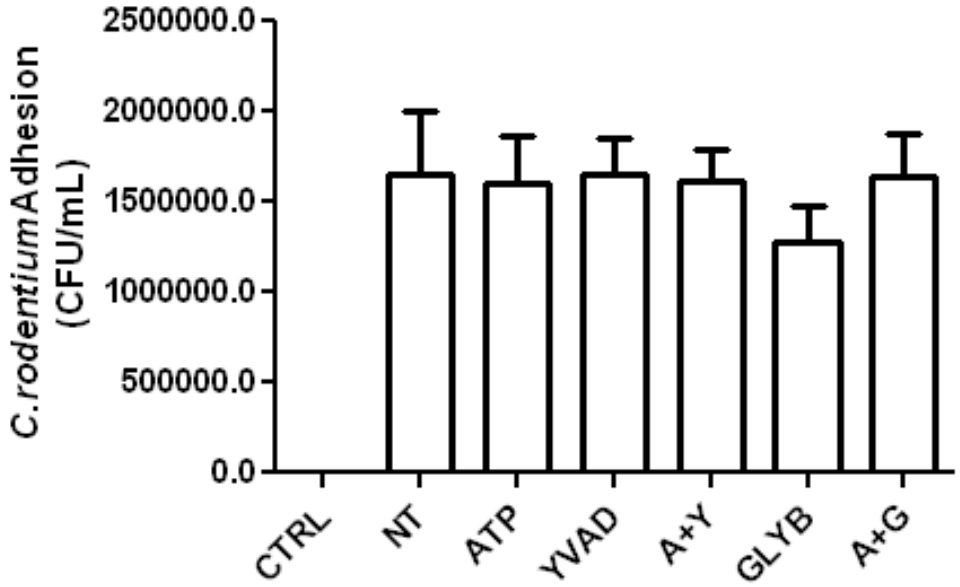

Apical Secreted IL-1β (pg/ml)
100
80
60
40
20
0
CNTRL
CR
J77
J77 ATP

Fig 5.2. Extracellular ATP increases epithelial healing after infection with *C. rodentium* by decreasing pathogen adherence. (**A**) Epithelial CMT-93 cells coated on a coverslip were infected with GFP – *C. rodentium* to determine adhesion/effacing lesion formation by the overlap of actin (red) and the bacterium (green). (**B**) CMT-93 coated Transwells were infected with *C. rodentium* at the apical membrane and macrophages activated with ATP-LPS or not added to the basolateral. CMT-93 tight junction integrity was measured using TEER over 24 hours (Cntrl= control, M= macrophages added, CR= *C. rodentium* infected CMT-93 cells, CR+M= *C. rodentium* and macrophages, CR+ATP= *C. rodentium* with ATP, CR+M (LPS+ATP induced)= *C. rodentium* infection in the presence of LPS+ATP induced macrophages, CR+M (LPS+ATP)+YVAD= *C. rodentium* infection in the presence of inflammasome inhibited macrophages exposed to LPS+ATP). N=3 independent experiments and shown as means ± SEM, * $p<0.05$. (**C**) TEER measurements of CMT-93 over 7 hours, macrophages and supernatants (from previously infected cells, diluted 10X) were added to the basolateral membrane. N=3 independent experiments and shown as means ± SEM. (**D**) Immunofluorescence showing the apical membrane of the transwells with the top row showing actin (red), DAPI for the nucleus (blue) and *C. rodentium* (green), the bottom row showing macrophage stain F4/80 (red), DAPI for the nucleus (blue) and *C. rodentium* (green). (**E**) After 24 hours, Triton X-100 was added to the transwells and the number of adherent *C. rodentium* was counted by serial dilutions and plating on LB at 37°C overnight. (**F**) Supernatants were collected after 24 hours and secreted IL-1β was measured using ELISA. N=6 independent experiments and shown as means ± SEM. *, $P < 0.05$ measured using ANOVA.

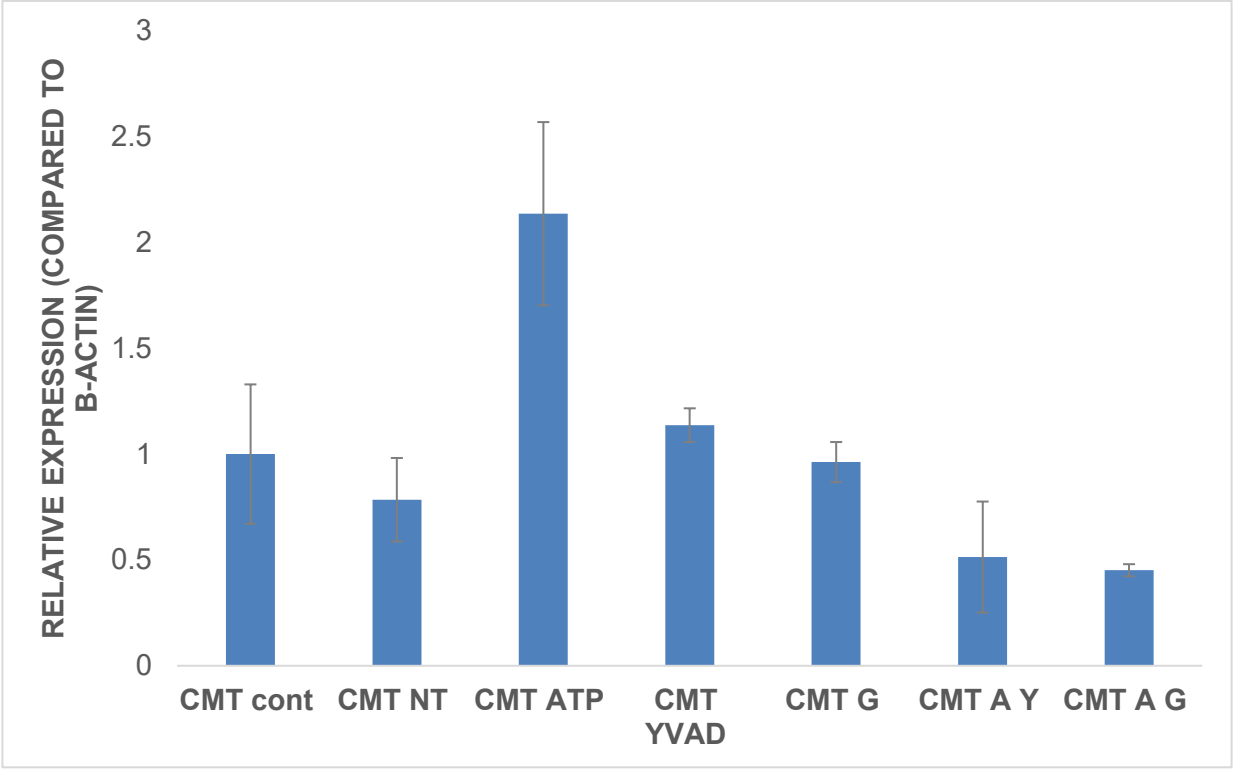
Epithelial cell NLRP3 inflammasome has no direct effect during *C. rodentium* infection

The addition of ATP to CMT-93 cells significantly increased expression of NLRP3 with YVAD and glyburide having no effect (**Fig 5.3A**). The addition of extracellular ATP did not affect *C. rodentium* adherence to CMT-93 cells (**Fig 5.3B**). The addition of ATP had no significant change in IL-18 or IL-1β secretion (**Fig 5.3C**). This suggests that the purinergic receptors in epithelial cells may have a different signalling pathway compared to macrophages that does not involve the inflammasome.

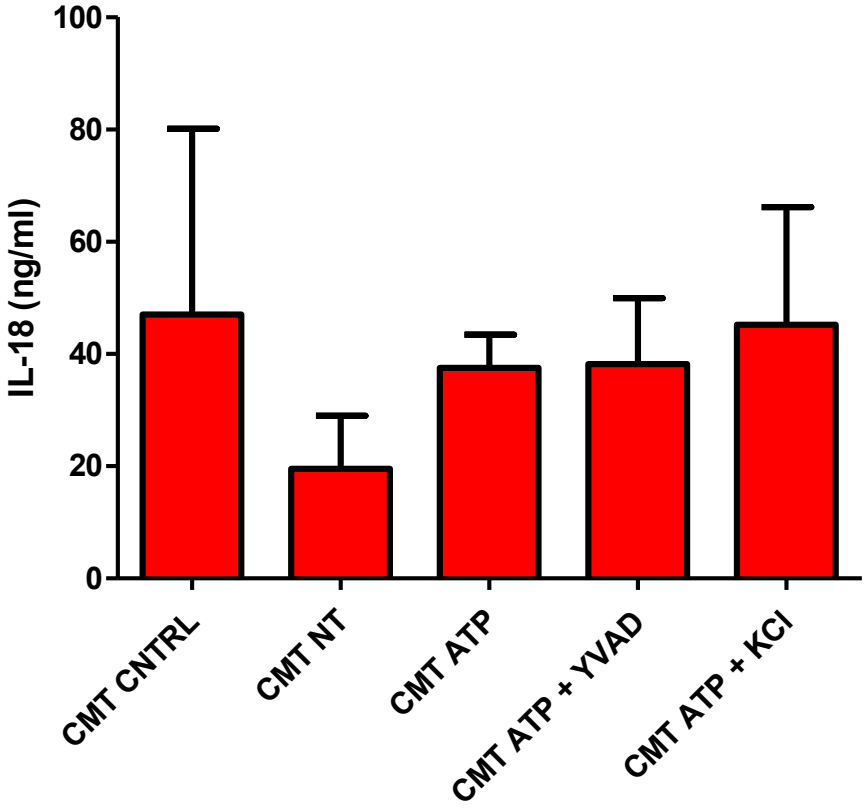
A


C.rodentium Adhesion (CFU/mL)
2500000.0
2000000.0
1500000.0
1000000.0
500000.0
0.0
CTRL
NT
ATP
YVAD
A+Y
GLYB
A+G

B


RELATIVE EXPRESSION (COMPARED TO B-ACTIN)
3
2.5
2
1.5
1
0.5
0
CMT cont
CMT NT
CMT ATP
CMT YVAD
CMT G
CMT A Y
CMT A G

C


100
80
60
40
20
0
IL-18 (ng/ml)
CMT CNTRL
CMT NT
CMT ATP
CMT ATP + YVAD
CMT ATP + KCl

D

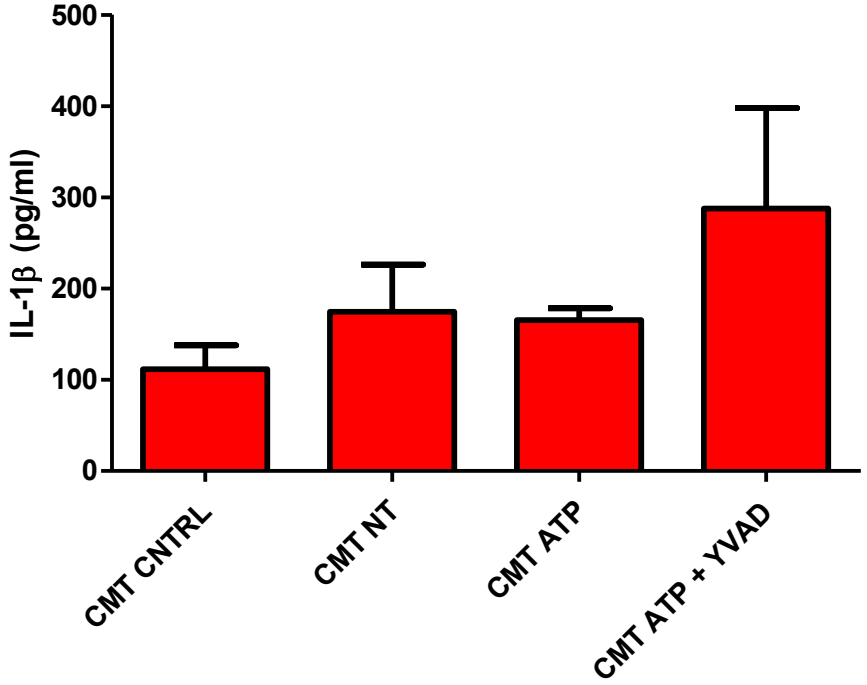

500
400
300
200
100
0
IL-1β (pg/ml)
CMT CNTRL
CMT NT
CMT ATP
CMT ATP + YVAD

Fig 5.3. NLRP3 activators have no effect on epithelial cells during *C. rodentium* infection. (**A**) The inflammasome of CMT-93 cells were either activated (2.5 mM ATP) or inhibited (40 μM, Glyburide, 25 μM YVAD, 45 mM KCl) and *C. rodentium* adhesion assessed after 24 hours. (**B**) *NLRP3* expression was analyzed after 24 hours using qPCR and normalized to actin expression. (**C and D**) Secreted IL-18 and IL-1β were measured from the supernatant collected after 24 hrs using ELISA. N = 3-5.

Appearance of ZO-1 rings during *C. rodentium* infection

Immunofluorescence staining of transwells showed ring-like structures consisting of ZO-1 during *C. rodentium* infection. This was independent of macrophages (**Fig 5.4**). This may allow for macrophage migration through the epithelial barrier to clear infection.

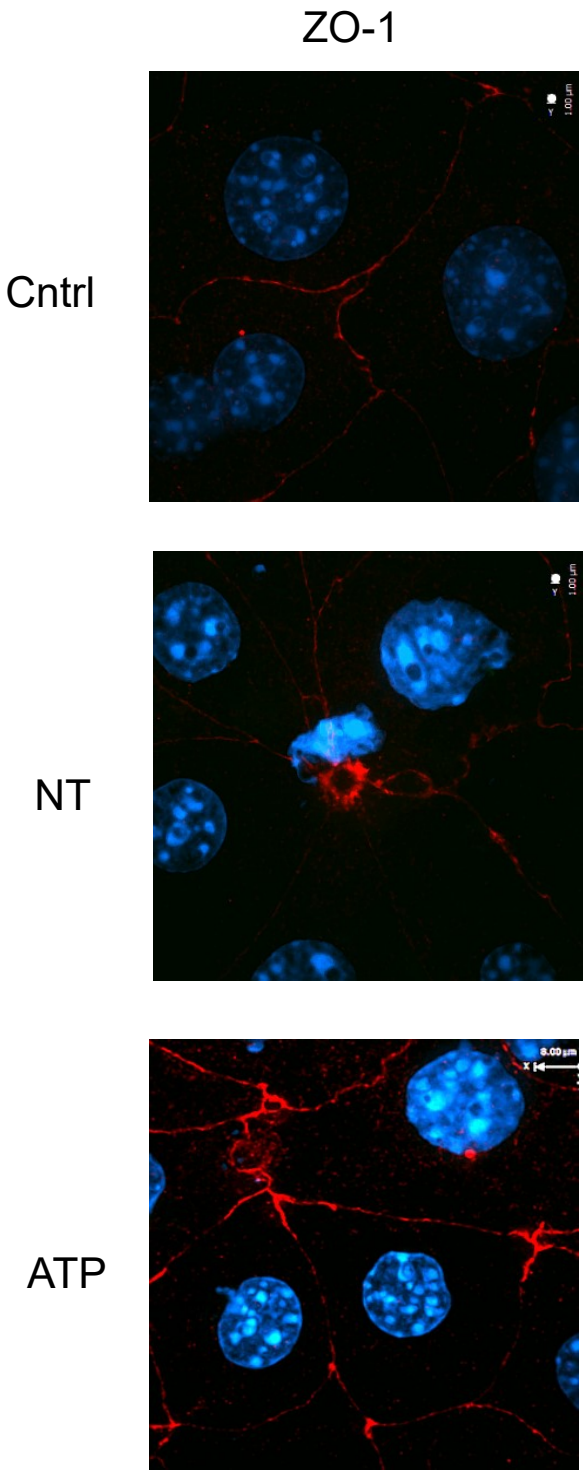
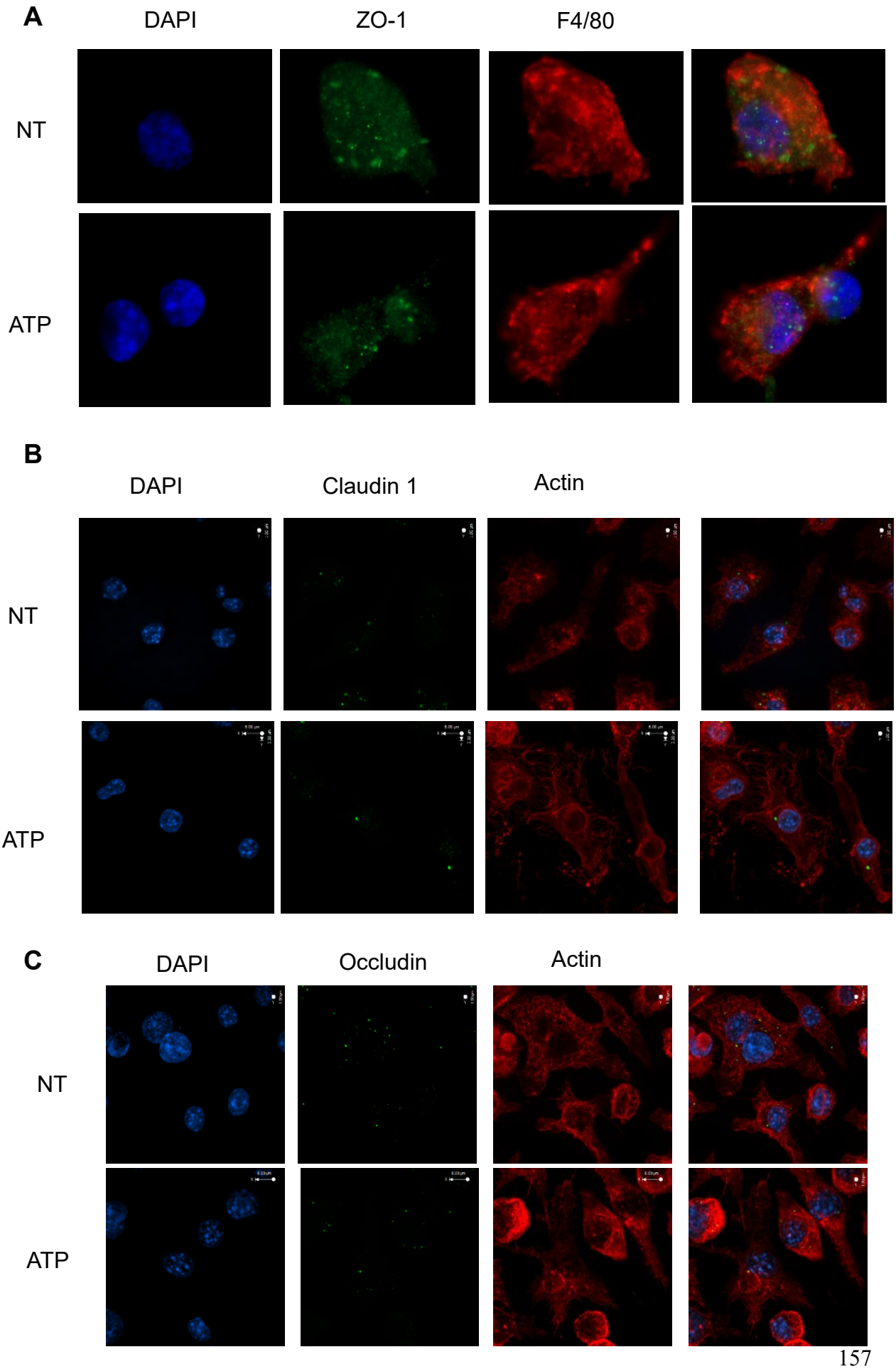


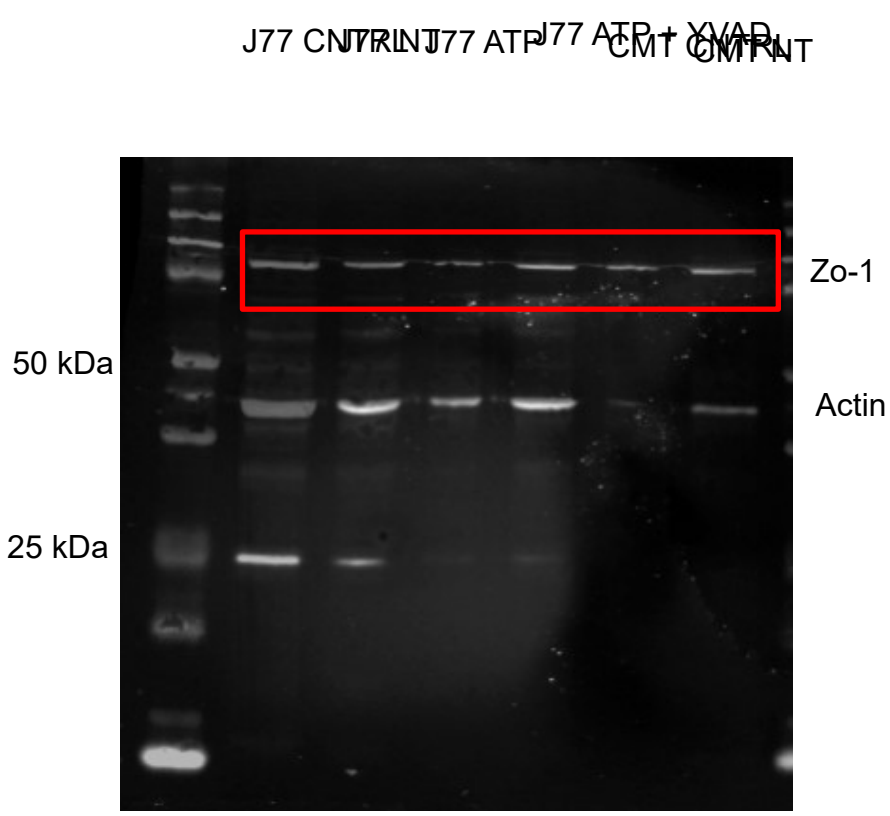
Fig 5.4. Formation of ZO-1 rings associated with *C. rodentium infection.* Transwells were infected with GFP-*C. rodentium* for 24 hrs, fixed and stained for ZO-1 and DAPI for DNA. Images were analyzed using spinning disk confocal microscopy.

J774A.1 macrophages express tight junction proteins

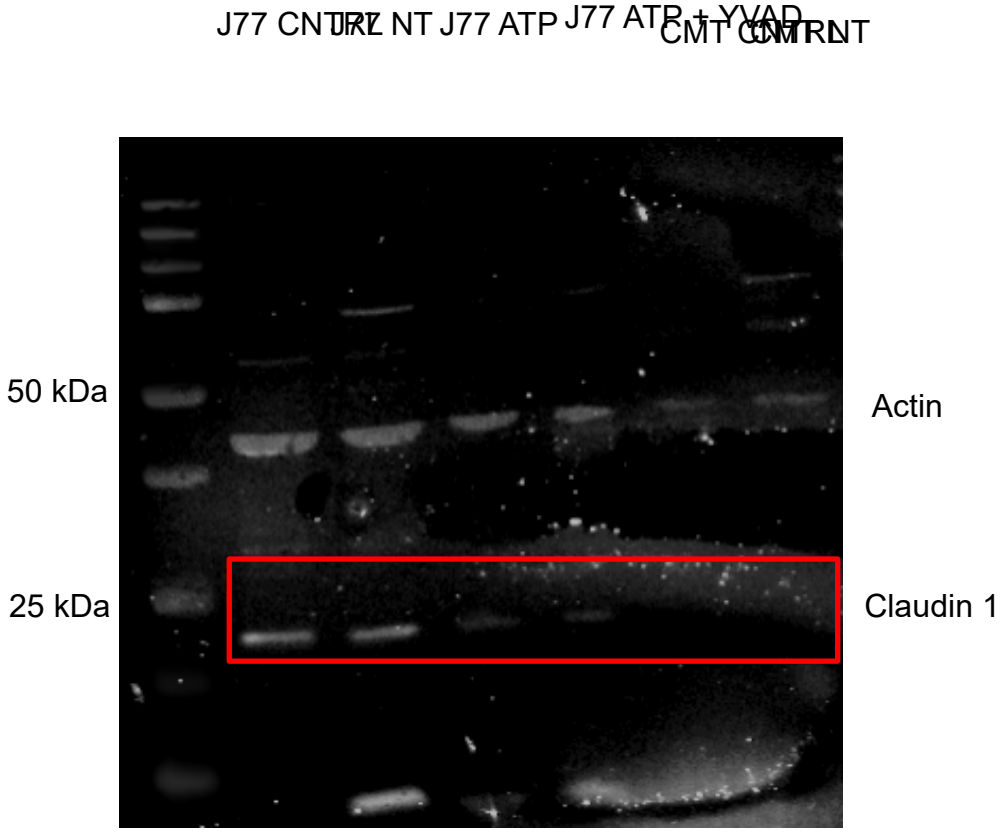
Immunofluorescence showed that J774A.1 macrophages may express tight junction proteins ZO-1, claudin-1 and occludin independent of infection (**Fig 5.5A-C**). Western blot analysis showed claudin 1 and ZO-1 in macrophage lysates (**Fig 5.5D-E**). Claudin-1 gene expression was increased during no treatment infection with *C. rodentium* but not with ATP treatment, whereas occludin and ZO-1 did not show any significant trend in expression (**Fig 5.5F-H**). Rer-1, Rpl-21 and Rpl-27 were used as house-keeping genes, with no significant changes seen (**Fig 5.5I-K**).


A
DAPI
ZO-1
F4/80
NT
ATP
B
DAPI
Claudin 1
Actin
NT
ATP
C
DAPI
Occludin
Actin
NT
ATP

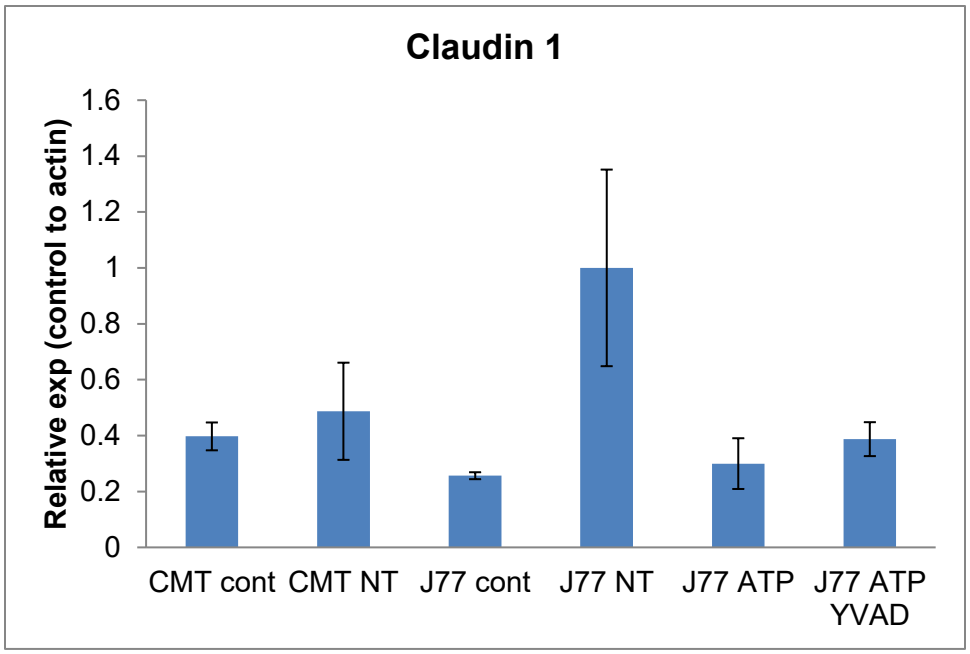
D


J77 CNTRL
J77 ATP
J77 ATP + YVAD
CMT CNTRL
50 kDa
25 kDa
Zo-1
Actin

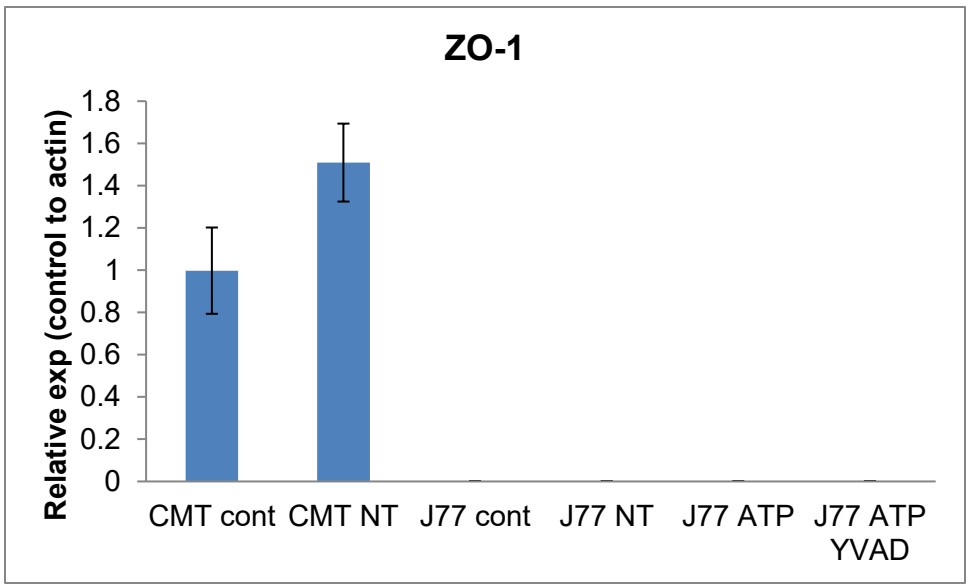
E


J77 CNTRL
J77 ATP
J77 ATP + YVAD
CMT CNTRL
50 kDa
25 kDa
Actin
Claudin 1

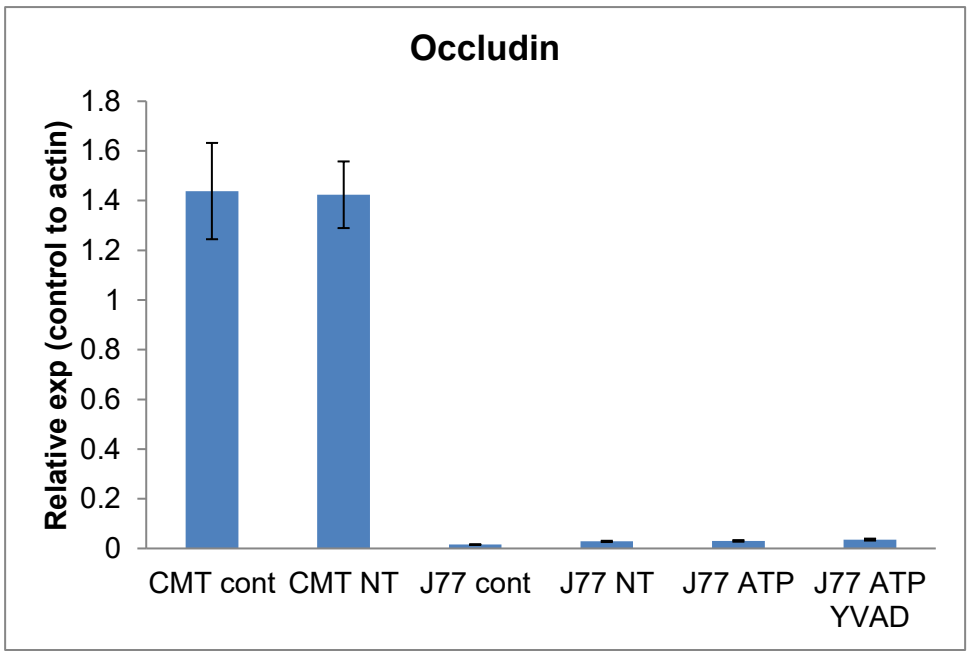
F


Claudin 1
Relative exp (control to actin)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT cont
CMT NT
J77 cont
J77 NT
J77 ATP
J77 ATP YVAD

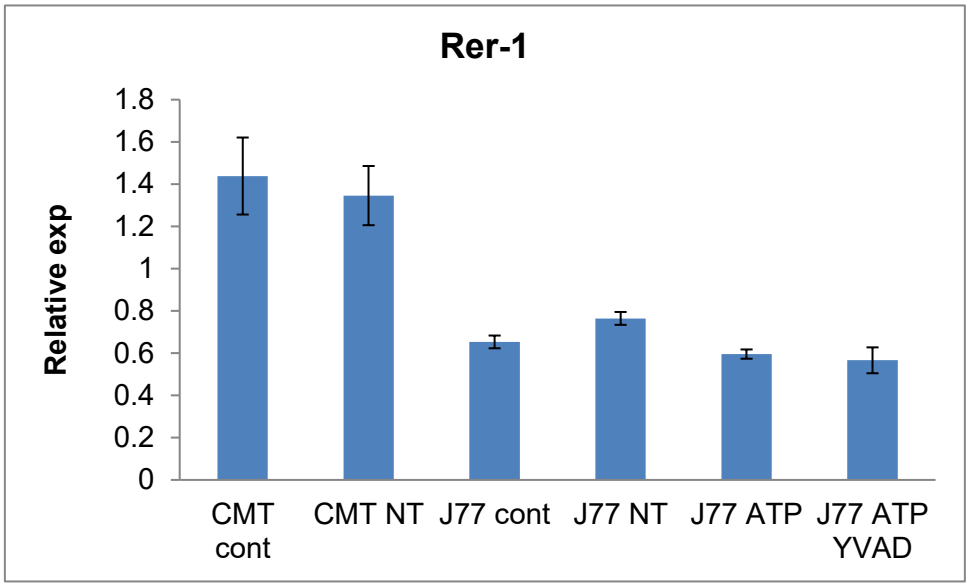
G


ZO-1
Relative exp (control to actin)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT cont
CMT NT
J77 cont
J77 NT
J77 ATP
J77 ATP YVAD

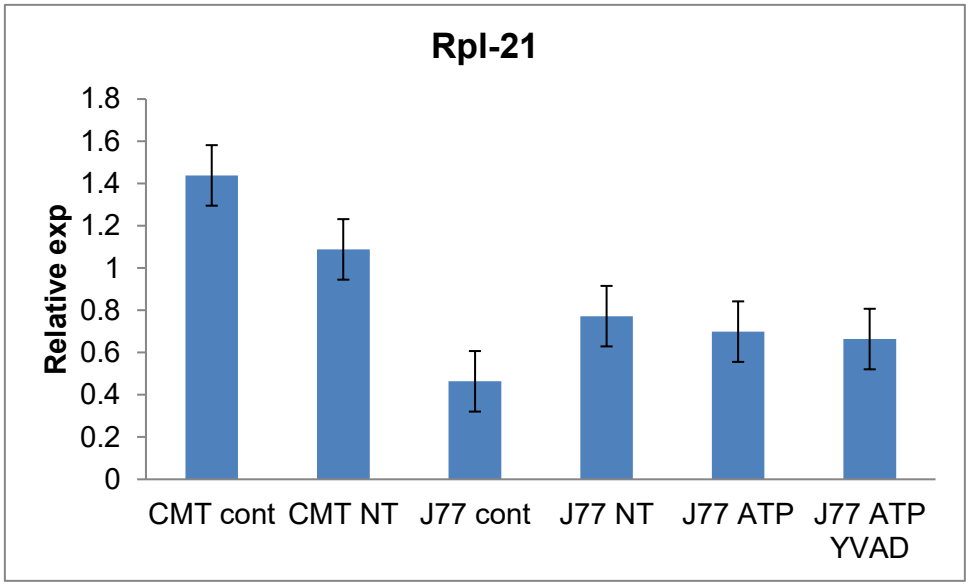
H


Occludin
Relative exp (control to actin)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT cont
CMT NT
J77 cont
J77 NT
J77 ATP
J77 ATP YVAD

I


Rer-1
Relative exp
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT cont
CMT NT
J77 cont
J77 NT
J77 ATP
J77 ATP YVAD

J


Rpl-21
Relative exp
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT cont
CMT NT
J77 cont
J77 NT
J77 ATP
J77 ATP YVAD

K

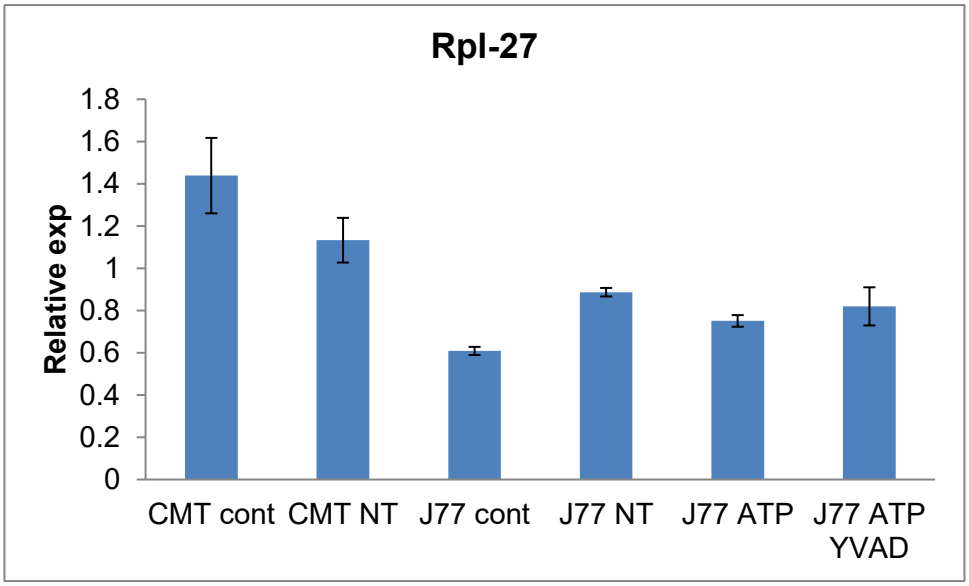

Rpl-27
Relative exp
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT cont
CMT NT
J77 cont
J77 NT
J77 ATP
J77 ATP YVAD

Fig 5.5. J774A.1 macrophages express tight junctions during infection with *C. rodentium.* (**A-C**) J774A.1 macrophages were infected with GFP – *C. rodentium* for 2 hours then activated with ATP (2.5 mM) for 30 min. Macrophages were then fixed with PFA (4%) and stained with claudin-1, occluding or ZO-1. (**D and E**) Western blot for claudin-1 and ZO-1 (green) and actin (red) was performed on cell lysates after the infection. (**F-H**) qPCR analysis was done for claudin-1, occludin and ZO-1 using mRNA isolated from macrophages and epithelial cells. (**I-K**) House keeping genes were Rer 1, Rpl 21, and Rpl 27. Statistical analysis was done using ANOVA. N=3.

Secreted factors from CMT-93 cells increase J774A.1 killing of *C. rodentium* independent of inflammasome activation

Using a gentamicin protection assay, it was determined that intracellular survival of *C. rodentium* in macrophages was significantly decreased in the presence of CMT-93 supernatant independent of either inflammasome activation or CMT-93 exposure to the bacterium (**Fig 5.6**).

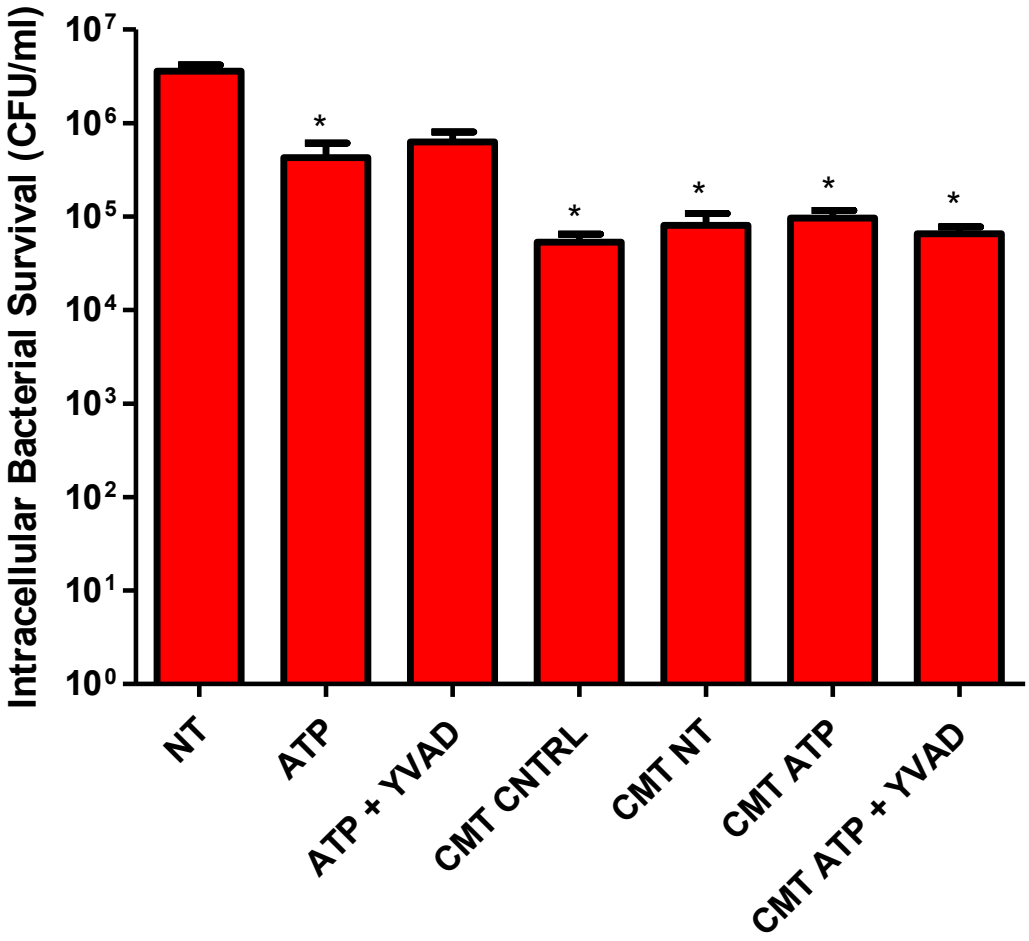


Fig 5.6. Epithelial cell supernatants increase macrophage clearance of *C. rodentium*. Macrophages were either inhibited (YVAD) or activated (supernatants from CMT-93 cells) for 1 hour before infected with *C. rodentium* for 2 hours. ATP (2.5 mM) was then added for 30 min. After infection, gentamicin (100 µg/ml) was added for 1 hour then the cells were lysed with Triton X-100 (1%). Supernatants were then plated on LB agar overnight at 37°C. Statistical analysis was done using ANOVA. *, $P < 0.05$ using ANOVA. N=6.

5.5 DISCUSSION

The intestinal epithelial barrier is known to be an important part of the innate immune system [14]. Epithelial cells secrete many factors that influence both the microbiota and the immune system such as cytokines, chemokines, and AMP. It has been shown that IL-7 secreted by these cells is critical for T and B cell development, particularly T helper (Th1) 1 and Th17 response in *C. rodentium* infection [170]. The role of the NLRP3 inflammasome has not been fully characterized in epithelial cells; However, evidence suggests that it plays a critical role in gastrointestinal pathophysiology, and in IBD [171]. Inflammasome activation has been studied extensively in other immune cells such as macrophages and neutrophils. In these immune cells, it has been observed that the inflammasome contributes to microbial clearance [49, 51, 82]. Individuals with IBD have a disrupted epithelial barrier that contributes to inflammation, but the cause of this disruption is currently unknown. There have been over 200 gene mutations associated with an increased susceptibility to IBD; one of those is *NLRP3* in CD [126]. These individuals have a defect that causes a hypoproduction of IL-1β when exposed to potential pathogens; this dysregulation of the immune system and inability to control potential pathogens may lead to a chronic inflammatory state [126]. Previous chapters have shown the inflammasome to be important for macrophage clearance of *C. rodentium* by extracellular ATP and mitochondrial ROS. The aim for this chapter was to look at the complex relationship between bacteria, macrophages and epithelial cells. In addition, we looked at the role of the NLRP3 inflammasome in epithelial cells and whether it affected *C. rodentium* infection.

First, we observed that ATP-induced macrophages improved epithelial barrier recovery after infection as compared to non-stimulated macrophages. This supports our hypothesis that NLRP3 inflammasome activation in macrophages can function in a protective manner during enteric infections, or when managing mucosa-associated bacteria in general. To assess if the recovery was due to the presence of the macrophages or a secreted factor, supernatants were collected from previously infected macrophages, filtered to remove bacteria, and added to the basolateral membrane; similar results were observed suggesting that it is a secreted factor responsible for this protection. Interestingly, ATP alone led to the greatest epithelial recovery percentage, suggesting that basolateral purinergic receptor activation induces tight junction formation. In addition, epithelial cells exposed to ATP had less adherent *C. rodentium* after 24 hours, suggesting that activation of the epithelial purinergic receptor may have an additional role to control infection; other than activation of the inflammasome this may be through increased AMP secretion. It has previously been shown that extracellular ATP acts as a chemoattractant for macrophage recruitment to the apical membrane of epithelial cells [172]. We did not assess ATP secretion from epithelial cells, but during *C. rodentium* infection macrophages were recruited to the apical membrane where ATP-induced inflammasome activation had no additional effect. This suggests that the increased recovery was not due to macrophage recruitment alone. In addition, there were less bacteria adhered in the presence of inflammasome activated macrophages. This finding agrees with a previous study where we showed an increase in bacterial killing by ATP-activated macrophages [82].

Next, we looked at the role of the inflammasome in epithelial cells, independent of macrophages. Interestingly, the number of bacteria adhered to the epithelium after inflammasome activation was no different, suggesting that the inflammasome may not directly affect epithelial response to infection. This set of experiments was conducted in a plate, not in a transwell, which may explain the contrasting results and suggest a membrane specific response requiring the cells to be polarized. *NLRP3* gene expression was increased in ATP activated cells, however there was no significant change in IL-1β or IL-18 secretion. This was again in contrast to that seen with the transwells where apical secretion of IL-1β was highest in infected without macrophages. These data suggest an apical membrane specific requirement for the NLRP3 inflammasome effect seen in the recovery of epithelial resistance. Perhaps the distribution of TLR4 is different when the cells are in transwells as compared to grown in a culture dish. These are questions that will need to be addressed in future studies.

Tight junction proteins anchor intestinal epithelial cells together and control paracellular trafficking by providing a semipermeable barrier. This barrier provides a boundary between apical and basolateral membranes as well as preventing any luminal bacteria from getting into the mucosa. It has been previously demonstrated that macrophages and dendritic cells can express tight junction proteins in an airway model, but this has not been described in the gut or gut-derived cells [173]. Therefore, we looked at gene and protein expression of claudin-1, ZO-1 and occludin as these were the same ones identified in that study. All three were observed using immunofluorescence, however Western blot and qPCR only showed expression of

claudin-1. Claudins are an integral part of the intestinal epithelial barrier, in which they can determine the integrity of the barrier [174]. Leaky barrier is considered a hallmark in IBD and a possible link to microbial-induced inflammation of the submucosa; claudin-1 has been shown to be dysregulated in Crohn disease leading to this leaky barrier [175-177]. Claudin 1 production in macrophages has a similar role in that it is expressed in M2 macrophages, those that are immune regulatory and help resolution of inflammation; this expression has been shown to be induced by epithelial cell secreted transforming growth factor beta (TGF-β) [178, 179]. Our previous work illustrated that ATP-induced inflammasome activation leads to a shift towards an M2 macrophage during infection with *C. rodentium* [82]. This shift may explain why these macrophages are increasing epithelial barrier recovery even though there is no significant difference in the abundance of macrophages at the apical membrane.

Finally, we assessed whether inflammasome activation of the epithelial cell will affect the ability of macrophages to clear *C. rodentium*. Interesting, not only was there no difference whether the inflammasome activator ATP was present but it did not make a difference if the epithelial cells were previously infected. Epithelial cells appear to secrete factors (yet unidentified) independent of infection that increase macrophage clearance of a pathogen. We did not determine what this factor was, but speculate that it could be a multitude of molecules such as AMP's. In addition to activating the macrophages, it may be possible that the epithelial cell line we used could be secreting an antimicrobial peptide that is killing the *C. rodentium*. This would explain the response seen in the supernatant from control uninfected epithelial cells.

In summary, we observed that ATP-activation of the inflammasome in macrophages can have a positive impact on epithelial barrier recovery after infection with *C. rodentium.* These activated macrophages are recruited to the apical membrane, possibly through expression of claudin-1, which may enable clearance of infection. Inflammasome activation in epithelial cells still needs further investigation, however we did show that extracellular ATP at the basolateral membrane can elicit epithelial barrier recovery greater than the activated macrophages. In addition, epithelial cells secrete factors constitutively that can enhance macrophage ability to clear infection. Immune modulation targeting the inflammasome (perhaps enhancing its activity in this case) may be a benefit to patients with IBD as this may lead to more efficient clearing of invading bacteria in addition to a recovery of the epithelial barrier.

Chapter 6

Increased Inflammasome Activation in Macrophages Exposed to Anaerobic Bacteria Isolated from Pediatric IBD Patients

CHAPTER 6. INCREASED INFLAMMASOME ACTIVATION IN MACROPHAGES EXPOSED TO ANAEROBIC BACTERIA FROM PEDIATRIC IBD PATIENTS

This manuscript is in preparation for submission

Authors are: Michael Bording-Jorgensen, Ghazal Danesh, Misagh Alipour, Eytan Wine

6.1 ABSTRACT

Introduction: Crohn disease and Ulcerative Colitis are two conditions belonging to a group of diseases termed Inflammatory Bowel Diseases (IBD). The etiology of these conditions is unknown; however, IBD has been shown to have over 200 associated genes. One of these polymorphisms is in the *NLRP3* gene resulting in a hypoproduction of the proinflammatory cytokine IL-1β. IBD is characterized by dysbiosis of the gastrointestinal microbiome with consequences not fully understood. It is becoming more evident that commensals may develop pathogenic features in these conditions (termed pathobionts) further inducing immune dysfunction. In this study we sought to identify potential pathobionts from IBD and non IBD patients by measuring their ability to induce IL-1β secretion and ROS production. Our hypothesis was that microbes from IBD patients would elicit increased inflammasome activation and ROS production compared to non IBD patients.

Methods: Anaerobic bacteria were isolated from pediatric IBD patients and controls undergoing colonoscopy and grown in an anaerobic chamber in Brain Heart

Infusion media. THP-1 monocytes were differentiated into macrophages using phorbol myristate on coverslips and infected with the isolated bacteria. IL-1β secretion was measured using an ELISA and reactive oxygen species generation quantified using DCFDA.

Results: Overall, bacteria isolated from Crohn disease patients induced increased IL-1β secretion and ROS production from macrophages. Lab strain *Bifidobacterium infantis* and non-IBD *Ruminococcus* spp showed reduced inflammasome activity in THP-1 cells whereas non-IBD *Bacteroides caccae* had increased activity.

Conclusions: Our observations show that some commensal bacteria from Crohn disease patients elicit an immune response through inflammasome activation. In addition, it was observed that *Ruminococcus* can mediate inflammasome activity. These results illustrate how the microbiome can affect gastrointestinal homeostasis through the immune system. In conditions such as IBD where dysbiosis and the potential for pathobionts is increased, this may explain the dysregulation of the immune system and chronic inflammation. Identifying these microbes through their functional effect on macrophages could lead to directed therapy such as antibiotics to reduce these microbes and restore the normal microbiome in these patients.

6.2 *INTRODUCTION*

IBD are a group of lifelong conditions that potentially affect the entire gastrointestinal tract [22]. [22]. The two main groups are Crohn disease and Ulcerative Colitis, where Crohn disease can affect the entire digestive tract whereas ulcerative colitis is restricted to the colon. The etiology of IBD is unknown; however, there have been over 200 IBD susceptibility genes identified. Treatment is focused on maintenance of the disease through suppression or modulation of the immune system, such as use monoclonal antibodies against TNFα therapy [22]. One of the genes associated with IBD is NLRP3; this protein is part of the inflammasome. The inflammasome is a multimeric protein complex that forms in the cytosol of many different cells but has mainly been found in neutrophils and macrophages [126].

This protein complex is responsible for the maturation and secretion of the proinflammatory cytokine IL-1β, which is unique in that it requires two signals for activation. The first signal is activation of TLR4 by LPS whereas the second signal arise from many different sources such as ATP, monosodium urate, or glucose and is not completely understood [180]. This pathway is found to be causative in many different diseases such as rheumatoid arthritis, familial Mediterranean syndrome, gout, asthma and obesity in a hyperproductive state however it has been found in IBD to be hypoproductive [126]. Villani *et al* showed that individuals with Crohn disease and the associated NLRP3 genotype secrete less IL-1β when their isolated macrophages were activated with LPS compared to those without this variant [126].

In addition to an inappropriate immune This dysbiosis is also characterized by what is referred to as a pathobionts, a condition where commensals have the potential to be pathogenic. There appears to be an enrichment of Firmicutes and Proteobacteria with a reduction in Bacteroidetes; however it is unclear what role the individual bacterial species are playing in this inflammatory state [62]. This dysbiosis can have wide ranging effects on host physiology such as inhibition of the intestinal epithelial Na^+/H^+ exchange as seen in IBD, shown by the transfer of microbes and subsequent exacerbation of colitis in germ free mice [181].Adherent invasive *Escherichia coli* (AIEC) is a example of a pathobiont – it is enriched in patients with Crohn disease and has been shown to induce alterations of the microbiome contributing to a loss in diversity [168].

Therefore, we investigated the inflammasome activation potential of anaerobic microbes isolated from the terminal ileum of patients with Crohn disease compared to those without IBD. Our hypothesis was that bacteria isolated from IBD patients would induce a greater proinflammatory immune response, reflected as an increase in IL-1β secretion. We observed that there was a general increase in IL-1β secretion when macrophages were incubated with bacteria from IBD patients and this was due to a select few bacterial species. In addition, these bacterial species had a different response depending on the patient they came from. This was a pilot project with multiple bacteria isolated from only 1 control patient and 2 patients with Crohn disease; therefore, a much larger cohort is needed to validate these findings.

6.3 MATERIALS AND *METHODS*

Chemicals

2, 7-dichlorofluorescein diacetate (DCFDA, Sigma), phorbol 12-myristate 13-acetate (PMA, Sigma)

Cell culture and bacterial cultures

THP-1 human monocytes (ATCC, TIB-202) were cultured in RPMI supplemented with 10% FBS for a maximum of 25 passages.

E. coli HB101, Enterohemorrhagic *E. coli* and Adherent invasive *E. coli* were grown in Lysogeny broth (BD) aerobically at 37 °C. *Bifidobacterium infantis* was grown anaerobically using an anaerobic chamber in Brain Heart Infusion (BD) media

Isolation of patient bacterial strains

Patients aged 3 – 18 years, with histological and endoscopic confirmed diagnosis of CD or UC, based on the revised Porto criteria [182] and the Paris classifications [183] were eligible to participate; non-IBD controls underwent colonoscopy for abdominal pain and/or diarrhea but endoscopy and histology were completely normal. Detailed inclusion and exclusion criteria were described previously [62, 184]. UC subjects endoscopically or histologically diagnosed with backwash ileitis were excluded from the study. For all patients, bowel cleansing was standardized using Picosalax®: (sodium picosulfate and magnesium oxide) prior to endoscopy. Aspirate washes from the terminal ileum (TI) were collected from patients during endoscopy and before biopsies were obtained at the Stollery Children's Hospital, University of Alberta in

Edmonton, Alberta, Canada. Protease inhibitor (1% v:v; Sigma Aldrich) was added immediately to the aspirate, and further purified by filtration (40 µm filters) and centrifugation at 200 g (5 min, 4°C) to discard food particles and human cells. Bacterial pellet was resuspended in 1X PBS and cultured in an anaerobic chamber for 48 hours. Bacterial DNA was isolated using a DNEasy Blood and Tissue kit (Qiagen) and identified using 16S sequencing.

Infection

24 well plates were filled with 3 mL of BHI agar and each bacterial strain was cultured into separate wells in an anaerobic chamber at 37°C for 24 hours. THP-1 monocytes were seeded overnight at 5 x 10^4 (80% confluence) onto coverslips and treated with 75 nM PMA to induce differentiation into macrophages. The 24 well plates were taken out of the anaerobic chamber and the coverslips with adhered THP-1 were placed cells down onto the bacterial lawns; 500 µl of RPMI was added on top and left at 37°C for 2 hours. Supernatants were then collected and secreted IL-1β was measured using ELISA.

Reactive Oxygen Species

ROS was measured using a previously described method [21, 22]. In brief, THP-1 monocytes were seeded overnight at 5 x 10^4 (80% confluence) cells per well in a 96-well plate with 75 nM PMA for differentiation. DCFDA (1 µM, Sigma) was added for 30 min, then washed thrice with 1X PBS, and fresh DMEM was added for infection. Cells were infected with the indicated bacteria for 2 h and then washed thrice with 1X

PBS. Fluorescence was measured at Ex485 and Em535. Hydrogen peroxide (0.3 %) was used as a positive control.

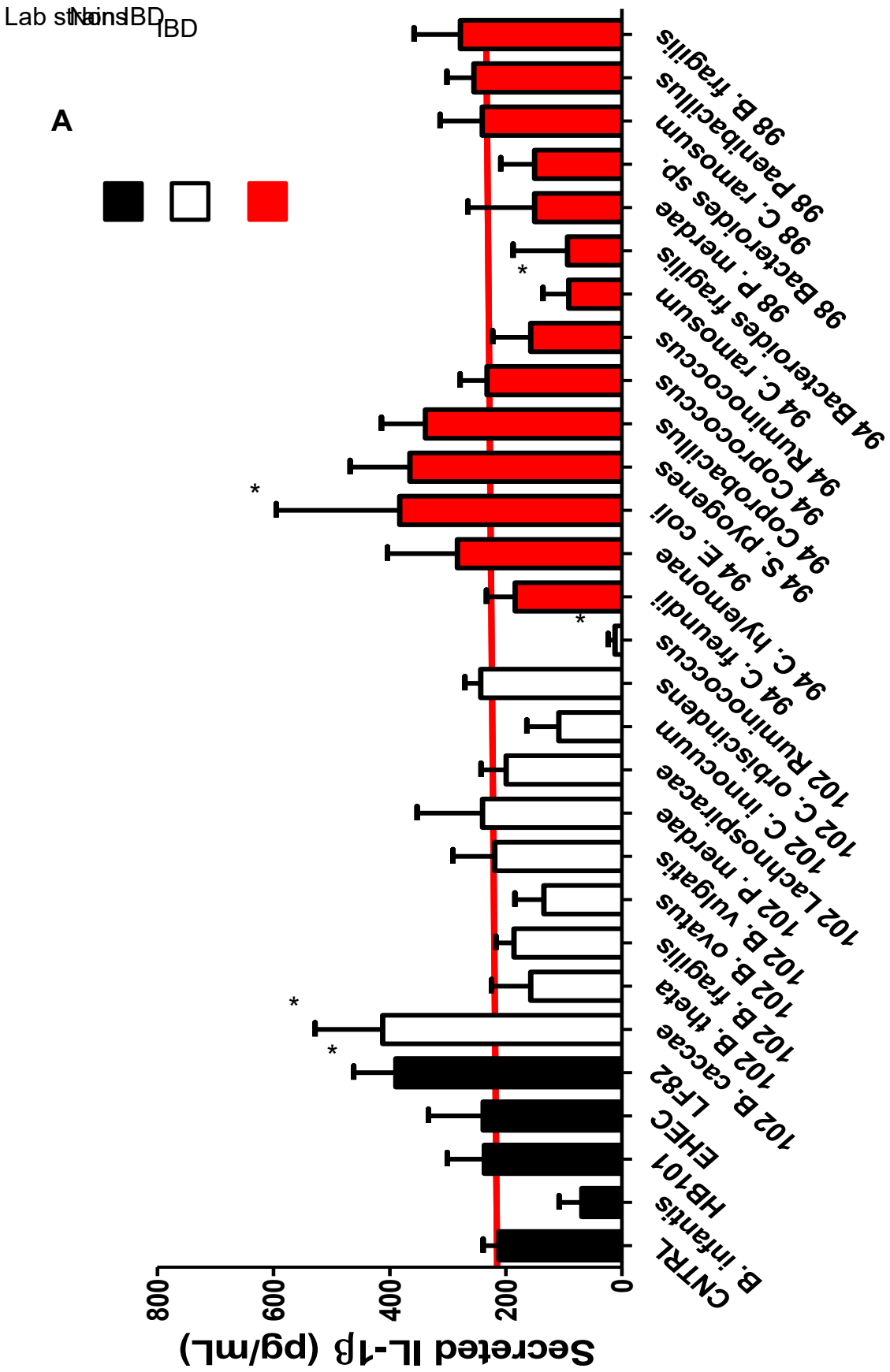
Statistical Analysis

Statistical analysis was performed using Prism5 for Windows (Graph Pad, San Diego, CA, USA). Kruskal-Wallis ANOVA with Dunn's Multiple comparison test were used for comparison. Bar graphs represent the mean $\pm$ SEM and all comparisons with $P < 0.05$ were considered significant.

6.4 *RESULTS*

Bacteria isolated from IBD patients induce inflammasome activation in macrophages.

Bifidobacterium infantis was used as a non-infection control bacterium that did not induce inflammasome activation in macrophages. The other control bacteria, 3 strains of *Escherichia coli*, all elicited an immune response similar or greater than control. Activation of the inflammasome varied between bacterial species in both IBD and non-IBD, however we found that several of those isolated from IBD patients tended to significantly ($p < 0.05$) activate the inflammasome (**Fig 6.1 A and B**). We also found that even between IBD patients there was a difference between the same bacterial species, as shown with *B. fragilis* in patients 94 and 98.


A
Lab strains
Non-IBD
IBD
Secreted IL-1β (pg/mL)
0
200
400
600
800
CNTRL
B. infantis
HB101
EHEC
LF82
102 B. caccae
102 B. theta
102 B. fragilis
102 B. ovatus
102 B. vulgatis
102 P. merdae
102 Lachnospiraceae
102 C. innocuum
102 C. orbiscindens
102 Ruminococcus
94 C. Freundii
94 C. hylemonae
94 E. coli
94 S. pyogenes
94 Coprobacillus
94 Ruminococcus
94 C. ramosum
94 Bacteroides fragilis
98 P. merdae
98 Bacteroides sp.
98 C. ramosum
98 Paenibacillus
98 B. fragilis

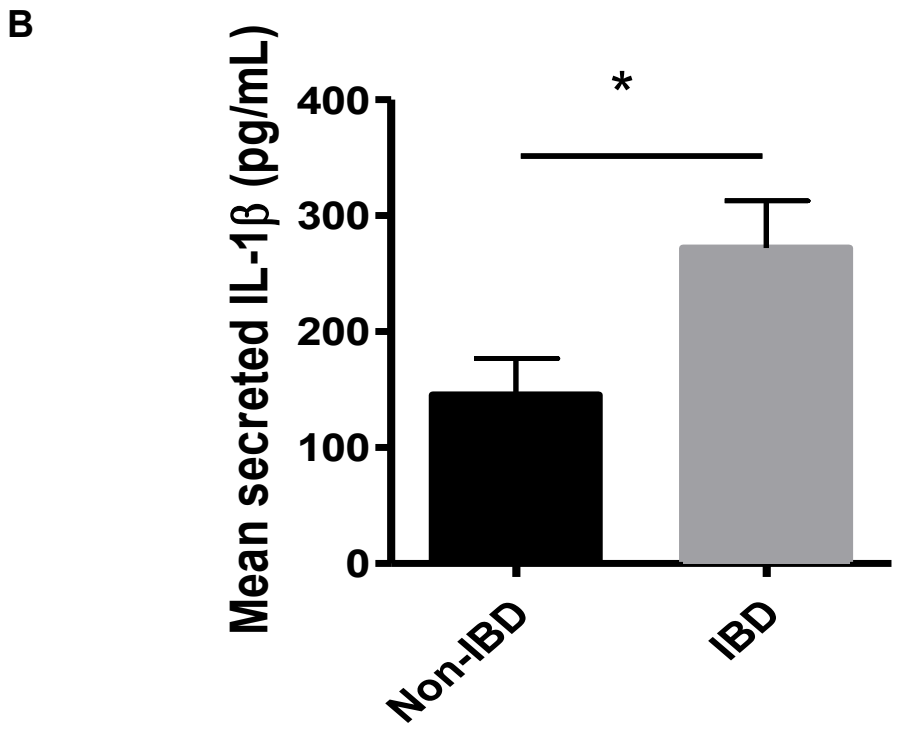
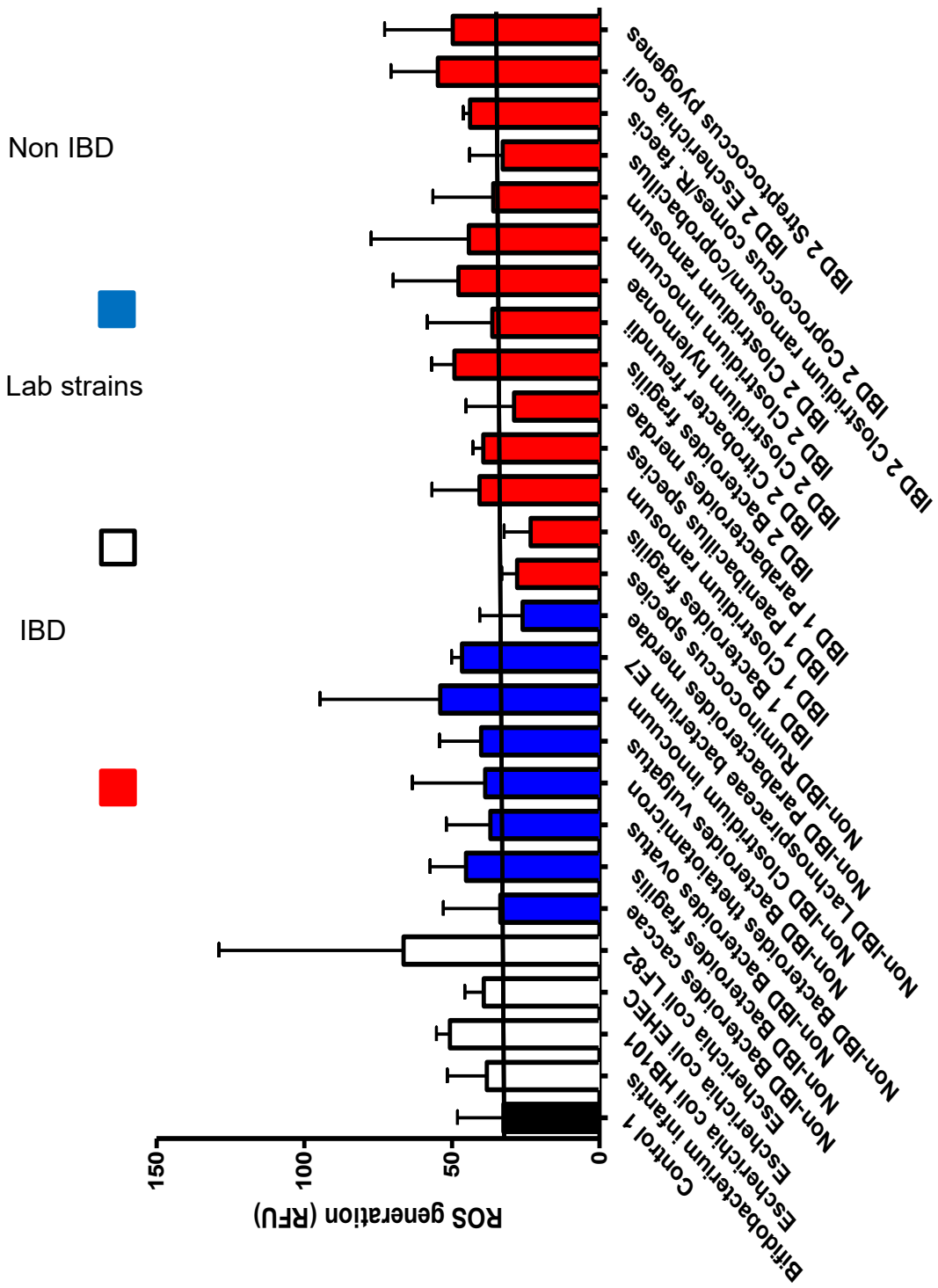


Fig 6.1. Inflammasome activation in macrophages during infection with bacteria isolated from IBD patients. (**A**) THP-1 monocytes were seeded onto coverslips and differentiated using PMA (75 nM) overnight. 24 well plates were filled with 3 mL of BHI agar and each bacterial strain was cultured into separate wells in an anaerobic chamber at 37°C for 24 hours. The 24 well plate was taken out of the anaerobic chamber and the coverslips with adhered THP-1 were placed cells down onto the bacterial lawns, 500 µl of RPMI was added on top and left at 37°C for 2 hours. Supernatants were then collected and secreted IL-1β was measured using ELISA. (**B**) Data from the IL-1β measured from each bacterial strain was compared between those collected from IBD and non-IBD patients. N= 6-8. *$p < 0.05$ measured using ANOVA.

Bacteria isolated from IBD patients induce ROS production in macrophages.

Non-IBD bacteria tended to either decrease ROS production or have no effect, notable exceptions are *Bacteroides ovatus, Bacteroides vulgatus, Lachnospiraceae,* and *Ruminococcus*. On the other hand, IBD bacteria tended to induce ROS production except *Parabacteroides merdae* (**Fig 6.2A and B**). N=6. *$p < 0.05$ measured using ANOVA.

A


Non IBD
Lab strains
IBD
ROS generation (RFU)
150
100
50
0
Control 1
Escherichia coli HB101
Escherichia coli EHEC
Escherichia coli LF82
Non-IBD Bacteroides caccae
Non-IBD Bacteroides ovatus
Non-IBD Bacteroides thetaiotamicron
Non-IBD Bacteroides vulgatus
Non-IBD Clostridium innocuum
Non-IBD Parabacteroides bacterium E7
Non-IBD Lachnospiraceae Ruminococcus
IBD 1 Bacteroides merdae
IBD 1 Clostridium ramosum
IBD 1 Parabacteroides fragilis
IBD 2 Bacteroides merdae
IBD 2 Citrobacter freundii
IBD 2 Clostridium hylemonae
IBD 2 Clostridium innocuum
IBD 2 Clostridium ramosum/coprobacillus
IBD 2 Coprococcus comes/R. faecis
IBD 2 Escherichia coli
IBD 2 Streptococcus pyogenes

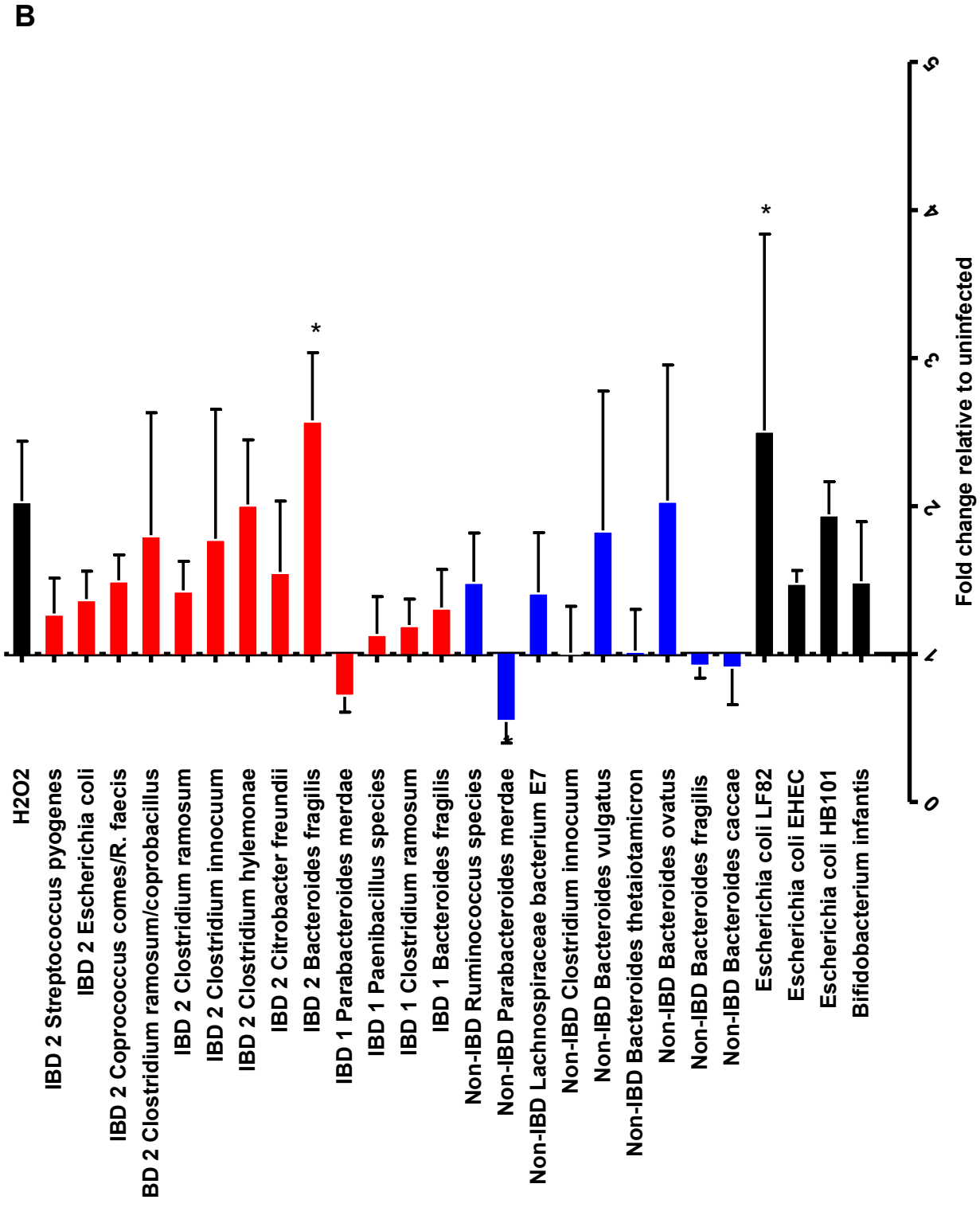

B
H2O2
IBD 2 Streptococcus pyogenes
IBD 2 Escherichia coli
IBD 2 Coprococcus comes/R. faecis
BD 2 Clostridium ramosum/coprobacillus
IBD 2 Clostridium ramosum
IBD 2 Clostridium innocuum
IBD 2 Clostridium hylemonae
IBD 2 Citrobacter freundii
IBD 2 Bacteroides fragilis
IBD 1 Parabacteroides merdae
IBD 1 Paenibacillus species
IBD 1 Clostridium ramosum
IBD 1 Bacteroides fragilis
Non-IBD Ruminococcus species
Non-IBD Parabacteroides merdae
Non-IBD Lachnospiraceae bacterium E7
Non-IBD Clostridium innocuum
Non-IBD Bacteroides vulgatus
Non-IBD Bacteroides thetaiotamicron
Non-IBD Bacteroides ovatus
Non-IBD Bacteroides fragilis
Non-IBD Bacteroides caccae
Escherichia coli LF82
Escherichia coli EHEC
Escherichia coli HB101
Bifidobacterium infantis
*
*
0
1
2
3
4
5
Fold change relative to uninfected

Fig 6.2. ROS production in macrophages during infection with bacteria isolated from the gastrointestinal tract of pediatric patients. THP-1 monocytes were seeded into black 96 well plates and differentiated using PMA (75 nM) overnight. Each bacterial strain was grown in BHI broth in an anaerobic chamber. DCFDA was added to macrophages, which were then infected with each bacterial strain for 2hrs followed by ROS measurement, hydrogen peroxide was used as a positive control. ROS was determined either as (**A**) relative fluorescence units RFU) or (**B**) fold change compared to uninfected control. N=3. *$p < 0.05$ measured using ANOVA.

6.5 DISCUSSION

IBD are chronic conditions in which the microbiota has been changed into a state of dysbiosis with changes in the major phylogenetic groups [185]. Studies have shown through genetic analysis there is an increase in *Firmicutes* and *Proteobacteria* with a decrease in *Bacteroides* in patients with IBD [62] . This dysbiosis can have multiple effects on the host such as nutrient breakdown and absorption, loss of barrier integrity and changes to the immune response. IBD is also known to have a dysregulated immune system where it has an inappropriate response to commensals [186].

Not only did the immune response differ between IBD and non IBD patients in our study, but there was also a difference between the same bacterial species in the two IBD patients. This was seen with *Ruminococcus*, *Bacteroides fragilis,* and *Clostridium ramosum*. This supports our hypothesis that commensal bacteria from IBD patients have the potential to become pathobionts. A well characterized pathobiont is the mucosal-associated AIEC, first isolated from Crohn disease patients, which we used as our positive control [169]. Unfortunately, we were unable to get the same bacteria in the non IBD and IBD groups (cultured bacteria from each sample were difficult to predict and reflect the diversity between individuals), but the microbiome is mostly unculturable and what we were able to isolate varied greatly. A larger cohort would provide more bacteria to analyze and potentially get the same species.

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B. fragilis is an example of a bacterium that has the potential to be a commensal in some case or cause disease in others. *B. fragilis* is responsible for many beneficial functions such as nutrient absorption, mucous secretion, and antimicrobial peptide synthesis [187]. The polysaccharide of capsular *B. fragilis* has also been shown to induce production of IL-10, a potent anti-inflammatory cytokine that is decreased in IBD [187]. On the other hand, *B. fragilis* can also be pathogenic as the capsule produced by this bacterium can induce abscess formation with a high rate of mortality in cases of untreated infections [187]. As this bacterium is decreased in IBD, perhaps this change in environment causes upregulation of potential virulence factors making it a pathobiont. This phenomenon is also demonstrated in our results as only one of the two IBD patients showed an increase in IL-1β secretion..

Ruminococci are commensal bacteria found predominantly in the cecum and colon and are known to produce short chain fatty acids (SCFA). These SCFA are an important nutrient source for colonocytes and can regulate immune response through regulatory T cell activation and IL-10 production [188]. Here we show that this genus can also reduce inflammasome activation and secretion of IL-1β. The species we isolated from the non IBD patient was not identified therefore we are unable to directly compare between the IBD and non IBD genus. *R. gnavus* has recently been identified as a pathobiont increased in IBD patients, which may potentially be our isolates[189].

ROS production by macrophages is considered a double-edged sword where on the one hand it is critical for the clearance of pathogens but can also cause oxidative stress and damage if dysregulated. There have been several rare mutations

identified in pediatric patients that result in the reduced capability of neutrophils to produce ROS [161]. Interestingly, it was the same *B. fragilis*, which caused increased IL-1β secretion and also suppressed ROS production. These findings suggest that we may have isolated a pathobiont from one of our patients, which elicits a strong immune response but suppressed ROS production and may be able to survive killing within the macrophage; this was also observed with the pathobiont AIEC.

In conclusion, this study illustrates the importance of a healthy microbiome and some potential consequences of dysbiosis. Increased IL-1β combined with a suppressed ROS production may be a tool used to identify potential pathobionts. In addition, we observed there are differences between the same species isolated from IBD patients illustrating the complex and individualistic nature of the microbiome. This was a pilot project and thus major conclusions can not be drawn at this time, but it does highlight the importance of the inflammasome in this disease. Fecal microbial transplants have not shown a high degree of success in IBD; however, this may be because we do not fully understand the microbiome yet and may not be targeting the right patients and/or bacteria. Current research shows that affecting the diet of the individual to be more effective in manipulating the microbiome and effective in treating pediatric patients with CD [190]. Identifying pathobionts and how they interact with the immune system will allow for the development of new therapies for conditions such as IBD.

CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS

CHAPTER 7. DISCUSSION AND FUTURE DIRECTIONS

7.1 DISCUSSION

IBD are a group of diverse chronic inflammatory conditions for which there is no cure; therapies are available but are not always effective and carry potential for significant side effects; most therapies focus on suppressing the immune response and can be associated with increased risk of infection. The etiology of IBD is unknown; however, research has shown an association between genetic susceptibility, environmental factors, and the microbiome, all leading to an uncontrolled immune response. More than 200 loci have been identified showing the complexity of these diseases. Furthermore, dysbiosis of the microbiome has become the focus of research as more evidence indicates these changes may play a role in the causation. Treatment for these conditions varies between patients and severity with most therapies acting as immune modulators. Central to IBD is the dysregulation of the immune system and possible overactive immune response to commensal bacteria. One of the main drivers of this project is the need to identify new ways to treat these conditions – instead of suppressing the immune response making it more effective through improving the ability of the immune system to contain bacteria. Almost all of the IBD susceptibility loci identified are linked to interactions between the innate and/or adaptive immune systems and microbes and several important ones are involved in autophagy, NOD2, IL-10, and the NLRP3 inflammasome.

The NLRP3 inflammasome is part of a group of innate immune complexes that form in the cytosol in response to antigens. There are several different inflammasomes, all of which are result in the maturation and secretion of the proinflammatory cytokines IL-18 and IL-1β. This process requires two signals, the first being activation of $NF_{\kappa}B$, leading to the transcription and translation of NLRP3, pro IL-18, and pro IL-1β. The second signal can include many different stimuli, in which the mechanism is not fully understood; however, this activation leads to formation of the inflammasome complex, recruitment of ASC, and cleavage of procaspase 1. Mature caspase 1 can then cleave pro IL-18 and pro IL-1β into their mature forms, which are secreted from the cell; the secretion is also not understood as neither protein has a signal peptide. This protein complex is an important part of innate immunity in both health and disease. In normal physiology this complex is involved in the clearance of many pathogens [82, 191-193]. However, when there is dysregulation, it can also be central to many diseases, such as rheumatoid arthritis, gout, diabetes, and Familial Mediterranean Fever. These conditions are considered sterile inflammation settings, and all have significantly high IL-1β. In contrast, the single nucleotide polymorphisms involved in IBD show a hypoproduction of IL-1β; this is important because IBD occurs in the gut, which certainly is not a sterile environment. However, IL-1β is found to be increased in patients with other polymorphisms, further illustrating the importance of a regulated and balanced immune system. The work done in this thesis illustrates the importance of the NLRP3 inflammasome in clearance of the mouse pathogen, IBD infection model organism, *C. rodentium* by macrophages summarized in **Fig 7.1**. This

chapter will discuss each of my findings, how they relate to each other, the potential implications and translation of these findings, and what future directions can be done.

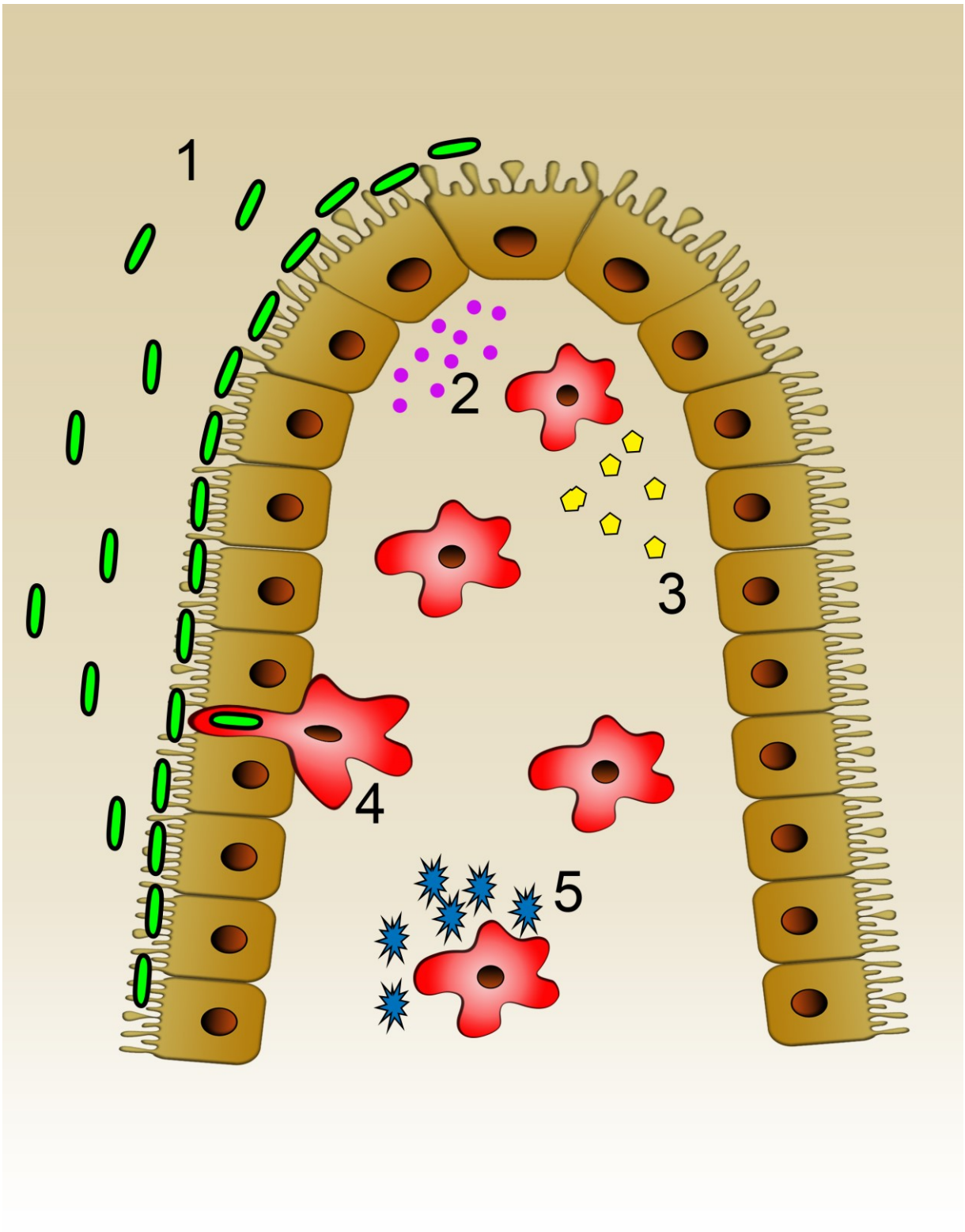

FIG. 7.1. Schematic of inflammasome activation in macrophages during *C. rodentium infection* of the intestinal epithelial barrier. **1.** *C. rodentium* infection of the epithelial barrier through attaching effacing/lesions causes barrier disruption. **2.** Release of cytokines, bacterial components, and damage-associated molecular patterns (*e.g*. ATP) into the gut submucosa. **3.** These will activate the NLRP3 inflammasome in macrophages and induce the release of other cytokines such as IL-1β. **4.** Activated macrophages will migrate to the apical membrane of the epithelial barrier to clear the infection as well as contribute to wound healing. **5.** Factors secreted by the activated macrophages stimulate infiltrating macrophages to continue this effect until the pathogen has been cleared.

The first two Results chapters looked at activating the inflammasome using different signal 2 stimuli in different macrophage cell lines. This work was done using *in vitro* cell lines in order to provide mechanistic insight into work that we previously published using a reductionist model. In the previous study we used *NLRP3* knockout mice infected with *C. rodentium* and showed that a balanced inflammasome activation can lead to effective clearance of the pathogen and resolution of infection and related inflammation, whereas low or high activity led to tissue damage [49]; however, it remained unclear how the inflammasome mediated these effects. The first cell line used was RAW 264.7 macrophages (Chapter 2), which do not have the ASC protein and thus cannot activate the inflammasome. The toxin nigericin is a common compound used to study the inflammasome and our research suggests it can act in an as yet undefined inflammasome-independent pathway to clear infection. Better understanding of how nigericin improves bacterial killing could offer novel therapy options for immune-mediated conditions.

We used *C. rodentium* not only because it is a colitis model organism for IBD but also because it only activates NLRP3, unlike other bacteria [194]. For example *Legionella pneumonia* can also activate AIM2, which can induce unprocessed caspase 1 and 11 to activate the inflammasome [195]; this allowed us to increase the specificity of our model. The pathways identified in the gene array could be looked at in future studies to determine how the clearance is being enhanced in these cells. We did not find any increase in phagocytosis or macrophage cell death; therefore, there are likely other signalling pathways involved. ROS generation was not looked at in

this cell line as it is generally considered to be required for NLRP3 activation, as ROS cannot activate this inflammasome in this cell line.

However, this was not the case in the next chapter where J774A.1 macrophages with extracellular ATP were used. Results from this chapter (Chapter 3) allowed us to introduce a new model for the inflammasome pathway, whereby the ATP-activated NLRP3 complex can induce ROS production, not seen in the literature at that time. Not only did we show that ATP reduced intracellular bacterial survival (indicating improved macrophage function), we also illustrated a cytokine shift from proinflammatory to a regulatory IL-10 mediated response. This illustrates the importance of the inflammasome in IBD, where the dysregulated immune system leads to chronic inflammation and tissue damage. Our hypothesis is that hypo-functioning polymorphisms of the NLRP3 gene, linked to IBD, result in a reduced ability to control and kill bacteria. This leads to over-activation of other immune pathways, resulting in a proinflammatory response, as seen in IBD. This dysregulation of the immune system also leads to disruption of the intestinal barrier, may contribute to microbial distribution, which ultimately leads to an inability to control microbes that get into underlying tissue. Granulomas, one of the prototypical microscopic features of Crohn disease, are a mixture of macrophages and T cells that form due to their inability to control an inflammatory condition, such as infection [196]. They are most known for their role in tuberculosis, where *Mycobacterium tuberculosis* is encapsulated within the granuloma where it cannot multiply but the immune system is unable to clear the infection [197]. Patients with severe Crohn disease may develop intestinal granulomas, with cause unknown as there is no infectious agent found

within these formations; studies linking inflammasome dysfunction with granulomas have not been conducted yet but would be a natural clinical extension of our research.

After identifying the ability of ATP, as an inflammasome activator, to improve bacterial clearance and demonstrating the important role of ROS, the next chapter (Chapter 4) was designed to determine a possible mechanism for where ROS was being generated and how it was responsible for clearance of *C. rodentium*; I was also interested in the immune pathways induced by this activity. I had already shown using ROS inhibitors (DPI, NAC, and Acteovanillone) that the ROS was not coming entirely from the NADPH oxidase complex. This complex is associated with the cell membrane and is part of the phagosome, where respiratory burst can kill the pathogen inside [198]. Recently, the mitochondria have been linked to inflammasome activation in macrophages. Priming of the inflammasome causes caspase 1-cardiolipin and NLRP3-cardiolipin to bind to the outer membrane of the mitochondria, resulting in mitochondrial stress and ROS production. Upon activation of the inflammasome by a second signal, ASC can bind to both complexes, forming the inflammasome and cleaving caspase 1 [153]. Therefore, extracellular ATP is activating the inflammasome through signal 2 and causing increased mitochondrial stress and increased ROS production.

In the experiment with live cell imaging and inert beads, there may be priming of the inflammasome and binding to the mitochondria; however, the ROS being produced by the mitochondria is either not detectable by the dye due to low concentrations or there is another signal not being activated. An alternative approach

could be to treat the cells with LPS instead of using inert beads and assess if this will trigger the priming mitochondrial ROS production, but this would activate signal 1. I showed that extracellular ATP caused a cytokine shift after infection to an IL-10 mediated response but I didn't initially analyze the cytokines that were being secreted during the infection. Using the proteome profiler, I identified a few proteins that were increased with the addition of ATP with the effects of these proteins already discussed.

The last set of experiments shown in this chapter were the addition of the supernatants (conditioned media), that likely contain the proteins identified in the proteome profiler, on newly infected macrophages. Conditioned media from ATP-induced macrophages caused increased IL-1β and ROS production in naïve macrophages; yet, this had the same effect on intracellular bacterial survival compared to conditioned media from nontreated macrophages in infected cells. This set of experiments illustrates how a dysregulated immune system can induce long term chronic inflammation through this positive feedback loop. The naïve macrophages have no infection to clear but are responding as if the bacteria were present. In IBD where both a dysregulated immune system and a constant supply of antigens from commensal bacteria are present, it is therefore not surprizing that conditions will facilitate the chronic inflammation seen and make the treatment so difficult.

In a physiological system, such as the gut, before any bacterium, such as *C. rodentium*, interacts with macrophages it comes in contact with colonic epithelial cells. The NLRP3 inflammasome in these cells is not as well understood as it is in

macrophages, but there is some evidence that it may be protective against pathogens [166]. Therefore, in Chapter 5, I used transwells to investigate the NLRP3 inflammasome in epithelial cells during infection and in the presence of macrophages. The observation that ATP-activated macrophages were able to aide in epithelial barrier recovery is most likely related to my earlier findings, where these macrophages change their cytokine profile into a more regulatory, IL-10 mediated response. This response would potentially represent a shift from M1 to M2 macrophages in a living animal/human, whereby they help reduce inflammation and induce wound healing [199]. Importantly, to understand what state these macrophages are in, analysis of their cell surface markers would need to be done, which was not feasible for my study.

The presence of tight junction proteins on macrophages is not a completely novel finding as this has been shown in an airway model but to our knowledge we are the first to show this in a model including epithelial cells originating from the gastrointestinal tract [173]. Monocytes and macrophages can also express claudin 2 which can form pores in the tight junctions of epithelial cells and cause redistribution of ZO-1 [200]. Therefore, the ZO-1 rings formed during infection could be due to the expression of claudin 2 by the macrophages and I speculate that this pore is what macrophages use to get to the apical membrane where *C. rodentium* is located. This only occurs during infection, suggesting the epithelial cells are secreting a cytokine that initiates this cascade.

The role of epithelial cells during infection is more than just a barrier to prevent the bacteria from getting into the underlying tissue. They are also part of the innate

immune system, secreting cytokines and chemokines as well as the production of AMP. In addition, they also express TLR's and have cytosolic microbial sensors such as NOD2 and inflammasomes. Inflammasome activation in epithelial cells is not fully understood and we found their role to be minimal during *C. rodentium* infection when looking at epithelial cell in isolation, without macrophages. The secreted IL-1β and IL-18 were not different during infection even though we saw an increased expression of NLRP3 when exposed to extracellular ATP. Colonocytes do express the purinergic receptor P2X7 and during infection will promote inflammation, this has been studied in IBD showing patients have increased expression of this receptor [201]. In contrast to what we found, most studies claim that extracellular ATP is linked to higher inflammation and apoptosis instead of barrier recovery [201]. This could be due to expression levels and another example of how important balance is to have an appropriate immune response.

The last results chapter of this thesis (Chapter 6) was using anaerobic bacteria cultured from patients and assessed their ability to activate the NLRP3 inflammasome and lead to ROS generation. Although the etiology of IBD is unknown, it is known that the microbes are involved. Whether the dysbiosis observed in IBD is a cause or a consequence is also not completely understood but commensals can change into pathobionts, as seen with AIEC in Crohn disease. In general, there was an increase in IL-1β secretion when macrophages were incubated with bacteria isolated form children with IBD, suggesting that commensal bacteria in IBD are more likely to cause inflammation. Interestingly, a few of the bacterial species behaved differently between patients. This suggests that while the commensals may be involved in IBD, how they

interact with the immune system may be unique to the individual person. One mouse model for IBD is phosphatase and tensin homolog on chromosome 10 (PTEN), which regulates TLR stimulation and is associated with an increase in *Bacteroides;* this is one of the genus identified as having increased IL-1β secretion by THP-1 cells [202].

Bacterial composition in the gastrointestinal tract is dependent on the environmental stimuli an individual is exposed to during childhood, as those living in a more microbe-rich environment such as farms tend to have less inflammatory disorders [203]. If a child has genetic predisposition to IBD such as NLRP3, NOD2 or NADPH oxidase mutations and combined microbe-poor environment, they would be more likely to get a chronic condition such as IBD. This has been long proposed as the “hygiene hypothesis’” where exposure to infectious stressors in early childhood is beneficial in preventing chronic inflammatory conditions in childhood and adulthood [204]. If we can identify which microbes are more likely to cause inflammation in an individual, then perhaps we can target those microbes either through more specific antibiotics or diet changes. Alternatively, identifying and enriching microbes that decrease inflammatory cytokines such as *B. infantis* or *Ruminococcus* in a personalized medicine approach may be more beneficial for the patient.

In conclusion, this thesis illustrates the importance of the inflammasome in macrophages during enteric infections and how this relates to homeostasis and inflammatory conditions. The pathway is not completely understood, but this work has shown a link between ATP-activation of the inflammasome, ROS production, and clearance of intracellular bacteria. Epithelial cells and macrophages form a multifaceted relationship to maintain homeostasis in the gastrointestinal system. As

they are both part of innate immunity, they work together to both clear the infection and in wound healing. A single model cannot describe the multifaceted nature of IBD; however, using *C. rodentium* in an *in vitro* environment allows for the intimate study of this pathogen on specific cell models. Further study in the inflammasome, and its relationship to pathobionts and dysbiosis in the gastrointestinal tract, has the potential for the development of new therapies in IBD.

7.2 FUTURE DIRECTIONS

As this study was done *in vitro*, and like any research – can raise more questions than answers, I will highlight some of the many experiments that still remain to be done. ATP and Nigericin both caused a decrease in intracellular bacterial survival and a possible mechanism was proposed. There were several genes identified to be expressed by RAW 264.7 macrophages during treatment with nigericin. Each of those proteins could be analyzed by Western blot to determine if they are being translated and with ELISA for quantification. Western blot would also determine activity of the proteins such as IL-12, IFNγ, Mapk11 (p38), and Nlrp4e. ROS generation should also be detected as this was seen to be increased in exposure to ATP with J774A.1 cells. In addition, iNOS is another important molecule used by macrophages to kill intracellular bacteria, which can be analyzed either using radiochemical HPLC or spectrophotometry [205, 206].

Further quantification of mitochondrial ROS generation could be done using MitoSOX or DCFDA using flow cytometry. Furthermore, inhibition of other pathways known to be involved in ROS production such as Rac2 could be done. In order to validate that my findings are not due to the specific cell lines, primary cells, organoids (see below), or animal models could be used. Primary cells such as peritoneal macrophages or bone marrow derived macrophages from knockout and wildtype mice (*NLRP3, caspase-1,* or *Nox*) would confirm these pathways. In addition, the experiments where conditioned media was used showed insights into the paracrine effects of ATP-induced inflammasome activation. Mass spectrometry on the conditioned media would allow further insight into what these cells are secreting.

Organoids are stem cells taken from the small or large intestine and cultured *in vitro* to develop into the differentiated cell types found in the gastrointestinal tract. These stem cells have a polarized epithelial layer and a fully functional lumen grown in suspension to develop a 3D structure. The apical membrane is on the inside of the organoid with the basolateral attached to the collagen found in Matrigel. In order to study pathogen/pathobiont activity on epithelial cells, the bacteria need to be microinjected into the lumen. A coculture system could then be set up using primary monocytes or macrophages in the Matrigel with the pathogen microinjected into the lumen. All of the previous experiments could then be used such as ELISA, Western blot and qPCR for inflammasome activation. Organoids offer a new and exciting opportunity to study individual patients with IBD and their disease in a noninvasive environment, as this has already been done for patients with cystic fibrosis and drug response [207]. Perhaps using primary cells such as organoids, the role of the

NLRP3 inflammasome in epithelial cells could be determined. CMT-93 cells are colonocytes and therefore are not receiving any signals from the other specialized cell types found in organoids. These cells responded differently depending on whether they were in a polarized state and therefore organoids offer a better physiological response. A Ussing chamber or transwell can be used with organoids as they can be attached to a membrane and maintain their function as spheroids, in order to measure epithelial barrier integrity. Organoids can also be used for transfection to study the many genes associated with IBD and their effects on epithelial cell function. This project has already been started, we have successfully isolated organoids from Caspase $1^{-/-}$ and Wildtype mice as well as from pediatric patients.

The last chapter of my thesis was looking at identifying pathobionts based on increased IL-1β secretion by THP-1 cells. Peripheral blood mononuclear cell (PBMC) isolation from IBD patients and culturing them with the anaerobic bacteria would identify how each patient responds. As this was a small study (3 patients), many more need to be recruited to expand the number of bacteria isolated . The main limitation with this study is the isolation of anaerobic bacteria as the majority are unculturable. There are selective media available for certain groups of bacteria such as *Prevotella, Bacteroides, Bifidobacterium,* and *Clostridia*. *Faecalibacterium prausnitzii* is a Gram-positive anaerobe and one of the most abundant microbes found in the human gastrointestinal tract, which is also reduced in IBD patients. This bacterium produces the SCFA butyrate and has been found to regulate Th17/Treg populations [208]. As this bacterium is important for immune development and

tolerance, it may direct macrophages towards an anti-inflammatory or IL-10 mediated response after inflammasome activation. As several bacteria appeared to decrease IL-1β secretion compared to control, another experiment that could be done is coculture of those bacteria with those that showed elevated IL-1β and see if they help clear the infection and maintain an anti-inflammatory state. The other alternative is that by dampening the immune response, they allow for the pathobiont bacteria to multiply.

7.3 SIGNIFICANCE

The NLRP3 inflammasome complex is an important innate immune sensor during bacterial infections. The results shown in this thesis suggest that it not only aids in clearance of *C. rodentium*, but also regulates epithelial barrier healing by macrophages. Since the NLRP3 inflammasome is dysregulated in some individuals with IBD resulting in a hypoproduction of IL-1β, this presents a new potential therapy. Targeting the inflammasome through regulation rather then inhibition may mediate some of the dysregulation these patients have and may result in healing of the mucosal barrier. This is particularly important for Crohn Disease patients as they are more likely to have the susceptibility mutation.

Further experiments on the role of the microbiota and inflammasome activation may also provide significant insight into the immune dysregulation found in IBD. The observation of some bacteria activating the inflammasome in an IBD patient also illustrates the importance of the luminal environment. IBD is a complex set of diseases without any clear etiology, therefore it may be critical to analyze patients individually. The microbiota plays a large role in determining immune tolerance,

therefore dysbiosis in individuals with IBD may be due to commensal bacteria losing the capacity to maintain this relationship. A personalized medicine approach may be the best method in dealing with the microbiome in IBD. There are not many published studies looking at fecal microbial transplants in IBD, unlike treatment for *Clostridium difficile* infections. Fecal microbial transplants work on the principal of replacing ones displaced microbiome with another individuals stable/normal microbiome. The problem with IBD is that we do not know whether the change in the microbiome is cause or effect of the disease. However, furthering what I have already started (Chapter 6) will allow for a greater understanding of an individual's relationship between their microbiome and immune system resulting in a therapy for some individuals.

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Appendix (other papers)

1. Armstrong H., **Bording-Jorgensen M**., Dijk S., Wine E. The Complex Interplay between Chronic Inflammation, the Microbiome, and Cancer: Understanding Disease Progression and What We Can Do to Prevent It. Cancers. 2018 Mar 20;10(3). pii: E83. doi: 10.3390/cancers10030083.

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4. Deenaz Zaidi, **Michael Bording-Jorgensen**, Hien Q. Huynh, Matthew W. Carroll, Jean-Francois Turcotte, Consolato Sergi, Julia Liu, Eytan Wine: *Increased Epithelial Gap Density in the Non-Inflamed Duodenum of Children with Inflammatory Bowel Diseases*. Journal of Pediatric Gastroenterology and Nutrition 02/2016;, doi:10.1097/MPG.0000000000001182

5. Misagh Alipour, Yuefei Lou, Daniel Zimmerman, **Michael W Bording-Jorgensen**, Consolato Sergi, Julia J Liu, Eytan Wine: *A Balanced IL-1β Activity Is Required for Host Response to Citrobacter Rodentium Infection*. PLoS ONE 12/2013; 8(12). doi:10.1371/journal.pone.0080656