Characterization of Perforin-2, a Novel Anti-bacterial, Pore-forming Protein of the Innate Immune System

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CHARACTERIZATION OF PERFORIN-2, A NOVEL ANTI-BACTERIAL, PORE-FORMING PROTEIN OF THE INNATE IMMUNE SYSTEM

By

Lesley R. de Armas

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CHARACTERIZATION OF PERFORIN-2, A NOVEL ANTI-BACTERIAL, PORE-FORMING PROTEIN OF THE INNATE IMMUNE SYSTEM

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Pore-forming proteins such as the membrane attack complex (MAC) of the complement system and Perforin of cytotoxic lymphocytes are potent host defense effectors employed by the immune system. These proteins share a structurally conserved domain, MACPF that is responsible for ring formation and membrane insertion of these toxic molecules. The objective of the current study was to identify the function of a third MACPF-containing pore-former of the immune system, Macrophage expressed gene 1 (Mpeg1). Mpeg1 encodes a highly conserved, membrane-bound protein which we have designated Perforin-2 (P-2). P-2 is induced upon monocyte maturation, is constitutively expressed by macrophages and upregulated by lipopolysaccharides (LPS) and interferon-γ (IFN-γ). Evidence is presented herein that macrophages employ P-2 to mediate intracellular killing of bacteria. Gene silencing was performed using P-2-specific siRNA which led to uncontrolled Salmonella typhimurium growth. To further characterize the protein, a P-2 fusion protein with a fluorescent tag was transfected into macrophages and analyzed for localization and function. P-2 in
macrophages resides in the endoplasmic reticulum, Golgi network and recycling endosomes where it would be available for trafficking to phagocytosed microbes. Combined inhibition of the established bactericidal mechanisms, reactive oxygen species and nitric oxide, with P-2 resulted in bacterial survival equal to that of P-2 inhibition alone suggesting that oxidative pathways of toxicity are downstream of P-2 action. Finally, site-directed mutagenesis of a conserved tyrosine in the cytoplasmic domain of P-2 rendered the protein non-functional in killing against *Mycobacterium smegmatis* suggesting that phosphorylation of this residue is important for activation of P-2. Therefore, P-2 represents a novel, highly conserved cytotoxic effector molecule expressed by macrophages with important implications for the control of microbial infection and host defense.
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CHAPTER 1: INTRODUCTION

Pore-forming proteins and the MACPF domain

Pore forming proteins solve the fundamental problem of how to insert soluble proteins into impermeable lipid membranes. The formation of pores on lipid membranes achieves several goals such as, transfer of materials from one side of a membrane to the other and membrane destabilization which may lead to osmotic lysis of cells or vesicles. Pore forming proteins of the mammalian immune system include 4 of the proteins from the membrane attack complex (MAC) of the serum complement system, C6, C7, C8 αβ and C9 (Podack, et al. 1979), and perforin of cytotoxic lymphocytes (CTL) and natural killer (NK) cells (Podack and Konigsberg 1984). Pore formation by C9, the terminal effector of the MAC, and perforin is mediated by the protein domain membrane attack complex/perforin (MACPF) that is required for monomer interactions leading to polymerization and membrane insertion (Baran, et al. 2009; DiScipio, et al. 1984; Young, Nathan, et al. 1986). The crystal structure of the MACPF domain has been resolved from several proteins including the insect pathogen, Photobactdus luminescens (Plu)-MACPF (Rosado, et al. 2007), human C8α (Hadders, et al. 2007; Slade, et al. 2008), and murine perforin (Law, et al. 2009). The structural analysis confirmed the importance of specific regions within the MACPF long-believed to be responsible for pore formation (Peitsch, et al. 1990). Interestingly, MACPF structure is remarkably similar to the structure of domains I-III of cholesterol-dependent cytolysins (CDC), a class of pore-forming toxins expressed by gram-positive bacteria (Rosado, et al. 2007). CDC proteins, such
as Perfringolysin O (PFO) from *Clostridium perfringens* form large transmembrane pores (diameter 25-30 nm). Membrane binding triggers oligomerization and pre-pore formation by means of a conformational change resulting in two α-helical bundles extending into amphipathic β-hairpins (Tweten 2005). In a similar fashion, MACPF domains also contain two sets of amphipathic α-helical bundles that execute pore formation through conformational changes to β-hairpins, albeit from the opposite orientation.

![Figure 1.1. Schematic comparison of pore formation by MACPF and CDC proteins. a-d. Perforin. a. Soluble monomer structure (the MACPF domain is in blue, red (central β-sheet) and orange (CH1 and CH2), the EGF domain in green and the C2 domain in yellow). b. Structural model of monomer with 2 CH helices extended into β-hairpins upon pore formation. c,d. Diagram of perforin sub-domains in colors corresponding to colored domains in a, b. g-j. Equivalent views of pneumolysin as a representative CDC. (Law, et al. 2009)
compared to CDC (Figure 1.1 from (Law, et al. 2009). MACPF proteins do not exhibit pre-pore formation following target recognition.

The MACPF domain family, which merged with the CDC family following the discovery of their structural similarity, has over 500 members found in a wide range of organisms (Rosado, et al. 2008), however only 12 of these proteins are expressed in humans. The pore-forming proteins of the immune system, the MAC of complement and perforin-1 are by far the best-characterized MACPF proteins. The remaining human MACPF proteins have not been extensively studied. Five of them have been suggested to play roles in neuronal biology; astrotactin-1 and -2 are important for neuronal cell adhesion and migration (Wilson, et al. 2010) and BMP/RA-Inducible Neural Specific Protein 1, 2, 3 (BRINP1, 2, 3) proteins seem to be involved in neuronal differentiation (Kawano, et al. 2004; Terashima, et al. 2009). However, it has not yet been determined whether these proteins are lytic or not.

**Pore formers of the Immune System**

**Membrane Attack Complex**

MAC pores are formed on the surface of bacteria and represent the final step in a cascade of events involving more than 20 proteins, known as the classical, alternative and mannose-binding lectin pathways of complement. This system alerts the innate immune system of bacterial presence in the serum and leads to opsonization of bacteria and bacterial membrane disruption via pore
formation. Complement proteins are synthesized in the liver and secreted into plasma, upon recognition of bacteria they are deposited on the bacterial outer membrane in a stepwise manner mediated by multiple proteolytic cleavage events. The MAC, also called the terminal complex, is formed when C5b on the bacterial membrane recruits C6, followed by C7, and C8 at which point the complex gains the ability to pierce the lipid membrane and act as an anchor (Podack, et al. 1979; Preissner, et al. 1985). Formation of the complex, C5b-C8, allows for recruitment of multiple soluble C9 monomers to simultaneously polymerize, unfold and insert into the bacterial membrane creating a pore containing 12-16 subunits and an inner diameter of 10-11 nM (Schreiber, et al. 1979). Purified C9 is capable of self-polymerizing in vitro at 37°C without the C5b-C8 complex (Podack and Tschopp 1982; Tschopp, et al. 1982) however; presence of the complex accelerates polymerization and insertion into membranes of gram-negative bacteria (Young, Cohn, et al. 1986). In vivo, lysis of bacteria is achieved through the combined presence of MAC pores and lysozyme, a secreted enzyme found in plasma, tears, mucus, and secretory granules within cells (i.e. polymorphonuclear cells) that can hydrolyze bacterial peptidoglycan (Glynn 1969). Destruction of the peptidoglycan layer is devastating for the bacteria, leaving them structurally unstable and vulnerable to osmotic lysis.

Lytic, pore forming proteins are inherently hazardous proteins to have floating around the extracellular milieu. Therefore, tightly regulated mechanisms must exist to target the effector molecules to the proper membranes. This is
accomplished through specific protein inhibitors of various members of the complement system. Complement regulatory proteins are present in the plasma and on the cell surface and target the components of the complement activation pathways as well as the MAC (also called the terminal complex). Serum inhibitors specific for the C5b-C6 complex (SP-40,40) and the C5b-7 complex (Vitronectin/S protein) prevent complete formation of the activation complex, while C8-binding protein and CD59 are expressed on the surface of all cells and bind C8 thereby preventing C9 recruitment and subsequent pore formation onto host membranes (Podack and Muller-Eberhard 1979; Schonermark, et al. 1986; Zalman, et al. 1986). A genetic defect in CD59 expression leads to the disease paroxysmal nocturnal hemoglobinuria (PNH) and is characterized by complement-induced hemolytic anemia, red urine, and thrombosis (Yamashina, et al. 1990). Genetic defects in each of the terminal complex components have been reported in humans and patients display a marked increased susceptibility to infections with the gram-negative bacteria Neisseria meningitidis (Figueroa and Densen 1991). This bacterial species is especially resistant to phagocytosis by complement-mediated opsonization making MAC formation a key mode of innate defense against this pathogen (Schneider, et al. 2007).

Perforin

Perforin-1 was first characterized as the lytic effector present in dense cytoplasmic granules of cytolytic lymphocytes (CTL) (Dennert and Podack 1983; Podack and Dennert 1983; Podack and Konigsberg 1984). Isolated granules
from CTL cell lines demonstrate rapid and unspecific lysis of cells, while the
different CTL lines showed restriction in tumour cell lysis. Rapid cytolysis of
tumour cells and sheep erythrocytes by the isolated granules was found to be
both Ca$^{+2}$-dependent and temperature sensitive (optimal at 37°C and neutral pH).
Perforin protein was ultimately isolated from the granules and displayed all the
characteristics of the granules themselves (i.e. hemolytic activity against sheep
erythrocytes in the presence of Ca$^{+2}$ and at the optimal temperature). Perforin is
a 70kDa protein that contains multiple domains, most with important biological
properties that have been conserved from fish to humans (Hwang, et al. 2004).
Poly-Perforin pores are visible on the lysed membranes under the electron
microscope as tubular pores made up of 12-16 protomers with an inner diameter
of approximately 16 nm (Lichtenheld, et al. 1988; Podack and Dennert 1983).
The contents of the cytoplasmic granules are secreted at the immunological
synapse between cytotoxic lymphocytes and target cells, identified by CTL or NK
through cell surface receptor:ligand interactions. Poly-perforin pores lead to cell
death in vitro via several pathways, first; pore-mediated membrane disruption
leads to an influx of small molecules and the induction of colloid osmotic lysis
within the target cell. Second; exocytosis of lysosomal granules delivers
granzyme molecules along with perforin, which are a family of lymphocytic serine
proteases that make up the bulk protein mass (90%) of cytolytic granule
contents. Upon perforin-mediated entry into cells, granzymes induce DNA
fragmentation and an apoptosis-like death of the target cell (Nakajima and
As is true for the MAC, regulatory mechanisms for perforin also exist to protect host cells from inadvertent pore formation. The C-terminal domain of perforin contains a proteolytic cleavage site, which upon enzymatic cleavage renders the protein active by revealing a phospholipid-binding C2 domain (Uellner, et al. 1997). Cleavage of perforin occurs during the biosynthesis of the lytic granules, so that once the secretory granules are formed the proteins contained within them will not necessitate further modification—allowing rapid killing upon granule exocytosis. The cleaved peptide contains an aspartic acid residue which is heavily glycosylated during perforin biogenesis in the Golgi serving to obscure the cleavage site and prevent premature activation (Voskoboinik and Trapani 2006). The acidic pH of the secretory granules (pH 4.9-5.2) ensures protonation of aspartate residues of the C2 domain that would otherwise bind free calcium and lead to pore formation. Upon target binding and granule release, the presence of a neutral pH and free Ca$^{2+}$ allow perforin to bind lipid membranes and induce the conformational changes within the MACPF to support polymerization and insertion into the membrane. The mechanism of pore formation involves oppositely charged residues within the MACPF domain that are revealed upon calcium-induced conformational changes within the monomer (Baran, et al. 2009). The oppositely charged residues form hydrogen bonds and catalyze the polymerizing reaction and simultaneous insertion into pores. In order to protect the killer cell from ‘self’-targeted pore formation, the granules contain a membrane-bound protease, cathepsin B, that has been
proposed to effectively shield the membrane by inactivating perforin monomers that have diffused back toward the killer cell (Balaji, et al. 2002).

The importance of perforin in the immune response against virus-infected and transformed cells was best demonstrated upon analysis of a perforin knockout mouse (Kagi, et al. 1994; van den Broek, et al. 1996). Perforin gene deficiency or dysfunctional perforin trafficking (also by genetic mutation) in humans leads to a disorder called familial hemophagocytic lymphohistiocytosis (FHL) characterized by hyper-proliferation and activation of macrophages mimicking acute responses to infection and presenting as fever, hepatosplenomegaly and enhanced hemophagocytosis in spleen, liver and bone marrow usually early in life (Fadeel, et al. 2001; Goransdotter Ericson, et al. 2001).

**Mpeg1**

Macrophage expressed gene 1 (Mpeg1) was first described as a novel genetic marker for macrophage differentiation (Spilsbury, et al. 1995). The gene was discovered using a differential cDNA screen of murine peritoneal macrophage derived library against a number of myelomonocytic cell lines representing immature monocytic and mature macrophage phenotypes. Mpeg1 was found to be absent in monocytic cell lines but was upregulated during macrophage differentiation. Initial sequence analysis of Mpeg1 identified local homology to perforin. Mpeg1 is an ancient gene which has been conserved in
Figure 1.2. Alignment of Mpeg1 sequences across species: Homo sapiens (human), Mus musculus (mouse), Bos taurus (domestic cow), Danio rerio (zebrafish), Haliothis corrugata (pink abalone/snail), Crassotrea gigas (pacific oyster), and Suberites domuncula (sponge).
Figure 1.2. (cont) Red font with yellow highlight denotes identical residues, blue font with teal highlight denotes conservative replacement, black font with green highlight identifies a block of similar residues, green font with no highlight denotes weakly similar residues, and black font with no highlight denotes non-similar residues.
humans all the way from sponges (Porifera), its origin predates the divergence of protostomes and deuterostomes. Interestingly, *Mpeg1* is not present in *Drosophila* or *Caenorhabditis elegans* genomes indicating it was lost in protostomes following this divergence. There is also evidence that the perforin gene originated following a duplication of an early *Mpeg1* gene as the 2 genes are adjacent and in the same orientation in the fish genome, where perforins earliest homologs are present (D'Angelo, et al. 2012). Figure 1.2 shows an alignment of *Mpeg1* protein sequences from a number of species.

Murine *Mpeg1* encodes a 713 amino acid protein, which is characterized by the domain organization shown in Figure 1.3. All *Mpeg1* proteins have an N-terminal MACPF domain that is highly conserved suggesting functional importance. Interestingly, the MACPF domain of *Mpeg1* shares only 15% sequence identity each with the MACPF domains from C9 and Perforin. Following the MACPF domain is a stretch of approximately 300 amino acids which is also highly conserved within *Mpeg1* proteins although it does not share homology with any other known protein domain. This region is however, cysteine-rich containing 16 of the 20 total cysteine residues in the protein which is reminiscent of the EGF-like domain present in perforin and complement C9 directly adjacent (downstream) to the MACPF domains. In perforin, the EGF-like domain is only 30 amino acids and contains 6 cysteine residues. The disulfide bonds formed between the cysteines allow it to make a shelf upon which the MACPF domain sits and separates it from the calcium-dependent membrane binding, C2 domain (Law, et al. 2009). A putative transmembrane domain
separates a short cytoplasmic tail from the lumenal portion of this type I membrane protein (sponges do not have transmembrane and cytoplasmic domains). The mammalian cytoplasmic domain has several conserved and potentially regulatory residues and motifs (Figure 1.3). There is a conserved basic region adjacent to the transmembrane domain with the sequence RKX(R/K)K(R/K). Peptide sequences containing a string of basic amino acids such as lysine (K) and arginine (R) can be sites for proteolytic cleavage by
enzymes such as trypsin or furin. Downstream of this basic region there are 2 residues conserved in all mammals that have the ability to be phosphorylated, a tyrosine and serine. Phosphorylation is an important mechanism of post-translational protein modification and functions in cell signaling pathways in a broad range of cellular processes including immunological function.

There have been a number of studies investigating Mpeg1 expression and function in marine invertebrate species. Mpeg1 from sponge (Wiens, et al. 2005) and abalone (Kemp and Coyne 2011; Mah, et al. 2004; Wang, et al. 2008) is expressed by innate immune cell types and the transcript is upregulated following treatment with lipopolysaccharide (LPS) or infection with bacteria. Interestingly, recombinant Mpeg1 (aa158-358) from Crassostrea gigas (Pacific oyster) had potent bactericidal activity (60-80% killing) against gram-positive and gram-negative bacteria, including Escherichia coli (E. coli) and Staphylococcus aureus (He, et al. 2011).

Macrophages and anti-bacterial immunity

Macrophages are heterogeneous innate immune cell types that play important roles in host defense, homeostasis, and embryogenesis.

Macrophages belong to a family of professional phagocytes including monocytes, myeloid dendritic cells and neutrophilic granulocytes. They are highly phagocytic and bactericidal which is believed to be through the production of large amounts of oxidative metabolites (e.g., superoxide and nitric oxide) and intracellular killing of phagocytosed bacteria (Chakravortty and Hensel 2003; Ding, et al. 1988).
Macrophages are found in lymphoid and non-lymphoid tissues and at sites of infection where they can clean up apoptotic cells and prevent spread of infection. This terminally differentiated cell type can arise from fetal liver, bone marrow and blood-derived monocytes. Similar to lymphoid cells, macrophages can display several different phenotypes based on origin, anatomical location, cell surface receptors, and functional characteristics including inflammatory, suppressive (regulatory), and tolerogenic (Auffray, et al. 2009; Geissmann, et al.; Gordon and Taylor 2005; Wright, et al. 2001). However, macrophages are mainly divided by their functional characteristics as cell surface molecules have not proved to be reliable in differentiating subtypes (Hume 2008). Classically activated pro-inflammatory macrophages, also called M1 macrophages, have been induced by LPS and IFN-γ, produce large amounts of IL-12 and TNFα, and are highly phagocytic and bactericidal (Gordon 2003; Mantovani, et al. 2004). Alternatively activated macrophages, or M2 macrophages, have been induced in the presence of cytokines IL-4 and IL-13, produce IL-10 to suppress immune activity, and promote angiogenesis and tissue microenvironment remodeling and healing (Allavena, et al. 2008; Varin, et al. 2009).

Macrophages mediate phagocytosis upon contact and recognition of an engulfable target (pathogen, or transformed or apoptotic cell) using any of a variety of cell surface receptors with specificity for a wide-range of ligands. The receptors are divided into non-opsonic receptors, also called pattern associated molecular pattern (PAMP) receptors; and opsonic receptors such as fragment crystallizable (Fc) and complement receptors for opsonized pathogens.
Following recognition, the membrane undergoes actin-dependent and drastic morphological changes as the cell engulfs the pathogen utilizing membrane donations from the ER and endosomal trafficking vesicles (Gagnon, et al. 2002). Once the phagosome is established it matures via fusion with endosomes and lysosomes. Acquisition of V-ATPases, which cause the pH in the lumen of the phagosome to drop to pH 5.0 within 15 minutes, and lysosomal hydrolases via fusion of endocytic vesicles lead to ultimate destruction of pathogens. Finally, toll like receptors (TLR) and other key innate signaling molecules are shuttled to the phagosome to determine whether an immune response, and thus antigen presentation should be initiated (Jutras and Desjardins 2005).

Especially interesting, given the important role that macrophages play in defending against bacteria, is that many bacteria depend on macrophages for their pathogenesis and survival within a host (de Chastellier 2009; Isberg, et al. 2009; Prost, et al. 2007). Pathogenic microbes, including bacteria, have evolved mechanisms to evade the immune system via disruption of host defense pathways. Indeed, some bacteria such as Yersinia species have mechanisms to avoid phagocytosis altogether to replicate and survive in extracellular spaces (Fallman, et al. 1995). There are three broad categories of immune evasion mechanisms utilized by intracellular bacteria to avoid the deadly phagolysosomal maturation process; 1) vacuole lysis, 2) escape from the endocytic pathway, and 3) maturation arrest (Meresse, et al. 1999).
Given the long evolutionary presence of P-2, it is likely that modern bacteria have evolved mechanisms to dampen or block the function of P-2 to promote survival in macrophages. Thus, we hypothesize that P-2 is a cytotoxic protein that contributes to the bactericidal activity of macrophages and other phagocytic cells.

Specific Aims

Specific Aim 1: Determine the role of P-2 in macrophage-mediated immunity.

Preliminary data indicate that P-2 is a pore-forming protein. The conditions required for P-2 polymerization and insertion into membranes will be investigated under this aim. In order to determine the role of P-2 in phagocyte-mediated killing of bacteria, bactericidal assays will be performed with P-2 expressing murine macrophage cell lines and primary cells. The possibility that P-2 is a specialized host defense pathway for a certain type of bacteria will also be explored as a part of this aim.

Specific Aim 2: Characterize the regulation of P-2 expression and mechanism in macrophages.

Experiments under this aim will focus on examining where P-2 is localized within the cell and what cellular signals regulate its expression. Known innate immune stimulatory signals will be investigated for their effects on P-2 protein expression. The proposed protein sequence of Mpeg1 contains several possible
sites for post-translational modifications including phosphorylation and proteolytic cleavage, which will be explored for regulatory roles in P-2 expression and function.
CHAPTER 2: RESULTS AND DISCUSSION

Characterization of Perforin-2-GFP fusion protein in HEK-293 cells

*Perforin-2 is a pore-forming protein*

In order to characterize the function of Perforin-2 (P-2), the coding sequence of *Mpeg1* was assembled from several expressed sequence tag (EST) clones and tagged at the C-terminus with the green fluorescent protein (GFP) using the EGFP-N3 plasmid (Clontech) (M.Shiratsuchi). Transfection of the P-2-GFP plasmid into 293 cells is toxic and required extensive selection with G418 (geneticin) and flow cytometry sorting to reach high expression levels. The resulting cells showed strong fluorescence in a few, large punctate bodies by immunofluorescence (Fig. 2.1a) and by flow cytometry were greater than 90% positive (Fig. 2.1b).

The stably transfected cell line provided a rich source of P-2-GFP protein that could potentially be isolated in large quantities.

Figure 2.1. P-2-GFP expression in HEK-293 cells. a) Monolayer of 293-P-2-GFP cells showing bright GFP fluorescence in punctate staining pattern. Image taken with 20X objective lens. b) Flow cytometric analysis comparing 293 (left) and 293-P-2-GFP (right) cells.
for further characterization. Dr. Podack and Motoaki had previously isolated and analyzed membranes from these cells and identified pores by electron microscopy but I sought to confirm the presence of pores in my own isolations. The gentle method of Dounce homogenization was used to lyse the cells after a brief incubation in hypotonic buffer which was followed by centrifugation over a discontinuous sucrose gradient. Figure 2.2 shows the distribution of GFP among the fractioned 293-P-2-GFP cell lysate as determined by fluorometric analysis (green bars) with the corresponding protein concentration (hatched bars) from each fraction. GFP fluorescence peaked in the lower fractions corresponding to heavy organelles or membranes, as predicted given the putative transmembrane domain. The peak in the last fraction (#15) corresponds to nuclei and unlysed cells, the heaviest material in

Figure 2.2. Fractionation of 293-P-2-GFP lysates. Green bars represent the percentage of GFP fluorescence in each fraction. Hatched bars represent the protein concentration in each fraction. Fraction 1 corresponds to the first fraction removed from the top of the sucrose gradient (15%).
the lysates. Fractions 11 and 12 were pooled and the membranes were pelleted for negative staining and electron microscopy analysis.

In order to strip bulky carbohydrates from the membrane surfaces and aid in visualization of the membranes and pores, the pelleted membranes were treated with trypsin prior to negative staining with phosphotungstic acid. Figure 2.3a shows electron microscopy images of pore-like structures from these membrane fractions shown at 143,000 fold magnification. Poly-P-2 pores have an average diameter between 8 and 10 nm, which is comparable in size to MAC pores and smaller than perforin pores (16 nm). Some pore complexes are visible at a side view showing extension off the surface of the membrane (Fig. 2.3a upper right panel). Similar to initial observations of poly-perforin pores, incomplete rings were visible amidst the well-formed circular pores which highlight the oligomeric nature of poly-P-2 (Fig. 2.3a lower right panel). As a control, we used P-2-GFP enriched membrane fractions that were not treated with trypsin and found no evidence of pores. Previously, controls were performed using untransfected 293 cells and pores were not observed on these membranes either (M. Shiratsuchi, unpublished data). For comparison, polymerized pore structures from perforin (Dennert and Podack 1983) and poly-C9 (Podack and Tschopp 1982) are shown. Poly-P-2 pores look remarkably similar to poly-perforin pores and support the hypothesis that P-2 is a pore-forming, cytolytic protein.
Figure 2.3. P-2-GFP forms membrane pores visible by electron microscopy. a. (main panel) Membrane fragment covered in doughnut-shaped poly-P2 pores. Membrane is stained black and P-2 remains unstained (white). (upper right panel) Arrow points to a side-view of a ring shaped structure projecting outward from the membrane. (lower right panel) Arrowheads point to open rings in which polymerization is incomplete. b. Figure from Dennert and Podack, 1983 showing the first electron microscopy analysis of poly-perforin pores in CTL-susceptible target membranes Scale bar=57 nm. c. Figure from Podack and Tschopp, 1982 showing images of poly-C9 pores in the absence of membranes. Scale bar=40 nm.
Perforin-2 cytoplasmic domain is susceptible to proteolytic cleavage by trypsin

Any pore-forming, potentially lytic molecule, must have regulatory mechanisms in place to prevent pore-formation at the wrong time or in the wrong place. It was unclear whether the poly-P-2 pores observed by electron microscopy represented the true nature of P-2-GFP expression in 293 cells or whether manipulation and isolation of the membranes induced pore formation. This is an important question because in order to analyze polymerization of P-2 in vitro, we would need a source of monomeric P-2 protein. As described previously, analysis of the peptide sequence of P-2 revealed that the cytoplasmic domain contains several well-conserved potential regulatory motifs, such as a cluster of basic residues (RKYKKK) that could serve as a proteolytic cleavage site, and tyrosine and serine residues that could function as phosphorylation sites. Our hypothesis is that the cytoplasmic domain interacts with proteins in the cytoplasm while awaiting signals for activation and polymerization which may include phosphorylation or dephosphorylation of amino acid residues and/or cleavage of the cytoplasmic tail. These protein:protein interactions in the cytoplasm could send signals through the transmembrane region and P-2 domains ultimately triggering the required conformational changes in the MACPF domain to initiate polymerization and pore formation in adjacent membranes. To assess whether the proteolytic cleavage site and thus cytoplasmic domain was still intact in our P-2-GFP-enriched membranes they were treated with trypsin for increasing amounts of time up to 30 minutes and lysates were analyzed by
Figure 2.4. Validation of P-2-GFP transfection and protein expression. Western blot analysis of transfected P-2-GFP expression in 293 cells. P-2-GFP was detected using polyclonal antiserum raised against the cytoplasmic domain of P-2 (P-2 cyto), a commercial peptide antiserum (Anit-P-2), and an anti-GFP antibody. P-2-GFP migrated at the expected size of ~102 kD. Antibodies to P-2-cyto and GFP both recognized a 293-expressed protein that served as a loading control (non-specific band).

Figure 2.5. P-2 cytoplasmic tail is susceptible to proteolytic cleavage by trypsin. P-2-GFP enriched membranes were treated with 10μg/ml TPCK treated trypsin for increasing amounts of time. The lysates were run on SDS-PAGE in triplicate and exposed to Anti-P-2 (left), Anti-P-2-cyto (right), or Anti-GFP (not shown) antibodies for western blot analysis.
western blot. To distinguish between different parts of the protein, two antibodies specific for P-2 were used, the first is a commercially available polyclonal antibody which was produced in rabbits using recombinant full-length P-2 from rat as the immunogen (Abcam), and a second which was custom made, also produced in rabbits, and generated against a peptide from the cytoplasmic domain (21st Century). Both antibodies recognize P-2-GFP in whole cell lysates from transfected 293 cells (Figure 2.4). With increasing exposure of P-2-GFP-enriched membranes to trypsin there was an increase in the core protein detection (~75kD band) using the commercial antibody and not the cytoplasmic peptide antibody, and a corresponding increase in the cytoplasmic domain-GFP cleavage product (~30kD band) with the cytoplasmic peptide antibody and not the commercial antibody (Figure 2.5). The 30kD band identified with the cytoplasmic peptide antibody was also recognized using anti-GFP antibodies (not shown). These results validated the specificity of our cytoplasmic peptide antibody and indicated that the P-2-GFP isolated from 293 membranes has an intact cytoplasmic domain. Further, if cleavage of the cytoplasmic domain is required for polymerization then exposure to trypsin may have induced the pore formation we observed in P-2-GFP enriched membranes.

Based on these results we anticipated that the P-2-GFP enriched membranes would be useful in testing for lytic function of P-2 in standard hemolytic assays such as those described for measuring complement activity in serum (Podack and Tschopp 1982). P-2-GFP enriched lysates were incubated with sheep erythrocytes at several dilutions and hemolysis was evaluated. As
controls, the same fractions from 293 cells that had not been transfected were used. Experiments were carried out testing the enriched fractions for hemolytic activity in the presence of calcium and magnesium ions, EDTA, furin, trypsin, ATP, and acidic and neutral pH. The P-2 enriched fraction demonstrated dose-dependent hemolysis, however, the activity never exceeded 25% despite concentrated protein lysates and ultimately the difference between P-2-GFP enriched fractions and matched fractions from untransfected 293 cells was not great enough to pursue the assay further (Figure 2.6). We opted to focus our efforts on establishing a functional assay for P-2 through gene silencing in cells which constitutively express P-2, such as macrophages.

![Figure 2.6](image.png)

Figure 2.6. Hemolytic activity of P-2-GFP enriched membranes. P-2-GFP-transfected and untransfected 293 cells were lysed and fractionated as described in the methods. The GFP-rich fraction from transfected 293 and corresponding fraction number from untransfected 293 were serially diluted and incubated with sheep erythrocytes in the presence of calcium and magnesium ions (GVB++ buffer). Percent hemolysis for each sample was calculated relative to the positive control of water-induced hemolysis. Hanks balanced salt solution (HBSS) was used as a negative control.
**P-2 expression in macrophages**

The initial study describing *Mpeg1* reported gene expression in macrophages upon differentiation from monocytes (Spilsbury, et al. 1995). We hypothesized that P-2 message and protein expression would be modulated by pro-inflammatory factors such as interferons (type I and II) and TLR ligands if it was playing a role in macrophage immunity against bacteria. In the murine monocytic/macrophage-like cell line, RAW264.7 (RAW), P-2 protein was detectable by western blot at the correct size (~70 kD) in whole cell lysates from RAW cells and increased upon stimulation with LPS and IFN-γ over the course of 24 hours (Figure 2.7a). In contrast, a second murine macrophage-like cell line, J774A.1, exhibited strong P-2 mRNA and protein expression in the absence of activating signals with approximately 15 fold greater message levels than unstimulated RAW cells (Figure 2.7b). However, stimulation with LPS and IFN-γ did not yield an increase in message or protein expression (Figure 2.7b, c). Similarly, stimulation with type I IFNs (α and β), the synthetic TLR3 ligand, poly(I:C), zymosan A (yeast cell walls), nor the cytokines, TNF-α, and IL-4/IL-13 combination resulted in a change in protein expression (Figure 2.7d and not shown). It is possible that these two cell lines represent different stages of macrophage differentiation with the RAW cells more characteristic of monocytes and J774 exemplifying macrophage characteristics.

Since we found variable responses to LPS and IFN-γ in the macrophage cell lines, we analyzed P-2 expression in primary murine macrophages in
Figure 2.7. P-2 mRNA and protein expression in macrophage cell lines. a. Mpeg1 mRNA expression was measured by RT-PCR using Taqman assay. Message levels were normalized to GAPDH (housekeeping gene) and fold increase in expression is relative to RAW unstimulated cells. Error bars represent 3 replicates per condition. b, c. Western blot analysis of P-2 expression in J774 cells following the indicated treatments. Cytokines and chemicals were used at the following concentrations: IFN-γ 100U/ml, LPS 1ug/ml, TNF-α 50ng/ml, IL-4 25ng/ml, and IL-13 50ng/ml. d. Western blot analysis of P-2 expression in response to LPS and IFN-γ stimulation over a timecourse of 24 hours in RAW cells.
response to pro-inflammatory factors. We used two sources and techniques for macrophage isolation, the first is bone-marrow derived macrophages (BMM) and the second is peritoneal-elicited macrophages (PEM). BMM are differentiated from bone marrow cells in the presence of granulocyte- macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF) for 7-10 days (Manzanero 2012). GM-CSF treatment has been used to produce large numbers of bone marrow-derived dendritic cells (DCs) especially with the addition of IL-4 in media (Banchereau and Steinman 1998; Inaba, et al. 1992). However, (Fleetwood, et al. 2009) reported that GM-CSF and M-CSF-differentiated bone marrow cells behaved like M1 and M2 macrophages, respectively. To determine whether GM-CSF-differentiated cells were macrophages or DCs, phenotypic analysis of the GM-CSF derived cells was carried out and the majority (90%) were F4/80 and CD11b double positive identifying them as mature macrophages. Within this population was a small number (10%) of MHCII and CD11c^{hi} double positive cells, representing the classical phenotypic definition for dendritic cells. Thus, we concluded it was a mixed population of macrophages and DCs. Both factors induced Mpeg1 expression after 8 days of culture, coinciding with the appearance of typical macrophage morphology (Fig. 2.8a,c). P-2 protein was detectable by western blot around the same time as well, as shown in M-CSF differentiated macrophages (Fig. 2.8e). Upon stimulation with IFNs and TLR ligands for 48 hours, P-2 expression was further increased relative to unstimulated macrophages, as measured by quantitative RT-PCR (Fig. 2.8b, d). As a second
Figure 2.8. P-2 mRNA and protein expression in bone marrow derived macrophages. a, b. GM-CSF-differentiated macrophages. c, d. M-CSF-differentiated macrophages. a, c. Mpeg1 expression in differentiated macrophages relative to freshly isolated bone marrow cells. b, d. Mpeg1 expression in differentiated macrophages following 48 hours of stimulation as indicated relative to unstimulated cells. Mpeg1 was normalized to GAPDH expression in all experiments. e. Western blot analysis of P-2 expression in bone marrow cells in response to M-CSF over a timecourse.
source of macrophages, we analyzed PEM cells for their P-2 expression. PEM are obtained via peritoneal lavage 4 days following intraperitoneal (i.p.) injection with 4% Brewer’s thioglycollate medium. Brewer’s thioglycollate is a rich culture broth containing proteose peptone and beef the infusion and it recruits activated leukocytes to the site of injection by an unknown mechanism. On day 4, the majority of the cells in the peritoneum are macrophages, while at earlier timepoints granulocytes occupy the region. Using this method, 20 to 25 million cells were regularly isolated, which were then purified for macrophages by adherence on tissue culture dishes for 2 hours (Zhang, et al. 2008). To positively identify the adherent cells as macrophages, they were analyzed for surface expression of F4/80 and CD11b by flow cytometry (Fig. 2.9a). PEM lysates displayed a stronger band for P-2 expression when compared to RAW cells, and further increased P-2 mRNA and protein expression following LPS and IFN-γ treatment (Fig. 2.9b, c).

The P-2 expression data confirmed the findings reported in the Spilsbury paper, that Mpeg1 is expressed upon differentiation of macrophages. Also, we demonstrated that P-2 expression can be induced in macrophages in response to pro-inflammatory factors such as IFNs and TLR ligands (on the message and protein level). This supports the hypothesis that P-2 plays a role in macrophage-mediated immune defense especially given that IFN-γ is critical for macrophage activation and clearance of a variety of pathogens (Flynn, et al. 1993; Huang, et al. 1993; Reed 1988; Rosenberger and Finlay 2002).
**Cellular localization of P-2 in macrophages**

**Antibody availability for immunofluorescence**

We utilized the fluorescent reporter P-2 construct to image protein localization in cells because there is a lack of antibodies that are able to recognize native and fixed P-2. The commercial and peptide antibodies have...
been tested in fluorescence microscopy and flow cytometry analysis and were unsuccessful in detecting P-2. Both of the aforementioned techniques require permeabilization and fixation of the cells. Not all antibodies are able to recognize the antigen in its properly folded state and, moreover, fixation with paraformaldehyde can induce crosslinking of proteins (through the formation of methylene bridges between amino acids and/or nucleotides) that can alter antibody-binding epitopes. The Podack lab has made several attempts to produce an anti-P-2 monoclonal antibody without success. In the absence of purified P-2 protein, P-2-GFP-enriched lysates from 293 cells were used as immunogen for antibody production in Armenian hamsters. In the most recent attempt, a combination of P-2-GFP-enriched lysate and P-2-GFP DNA bound to dendrimers were used for immunization, as dendrimer technology has been shown to enhance the efficacy of DNA vaccines and therefore may elicit more robust antibody production (Daftarian, et al. 2011). The resulting hybridomas underwent two rounds of screening, first they were positively selected for IgG production by enzyme-linked immunosorbent assay (ELISA) and then subsequently negatively selected for non-specific binding. Since unpurified protein was the antigen, it was likely that the animals would generate antibodies to all proteins present in our preparation, including GFP and any contaminating 293 proteins. In order to select against antibodies with specificity to non-P-2 proteins, a flow cytometric assay was developed in which 293 and 293-P-2-GFP cells were mixed at a ratio of 1:1, permeabilized and stained with the hybridoma supernatant and fluorescently labeled secondary antibodies. Hybridoma clones
that recognized the GFP negative and GFP positive cells equally were eliminated and clones that recognized only the GFP positive cells were analyzed further. From 960 hybridoma clones, 168 were positively selected for IgG production, and 38 clones displayed recognition to 293 and 293-P-2-GFP cells alike while only 3 clones showed reactivity only against 293-P-2-GFP cells. Using flow cytometry again the clones were selected based on specificity to P-2 by removing the clones that displayed positive staining against 293-GFP cells, leaving only 1 clone. Unfortunately, this hybridoma showed equal binding in macrophages despite efficient siRNA-mediated knockdown of P-2 (described later). The difficulty in generating P-2 specific antibodies most probably lies in its high degree of conservation between species and our lack of purified protein as source of immunogen.

Use of P-2 fluorescent reporter construct for localization

The expression pattern of the P-2-GFP fusion protein in 293 cells was punctate by fluorescence microscopy (Figure 2.1a). We generated a RAW-P-2 GFP stable cell line so we could study the localization of P-2 in macrophages. The cells exhibited several different staining patterns under the fluorescent microscope; in some cells the GFP signal was dim and diffuse throughout the cell cytoplasm and in other cells it displayed a bright punctate expression. These bright ‘organelles’ were often localized around the nuclear membrane but also distributed throughout the cytoplasm. To determine the localization of these fluorescent bodies, RAW-P-2-GFP cells were stimulated with LPS and IFN-γ.
overnight and then labeled with ERTracker and LysoTracker reagents and imaged live using confocal microscopy and a heated stage. Given our hypothesis of P-2 as a cytotoxic molecule we supposed that it might reside in lysosomal vesicles where it could participate in bacterial destruction. However, we found that P-2-GFP expression does not colocalize with lysosomes but rather...
shares a localization pattern with the ER. This is especially evident in areas where P-2 displays diffuse cytoplasmic staining, while the bright bodies remain only green (not yellow). It has been reported that fluorescent proteins (i.e. GFP) can aggregate when expressed at high levels (Chudakov, et al. 2005; Shaner, et al. 2007) and the paucity of colocalization of the bright GFP positive regions with the ER or lysosomes suggested that this might be an aggregation of the protein and therefore an artifact of overexpression. In order to prevent aberrant localization of P-2 due to aggregation of the c-terminal GFP molecules, we opted for transient transfection of an alternative P-2 fusion protein with dsRed (also called monomeric RFP) into RAW cells and labeled cellular organelles to determine localization of P-2. RAW-P-2-RFP cells were stimulated with LPS and IFN-γ following transfection to ensure the cells displayed a characteristic macrophage phenotype and the cells were imaged within 24 hours of transfection to avoid deviant effects of over-expression. P-2-RFP displayed colocalization with the ER as seen previously and P-2-RFP fluorescence intensity was relatively even throughout the cell cytoplasm and displayed obvious structure throughout the cell as opposed to diffuse staining exhibited by RFP alone. Using an antibody to GM130, a Golgi matrix protein, we found colocalization with P-2-RFP although P-2-RFP staining extended beyond the golgi complex. Early endosomal antigen 1 (EEA-1), a marker of early endosomes, expression also colocalized with P-2-RFP, although not exclusively as some EEA1+ signals were not RFP+ and vice versa. As is evident from the brightfield image, P-2-RFP was excluded from the plasma membrane and nucleus. This was confirmed using an
Figure 2.12. Subcellular localization of P-2-RFP in RAW cells. Confocal images of P-2 RFP transiently transfected RAW cells co-labeled with various organelle markers as indicated in left column. Images were taken using a 40X oil objective lens and 6X zoom.
antibody against CD11b, an integrin family member expressed on monocytes and macrophages (as well as NK, neutrophils and granulocytes), to identify the plasma membrane in RAW cells and Hoechst dye to counterstain the nucleus. To identify lysosomes we used LysoTracker (not shown) and antibodies against lysosomal associated membrane protein 1 (LAMP1). P-2-RFP was excluded from lysosomal vesicles as evidenced by the absence of yellow fluorescence.

**Establishing an in vitro bactericidal assay**

The gentamicin protection assay has been used widely to measure intracellular bacterial killing by macrophages in vitro (Laroux, et al. 2005). In this assay, cells are cultured in medium free of antibiotics and allowed to co-incubate
with bacteria for a defined amount of time. The cells are then washed to remove extracellular bacteria and are recultured in the presence of gentamicin, an aminoglycoside antibiotic that is unable to penetrate the cell membrane. The antibiotic kills extracellular bacteria that remain following the washing procedure, thereby limiting additional infection events and concurrent bacterial replication in the supernatant that could interfere with the interpretation of colony forming units (CFU) over the course of the assay, especially for fast replicating bacteria such as *E. coli* which can divide every 20-30 minutes. To obtain CFU, the cells are lysed in water containing a low concentration of detergent and the lysate is diluted and plated on agar plates. Colonies are counted by eye or machine and multiplied by the dilution factor to determine live bacteria in the sample. A timecourse is used to determine whether the cells are able to kill intracellular bacteria or they replicate over time.

Small interfering RNA (siRNA) is a potent method for gene silencing in mammalian cells (Elbashir, et al. 2001). We used siRNA technology to knockdown P-2 in macrophages and then assess intracellular bacterial killing using the gentamicin protection assay. Three P-2-specific siRNA sequences were obtained from Ambion and used to generate siRNA duplex constructs for use in transient transfection experiments. Two of the constructs were specific for P-2 sequences in the 3’UTR and one construct was specific for a sequence in the P-2 coding region. Macrophages are notoriously difficult cells to transfect, especially by chemical methods, therefore, we used an electroporation method using the LONZA (previously AMAXA) system and reagents which has been
successful in transfecting macrophages with DNA and RNA (Chua and Deretic 2004; Liu, et al. 2007).

J774 macrophages were attractive initially to investigate P-2 function because they exhibited a strong P-2 band by western blot without the need for exogenous stimulation. Using the pool of siRNA constructs and a scramble siRNA control construct, P-2 was efficiently knocked down at 24 hours post-

transfection in J774 cells as determined by western blot and RT-PCR (Fig. 2.13). P-2 protein levels begin to increase again after 48 and 72 hours post-transfection so later experiments comparing P-2-silenced cells and controls were performed no later than the day following transfection. P-2 siRNA, scramble siRNA and untransfected macrophages were infected with the gram-negative organisms; *Salmonella enterica serovar typhimurium* (*S. typhimurium*), Enterotoxigenic *E. coli* (ETEC), or *Shigella flexneri* (*S. flexneri*). These bacteria are all intestinal pathogens, and *S. typhimurium* and *S. flexneri* are invasive organisms, meaning

![Figure 2.13. P-2 mRNA and protein silencing following P-2 specific siRNA transfection. a. Mpeg1 expression by RT-PCR. b. Western blot analysis of P-2 protein expression using Abcam anti-P-2 antibody.](image-url)
they do not depend on the cell autonomous mechanisms for entry, while ETEC is a non-invasive organism. Of the facultative intracellular pathogens tested, S. typhimurium depends on survival within macrophages specifically for its virulence, while S. flexneri is able to escape from the vacuole and induce apoptosis of macrophages (Sansonetti 2002). CFU were counted at 2 and 24 hours post-infection to determine whether P-2 would inhibit intracellular bacterial growth over time (Fig. 2.14). S. flexneri growth was not observed in macrophages within a 24 hour time period indicated by a ratio less than 1. This was expected due to its known effects on macrophage viability and also the attenuated virulence of the strain used (i.e. icsA deletion mutant). S. typhimurium and ETEC survived and demonstrated replication during the assay period. P-2 silencing had no measurable effect on S. flexneri intracellular growth, while in the infections with S. typhimurium and ETEC, a specific inhibitory effect of P-2 on bacterial survival was observed, however the results were not significant.
RAW macrophages require stimulation (greater than 12 hours) with LPS and IFN-γ to induce P2 expression (Fig. 2.7). Using S. typhimurium, we tested the killing capacity of RAW macrophages with and without pre-stimulation with LPS and IFN-γ in the gentamicin protection assay. Similar to other reports (Gulig, et al. 1997; Rosenberger and Finlay 2002), we found that RAW cells are bactericidal only after stimulation with LPS and IFN-γ (Fig. 2.15). At 48 hours post-infection there was still surviving S. typhimurium inside cells that had been pre-stimulated, however the untreated cultures succumbed to overwhelming infection such that CFU could not be determined at 48 and 72 hours post-infection. The upregulation of P-2 upon stimulation with LPS and IFN-γ, therefore correlated with killing activity in RAW cells. To determine whether P-2 was responsible for limiting S. typhimurium replication in RAW cells, P-2-specific siRNA or scramble siRNA were transiently transfected and gentamicin protection assays were performed. Lysates from P-2-knockdown cells yielded greater

![Figure 2.15. Effect of LPS and IFN-γ stimulation on intracellular S. typhimurium in RAW cells. Representative data showing the recovered CFU at the indicated timepoints. Skull symbol denotes too many colonies to count and death of the cells.](image)

CPU (x10^6)

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numbers of intracellular bacteria than lysates from control cells at 24, 48, and 72 hours post-infection (Figure 2.16). Cell viability was measured prior to infection with *S. typhimurium* by flow cytometry using the fluorescent DNA intercalating reagent, 7-aminomycin-D (7aaD). P-2-knockdown and control cells had comparable viability following transfection and overnight stimulation with LPS and IFN-γ (Fig. 2.17a). Cell viability was measured throughout the infection assay as
well to determine if macrophage death could be contributing to lower CFU from control cells. Scramble siRNA-treated cells consistently displayed lower viability compared to the P-2 knockdown macrophages at 24, 48, and 72 hr post-infection (Fig. 2.17b). Intracellular and extracellular bacteria can induce cell death in macrophages by a variety of mechanisms (Navarre and Zychlinsky, 2000), but we did not anticipate a link between P-2 and cell death. Given that RAW cells are immortalized tumor cells, which often have defects in cell death pathways, we investigated the effects of silencing P-2 in primary murine macrophages.

**Figure 2.17.** RAW cell viability following siRNA treatment and infection with *S. typhimurium*. a. Flow cytometric analysis of necrotic cells (7-AAD+) following P-2 knockdown in RAW cells. b. Cell viability by trypan blue exclusion in RAW cells at indicated timepoints after infection with *S. typhimurium*. Data from same experiments in Figure 2.16.

PEM were chosen over BMM for studies with primary macrophages due to a faster acquisition of usable cells (4 days vs. 7 days) and the lack of a requirement for expensive cytokine stimulation. PEM are superior to RAW cells in clearing intracellular *S. typhimurium* (Fig. 2.18a) using the gentamicin protection assay. *S. typhimurium* replicates and survives (approximately 5 fold
Figure 2.18. P-2 knockdown in PEM allows more intracellular bacterial growth.
a. Fold growth of S. typhimurium in untransfected, stimulated RAW and PEM.
b. Mpeg1 expression by RT-PCR following siRNA treatment in PEM.
c. Western blot analysis of P-2 expression following siRNA treatment in PEM.
d. CFU assay in siRNA-treat PEM following infection with S.typhimurium (MOI 100).
e. CFU assay in siRNA-treated PEM following infection with E. coli (MOI 100).
growth) in stimulated, untransfected RAW cells and on the other hand, is efficiently eliminated by PEM (approx. 90% killing) in 24 hours. In order to define the role of P-2 in PEM killing of *S. typhimurium*, peritoneal cells were transfected, prior to purification by adherence step, with P-2-specific or scramble siRNA and gentamicin protection assays were performed. P-2-specific gene silencing was equally efficient in PEM as in RAW cells (Figure 2.18b, c). The loss of P-2 in PEM led to significantly more growth of *Salmonella* at 4 hours post-infection. Viability over the course of the assay was comparable in knockdown and control cells, unlike what was observed in RAW cells supporting the notion that this may have been an artifact from using a tumor cell line. Non-pathogenic *E.coli* K12 was also protected from macrophage killing in the absence of P-2 (Figure 2.18e), suggesting that P-2 participates in clearance of different bacterial species.

**Genetic Complementation**

To confirm that P-2 is responsible for the bactericidal activity we observed in macrophages, I used the previously described P-2 fusion protein to complement cells in which P-2 was silenced and determined whether it was sufficient to rescue the killing defect. To perform this assay, only the two P-2-specific siRNA constructs that targeted the 3'UTR were used. P-2 silencing activity was analyzed by western blot (Figure 2.19). Although the gene silencing was inferior relative to the pooled constructs, the two siRNA constructs targeting the 3'UTR were sufficient to inhibit *Salmonella* killing in RAW cells compared to scramble control cells (Figure 2.21).
Co-transfection of siRNA and DNA expressing the P-2 fusion protein led to reconstitution of *S. typhimurium* growth inhibition in RAW cells compared to control cells complemented with the RFP vector alone (Figure 2.20b). Western
blot analysis of cells used for the CFU assay indicated that protein levels in cells complemented with the P-2 fusion protein greatly exceeded the levels of endogenous P-2 in scramble siRNA treated control cells, however this did not translate proportionally into more bacterial killing. One explanation could be that only 20-30% of cells are expressing P-2-RFP as determined by flow cytometry at the time of infection. Therefore, the dense band of P-2 RFP indicates that transfected RAW cells are expressing more P-2 on a per cell basis than is normally present. Despite having an excess of P-2, which appears to be functional, the transfected cells are competing in the assay with untransfected cells which are defective in bacterial killing due to siRNA knockdown of P-2. The CFU data from macrophage cell lines and primary macrophages suggest that P-2 is limiting; higher levels of P-2 expression correlate with more bacterial killing.

**P-2 mediated bacterial killing compared to other bactericidal pathways**

The bactericidal capacity of macrophages is attributed to the high production of oxidative metabolites such as reactive oxygen and nitrogen species and fusion of the bacteria-containing phagosome with lysosomal enzymes. We sought to determine how the bacterial killing ascribed to P-2 compared to killing by ROS and NO. Chemical inhibitors of each of the pathways were utilized to inactivate ROS and NO separately or in combination with P-2 inhibition. The ROS scavenger, N-acetyl cysteine (NAC) was used to inhibit ROS-mediated killing and N^G^-nitro L-arginine methyl ester (NAME), which inhibits inducible nitric oxide synthase (iNOS) production of NO, was used to inhibit NO-mediated killing.
ROS and NO are both reported to be important in protection against *S. typhimurium* infections in vitro and in vivo (Mastroeni, et al. 2000; Vazquez-Torres, et al. 2000). PEM produce NO in response to LPS and IFN-γ stimulation and its production is measured by detecting the byproduct, nitrite (NO$_2^-$) in the supernatant using the standard Griess assay (Zhang, et al. 2008). NO$_2^-$ is detectable in the supernatant beginning after 4-6 hours of stimulation and increases steadily over time. As expected, PEM produced appropriate amounts of NO$_2^-$ following 24 hours of stimulation and L-NAME inhibited this production in a dose-dependent manner (Figure 2.21). NAC did not inhibit NO production by PEM. siRNA treatment also did not affect PEM ability to produce NO.

To measure ROS in PEM, the cells were loaded with the dye, CM-H$_2$DCFDA. 2’7’-dichlorofluorescein (DCF) is a non-fluorescent molecule until it is oxidized by ROS inside the cell and then it becomes fluorescent (green). The chloromethyl (CM) group allows for better retention within the cell following oxidation, thus ROS production in loaded cells would lead to an accumulation of fluorescent DCF. Unstimulated and untransfected PEM were fluorescent as
visible under the microscope; however LPS or PMA with IFN-γ stimulation did not increase the ROS signal as measured on the flow cytometer (Figure 2.22). Pre-treatment with NAC reduced the fluorescence indicating that NAC does act as a scavenger of ROS. The iNOS inhibitor, NAME, had no effect on the ROS signal in the cells. Exogenous H₂O₂ is used as a positive control for ROS detection and pre-treatment with NAC exhibited a reduction in intracellular ROS following H₂O₂ addition in a dose-dependent manner (not shown). siRNA silencing of P-2 did not affect these baseline ROS levels in PEM (Figure 2.22).

The inhibitors were titrated in the gentamicin protection assay with untransfected PEM to determine the lowest concentration at which an effect on intracellular bacterial growth could be achieved (Figure 2.23). PEM treated with NAC at concentrations above 4 mM displayed a drastic defect in killing intracellular bacteria indicating that Salmonella are sensitive to ROS-mediated killing. NAME treatment was less drastic at the tested concentrations, although there was a delay in killing in PEM treated with 1 mM NAME. Based on these preliminary results we decided use a concentration of 10 mM for each of the
inhibitors in CFU assays with siRNA-treated PEM. We also determined that the chemical inhibitors alone were not toxic to *Salmonella* nor did they affect their ability to replicate (Figure 2.24).

![Figure 2.23. Effect of ROS and NO inhibition on intracellular *S. typhimurium* growth in PEM. Cells were treated with IFN-γ and infected at MOI 100. Inhibitors were added 30min before infection and maintained in the media throughout the assay. CFU were recovered from cells at 0, 6, and 24 hours after the infection period.]

Similar to what was observed in the titration experiment, pre-treatment of scramble siRNA treated cells with the inhibitors, NAC or NAME showed defects in bacterial killing relative to untreated cells indicating that *Salmonella* are vulnerable to ROS and NO-mediated toxicity (Figure 2.25). Interestingly, in PEM in which P-2 was silenced, the inhibitors had no additional effect on bacterial killing compared to P-2 knockdown in the absence of inhibitors. These data suggest that ROS and NO killing pathways, while effective against *Salmonella*, are likely acting downstream of P-2 killing. Further, P-2 may act in concert with
ROS and NO killing pathways enhancing their activity via pore-mediated access
to the bacterium proteoglycan layer and inner membrane.

Co-localization studies of P-2 with bacteria

Taken altogether, the data so far suggest that P-2 plays a role in defense
against bacteria, however we sought to confirm whether P-2 is mediating direct
effects on bacterial cells through pore formation as we hypothesized or not. In
order to better understand the interaction of P-2 and bacteria on a per cell basis,
co-localization studies were performed using fluorescence microscopy. To
visualize P-2, P-2-RFP was transiently transfected into RAW macrophages. In
order to visualize bacteria, we used a phage construct obtained from Dr. Bumann (University of Basel) that expresses GFP under the sifB promoter. SifB is a Salmonella pathogenicity island 2 (SPI-2) effector with unknown function. SPI-2 encodes a type III secretion system and many effectors which are essential for intracellular survival and pathogenicity of S. enterica (Cirillo, et al. 1998; Hensel, et al. 1998). SPI-2 genes are activated upon bacterial sensing of low pH and lack of nutrients within the host cell, so fluorescent bacteria expressing the plasmid should theoretically be intracellular. This system is advantageous for co-

![Figure. 2.26. Fluorescent imaging of P-2-RFP and Salmonella. Live P-2-RFP transiently transfected RAW cells 24 hours post infection with psifB::GFP S. typhimurium. Rows a, b, and c represent 3 different fields of view. Images were taken on Zeiss apotome using 63X objective lens.](image_url)
localization studies since it can be difficult to discriminate between intracellular and extracellularly-bound bacteria under the microscope. Wild-type *Salmonella* were transformed using the *psifB::GFP* construct and selected for on the basis of kanamycin resistance. Initially, RAW-P-2-RFP cells were analyzed at 24 hours post-infection since this is the timepoint where we begin to see an effect of P-2 on intracellular bacterial counts. Cells were infected with *psifB::GFP Salmonella* at an MOI of 100 and 24 hours later the cells were imaged live using the Zeiss Apotome fluorescent microscope with a heated stage. GFP+ bacteria were readily visible within the cells at 24 hours post-infection, as were P-2-RFP expressing cells. By flow cytometric analysis, P-2-RFP expression was quantified at approximately 40% of cells at this timepoint (48 hours post-transfection). Figure 2.26 shows co-localization signified by yellow staining in cells that both express P-2-RFP and are infected with *Salmonella*. To analyze the data on a per cell basis, GFP+ bacteria were quantified in P-2-RFP expressing cells that had been co-transfected with siRNA (3'UTR-targeted) 24 hours after infection compared to RFP expressing cells. Of approximately 100 RFP+ cells counted in each condition, 36% RFP+ cells were infected with at least 1 bacteria compared to 13% of P-2-RFP+ cells, corroborating the complementation CFU data. To confirm co-localization we performed the same assay and analyzed the samples by confocal microscopy instead of regular fluorescent microscopy to rule out the possibility that the P-2 signal and bacteria signal artificially overlapped in the z-axis without actual colocalization. Using confocal microscopy, I was able to detect yellow fluorescence indicating co-
localization of the green and red fluorescence of the bacteria and P-2 molecules, respectively at 24 hours post-infection (Figure 2.27). However, these results were disappointing because although we do detect some co-localization there is not observable translocation of P-2 as we expected. The staining pattern looks similar in the presence and absence of bacteria. I performed live cell, timelapse experiments with P-2-RFP expressing RAW cells and psifB::GFP-expressing Salmonella, some beginning at the time of infection, however I have not found a situation where P-2-RFP and Salmonella GFP signals co-localize followed by a disappearance of GFP (gradual or immediate) indicating death or lysis of the bacterial cell. The fluorescent microscopy studies therefore only show that P-2 molecules are present in the same location as bacteria and maintain the potential to interact. Further analysis of fixed cells at 4 hours post infection showed

Figure 2.27. Confocal analysis of P-2-RFP and Salmonella co-localization. Live P-2-RFP transiently transfected RAW cells 24 hours post infection with psifB::GFP S. typhimurium. Images were taken using a Zeiss LSM510 confocal microscope and 40X objective lens.
examples of bacteria within a P-2-RFP decorated vacuole inside the cell, however there was no co-localization visible and P-2-RFP displayed no specificity to the bacteria containing vacuole (not shown). Interestingly, co-staining the cells with anti-LAMP1 4 hours after infection revealed co-localization of P-2 with LAMP1 which was not present in activated but uninfected RAW cells (compare figures 2.12 and 2.28).

Figure 2.28. Confocal analysis of P-2-RFP subcellular localization during infection. Fixed P-2-RFP transiently transfected RAW cells imaged 4 hours post infection with psifB::GFP expressing S. typhimurium. Top left corner is bacteria (white), top right corner is P-2 (red), bottom left corner is LAMP1 (green) and bottom right corner is a merge of all 3 images with brightfield.

**P-2 cytoplasmic domain mutagenesis**

Upon determining a function of P-2, the next objective was to elucidate its mode of action. To address the question of whether the cytoplasmic domain plays a role in signaling and regulation of P-2, mutants were generated containing single amino acid or multiple amino acid modifications in the cytoplasmic domain. The mutations were designed based on the conserved regions as described previously (Figure 1.3b). The four constructs generated are
as follows: 1) KYKK→QFQQ (aa678-81), 2) Y→F (aa684), 3) S→A (aa692), and 4) S→D (aa692). The tyrosine to phenylalanine mutation will render the site incapable of phosphorylation but will maintain the bulky structure of the amino acid so as to not disrupt folding of this region. Similarly, the serine to alanine mutation maintains the size of the residue but removes the -OH group to prevent phosphorylation. Alternatively, the serine to aspartic acid mutation will mimic a constitutively phosphorylated version of the residue (Kaufman, et al. 1989). The purpose of the four amino acid mutations from KYKK to QFQQ was to disrupt the cleavage site sequence without disturbing the charge and steric characteristics of this motif. The constructs were generated using site-directed mutagenesis of the P-2-RFP vector that was used in all previous experiments. Primers were designed based on the schematic shown in figure 2.30. Originally, attempts were made to mutate the tyrosine using the primer design method that was successful for the serine mutations, however the primers were promiscuous and the resulting sequence was incorrect. Primers containing the mutation in the middle of both primers as opposed to the beginning of the forward primer produced the correct sequence for the Y→F mutant.
To address whether the constructs could be expressed properly by mammalian cells, they were transfected into 293 cells and analyzed for RFP expression. As mentioned previously, expression levels of transfected P-2-RFP is always lower than the RFP control, presumably because P-2 is a toxic molecule. All of the mutant constructs were expressed at very high levels 24 hours post-transfection displaying 50-80% of cells expressing RFP. RFP vector alone and non-mutated P-2-RFP were also transfected and analyzed to serve as controls. As expected, RFP was expressed at higher levels (both percentage cells positive and mean fluorescence intensity (MFI)) than P-2-RFP. Transfected cells were also imaged by confocal microscopy to determine the pattern of cellular localization and it was found to be analogous to P-2-RFP for all the mutations (not shown). Stable cell lines were established following 2 weeks of selection and the cells were analyzed again for RFP expression. Both of the serine mutant constructs were expressed at similar levels relative to non-mutated P-2 (approximately 30% RFP positive cells), while the lysine mutant showed expression levels resembling RFP alone, with almost 100% of the cells expressing the protein. The tyrosine mutation showed an intermediate level of expression (more than P-2-RFP and less than RFP) with approximately 60% of the cells displaying RFP positivity. The low expression of the serine mutations suggests that, in this context, the toxic function of P-2 is intact in these mutations. The uninhibited expression of the tyrosine and lysine mutants, on the other hand, suggests that the toxic effects of P-2 have been ameliorated by the alterations in the cytoplasmic domain sequence.
In order to characterize the functional defects of our mutants we analyzed them in the gentamicin protection assay with macrophages following transfection with P-2 specific 3'UTR-targeting siRNA and mutated P-2 plasmid DNA or in macrophages from mice containing a genetic deletion of P-2. For these complementation assays, I used *Mycobacterium smegmatis* (*M. smegmatis*), a
non-pathogenic, non-invasive acid fast bacteria instead of *S. typhimurium* to infect macrophages. The effects of P-2 are often more dramatic in vitro against this bacteria compared to *S. typhimurium* which are pathogenic, invasive bacteria and are more likely to be equipped with mechanisms to avoid attack by P-2.

![Graphs showing RFP expression](image1.png)

**Figure 2.31.** Complementation of P-2 deficient macrophages with cytoplasmic P-2 mutant (YF) results in intracellular bacterial growth. a. Histogram plots showing RFP expression 24 hours after transfection as an indicator of transfection efficiency (green solid lines). Black dotted line represents untransfected PEM control. Values represent MFI and percent positive in the texas red (RFP) channel. b. Fold growth of intracellular *M. smegmatis* in PEM at 5 hours post-infection relative to 1 hour post-infection. Data represent summary of 3 independent experiments.

The transfection efficiencies in macrophages were lower than in the 293 cells, however mutated P-2 was expressed to similar levels as P-2 RFP at 24 hours post-transfection. Transfection of the Y→F mutant resulted in intracellular bacterial growth over a 4 hour culture period which reflected the results in P-2-deficient cells, whilst cells transfected with a functional P-2 inhibited intracellular
bacterial growth in the same amount of time (Figure 2.31). The serine and lysine mutants have not yet been tested adequately in the gentamicin protection assay so it remains to be seen how they will affect P-2 dependent killing in macrophages.

The Y-F mutant resulted in a P-2 that was non-functional, suggesting as hypothesized that phosphorylation at this residue is required for P-2 polymerization and pore formation.

**Discussion**

Perforin-2 is distinct from the other immune pore-formers (C9 and Perforin) in that it is membrane-bound as opposed to secreted. The presence of a putative transmembrane domain in the protein sequence, its expression pattern in RAW macrophages, and the fractionation data of P-2-GFP in 293 cells all support this notion. Conceptually, a killer molecule that is tethered to the membrane would be very valuable in protecting the host cell from intracellular bacterial replication following invasion. However, the membrane-bound nature of P-2 makes it challenging to perform in vitro assays, mainly because purification of the full length protein would be arduous requiring extraction from the membrane and solubilization. According to Protein Chromatography literature, membrane proteins account for 20-30% of expressed proteins, yet comprise only 2% of listed structures in the protein data bank due to methodological difficulties with purifying membrane proteins in great quantities (Smith 2010). Further, even upon purification of large quantities of P-2, without knowing the signaling
pathways required for P-2 activation and polymerization, thoughtful and fruitful experiments would be difficult to design. We did not attempt to produce a recombinant P-2 made up of just the luminal portion (or the MACPF) for in vitro assays either, although this is a widely used method to purify large amounts of protein. Indeed, He, et al (2011) analysed the bactericidal function of recombinant P-2 in vitro and found that presence of the MACPF domain inhibited growth of gram-negative and gram-positive bacteria. However, the question remained: what is the physiologic relevance of P-2 in macrophage immunity?

Macrophage cell lines were readily available and demonstrated very efficient knockdown of P-2 with siRNA (Figures 2.13, 2.16, 2.18, 2.10). However, the tumor lines displayed moderate killing compared to primary macrophages, and although J774 cells were more bactericidal than the RAW cell line, their dependence on P-2 was not as easily demonstrated. The J774 bactericidal functional studies are by no means conclusive and the assay was more or less abandoned when a reproducible observation was established using the RAW cells. RAW cells were also easily transfectable with plasmid DNA expressing the P-2 fusion protein with efficiencies of greater than 40% unlike J774 and PEM which do not exceed 20%. Therefore, the RAW cells were better suited for imaging studies because the incidence of P-2 expressing cells was relatively high, but their sluggish bactericidal activity against Salmonella especially at early timepoints made the colocalization studies with bacteria more difficult to interpret.

Another difficulty interpreting the co-localization studies revolved around the bacterial GFP expression system. Salmonella induce SPI-2 expression upon
sensing the intracellular environment and produce effectors which allow them to avoid immune detection and establish residency inside the cell (Cirillo, et al. 1998). We hypothesize that Salmonella (and other intracellular bacteria) necessitate mechanisms to block P-2 expression and/or function in order to survive and replicate in macrophages. The GFP-expressing Salmonella in our co-localization studies had survived the RAW-mediated bactericidal pathways and initiated SPI-2 induction, therefore they might represent bacteria that had already successfully blocked P-2. This would explain the failure of timelapse experiments to demonstrate a loss of GFP expression indicating lysis following an interaction of P-2 and bacteria. In accordance with this explanation, recent experiments with E.coli-GFP (expressed constitutively) and P-2-RFP expressing BV2 macrophages have shown rapid translocation (5 minutes) of P-2 to the intracellular bacteria (R. McCormack, unpublished data).

The localization of P-2 within the ER is consistent with the presence of P-2 (Mpeg1 encoded protein) in latex bead containing phagosomes from J774 macrophages (Garin, et al. 2001). In this study, a proteomic screen was performed of phagosome-associated proteins and the proteins were differentiated by presence within the lumen of the phagosome and outside (cytoplasmic). As expected, the bulk of P-2 protein was found inside the lumen along with LAMPs 1 and 2 and several cathepsin molecules which are normally associated with lysosomes. Also identified in the phagosomal lumen was calreticulin, an ER-specific chaperone that is also present in secretory granules of CTL, and other ER-residing proteins. It is controversial, however ER
membranes are reported to contribute to the nascent formation of phagosomal membranes (Gagnon, et al. 2002). P-2-RFP was excluded from lysosomes in RAW cells that were activated but not in those infected with bacteria. In the presence of Salmonella, co-localization was observed with P-2-RFP and LAMP1 suggesting that in the context of phagocytosis and bacterial infection P-2 may associate with lysosomes. We hypothesize that P-2 acts early in the process of phagolysosomal maturation (i.e. before phagolysosomal fusion) so that the effects of damaging oxidative and non-oxidative molecules are maximized following membrane disruption mediated by poly- P-2. However, in order to identify this co-localization as a physiologically relevant observation it would be necessary to show the temporal acquisition of P-2 staining in lysosomes in the context of bacterial killing.

The current dogma in immune defense against bacteria proposes that phagocytic cells ingest and destroy bacteria through the production of various non-specific anti-microbial molecules, including oxidative metabolites such as reactive oxygen and nitrogen species and non-oxidative molecules such as hydrolytic enzymes present in lysosomes (Chakravortty and Hensel 2003; Ding, et al. 1988). Evidence supporting the significance of these molecules in immunity is derived from studies with animals deficient in the pathways that produce these molecules. ROS are produced by all cells as a byproduct of mitochondrial respiration; however a secondary mechanism for ROS production is achieved through a complex of 5 proteins called the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. There are 6 members in the NOX
family of NADPH oxidases and they are distinguished by activation requirements and tissue tropism. NOX2/gp91(phox) is the subunit that is expressed in phagocytes, and is responsible for the ‘respiratory burst’ (Bedard and Krause 2007). Defects in any of the subunits of the NADPH oxidase leads to pathology in humans and mice which is categorized under the disease chronic granulomatous disease (CGD). CGD patients exhibit recurrent infections most commonly to the bacterial species Staphylococci, Salmonella, Klebsiella, Aerobacter, Serratia and the fungal species Aspergillus (Segal 1997). Knockout of gp91phox or phox47 resulted in a diminished ability of mice to control S. aureus infections in vivo (Jackson, et al. 1995; Pollock, et al. 1995) as well as increased susceptibility to spontaneous visceral infections (with p47phox knockout). Interestingly, treatment of CGD patients and mice with IFN-γ ameliorates disease by reducing the incidence of infection (Jackson, et al. 2001; Muhlebach, et al. 1992).

Reactive nitrogen species (i.e. NO which generates peroxynitr ate upon reaction with superoxide) are produced by multiple cell types as well in response to inflammatory stimuli by one of three isoforms of the nitric oxide synthase (NOS). In phagocytes, the enzyme responsible is NOS2 (or inducible NOS (iNOS)). Defects in this gene lead to susceptibility to a different group of pathogens than NADPH oxidase deficiency such as, Mycobacterium tuberculosis, Leishmania, and Listeria (review in (Nathan 1997).

Mice which lack both NOX2 and NOS2 exhibited spontaneous infection to commensal bacteria even under pathogen-free conditions and antibiotic
administration and the incidence of infection increased with age demonstrating how essential they are in establishing tolerance of commensal organisms (Shiloh, et al. 1999). However, macrophages isolated from these mice displayed demonstrable killing of 5 out of 6 of the organisms tested, indicating that other bactericidal mechanisms are present and required for macrophages-mediated immunity.

The impact of P-2 function in immunity will also be demonstrated with the use of genetically deficient animals, which have been recently generated in our laboratory with the help of the Transgenic core. Initial studies show that P-2 deficient mice succumb to lethal infection of *S. typhimurium* using an inoculum of 100 CFU while heterozygote and wild-type littermates are capable of clearing the infection (R. McCormack, unpublished data), recapitulating the results generated in vitro with siRNA knockdown of P-2 in macrophage cell lines and primary cells.

The pore-forming attribute of P-2 is especially beneficial given the presence of antibacterial compounds stored in macrophages that may achieve greater penetration and substrate recognition with better access through the bacterium’s thick cell wall. Macrophages and phagocytic cells are clearly important for sensing and killing bacteria, however during an infection bacteria come into contact with many other cell types and many bacteria have evolved mechanisms to avoid phagocytes and phagocytosis altogether (Celli and Finlay 2002). In the initial paper describing *Mpeg1* (Spilsbury, et al. 1995) they analysed various tissues and cell types before concluding that *Mpeg1* was macrophage-specific, however, Jahir Ramos in our lab discovered that NIH/3T3
murine fibroblasts (3T3) upregulate Mpeg1 gene expression upon stimulation with type I interferons (IFNα and IFNβ) and the combination of type I and II interferons was very potent in P-2 upregulation (Figure 2.32). Resting 3T3 express undetectable levels of P-2 as measured by RT-PCR. It has been reported that 3T3 can kill S. typhimurium in vitro (Martinez-Moya, et al. 1998) and autophagy has been described as a defense mechanism against bacterial infection in non-phagocytic as well as phagocytic cells (for review, (Randow and Munz 2012). We found that fibroblasts indeed were able to clear infection by S. typhimurium and the clearance was expedited if the cells were pre-stimulated with interferons (McCormack, et al. 2012). Further, bacterial killing in murine embryonic fibroblasts (MEF) was dependent on P-2 using siRNA knockdown and the gentamicin protection assay. Many other non-phagocytic cell types have now been tested for induction of P-2 with interferons and it seems that all cells can express P-2 and further, using siRNA knockdown of P-2, all cells depend on P-2 for control of bacterial growth (R. McCormack, unpublished data).
Given P-2s ubiquitous expression, we propose that in all cell-types, P-2 is trafficked to the phagosome soon after its formation (or to the autophagosome in the case of non-phagocytic cells) and activated via signals mediated through its cytoplasmic tail. Kirill Lyapichev in the Podack lab carried out a yeast two-hybrid screen using the cytoplasmic domain of human P-2 as bait and a cDNA library from LPS and IFN-γ activated RAW cells as prey. His studies identified 6 potential binding proteins, GAPDH, Galectin-3, Ubc12, RASA-2, Ubiquitin C, and 20S proteasome subunit PSMA6. The next phase of the P-2 project is to determine the mechanism of P-2 translocation, activation and polymerization starting with confirming the interaction between P-2 and the aforementioned proteins. The cytoplasmic tail mutants will also be valuable tools in determining the mechanism since they address the importance of highly conserved regions in the P-2 sequence. So far the data with cytoplasmic tail mutants indicate that tyrosine phosphorylation is important for the function of P-2. The next questions to answer will be to determine the nature of the functional defect, whether it lies in an inability to translocate to the bacteria-containing phagosome or an inability to polymerize. Determination of the kinase responsible for phosphorylation of the conserved tyrosine will also shed light on P-2s mechanism. Indeed, a screen of the cytoplasmic domain sequence against a database of known serine, threonine, and tyrosine kinases identified Src as a potential candidate for phosphorylation of this site and will be a target for future investigation.

In conclusion, we have demonstrated that P-2 is constitutively expressed in macrophages, a vital component of the innate immune system, and inducibly
expressed in all cells, making it the third pore former of the immune system overall. The lytic effector functions of the other immune MACPF proteins, perforin and complement rid our bodies of viral infections and extracellular bacteria, respectively. P-2 represents a novel mechanism of host defense against intracellular bacteria (summary Figure 2.33).

Figure 2.33. The pore formers of the immune system. C9, Perforin, and Perforin-2 are potent host defense mechanisms against invasive pathogens covering extracellular bacteria, virus and intracellular bacteria, respectively. The pore-forming proteins cooperate with other molecules to carry out their function, C9 and lysozyme, Perforin and granzymes, and Perforin-2 and ROS, NO, and lysozyme.
CHAPTER 3: EXPERIMENTAL PROCEDURES

Eukaryotic cell culture

Human embryonic kidney (HEK)-293 and the murine monocyte/macrophage cell lines RAW264.7 and J774A.1 were obtained from ATCC (Manassas, VA). All cells were cultured in complete medium (Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% FBS (Life Technologies)) at 37°C in a humidified atmosphere containing 5% CO2 following ATCC recommendations. Murine primary macrophages were obtained from the peritoneum or bone marrow. Thioglycollate-elicited peritoneal macrophages: 1.5 ml of a 4% thioglycollate solution was injected i.p. into C57BL/6 mice. Four days later, peritoneal cells were harvested by injection of 10 ml of complete medium containing 100ug/ml Gentamicin (Life Technologies) into the peritoneal cavity via the thigh muscle using a 22 gauge needle. Cells were extracted with a glass Pasteur pipette and washed twice in complete medium with gentamicin. Macrophages were purified by adherence for 2 hours on tissue culture dishes immediately of following transfection as described later. Bone-marrow derived macrophages: bone marrow was flushed from the long bones of C57BL/6 mice. Red blood cells were lysed with Ammonium-Chloride-Potassium (ACK) buffer and the cell pellet resuspended (10^6 cells/ml) in complete medium containing 20 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ, USA). On day 4, the non-adherent cells were harvested and replated in fresh complete medium. Fresh medium was added every 3 days until cells were ready for experiments (usually between day 7 and 10).
P-2-GFP isolation from 293 cells

Stably transfected 293-P2-GFP cells (approximately $200 \times 10^6$ cells) were harvested by trypsinization and washed twice with Hank’s buffered salt solution (HBSS). The pellet was resuspended in 10X volume of the packed pellet using hypoosmotic buffer (10mM HEPES pH7.2, 10mM KCl, 1.5mM MgCl$_2$, 2 mM EGTA, 100X protease inhibitors, 1mM Na$_3$VO$_4$) and incubated for 15 minutes on ice. 10X isotonic buffer (100mM HEPES, 1.4M KCl) was added and cells were homogenized using dounce homogenizer (7mL) and motorized chuck until approximately 90% of cells had burst which was determined by visualization under the microscope. The homogenate was layered over a sucrose gradient containing the following fractions of 2ml each in a 15ml glass centrifuge tube; 15%, 25%, 35%, 45%, and 61.56% sucrose (buffered with 10mM HEPES) and centrifuged at 15,000 x $g$ for 3 hours at 4°C. The homogenate was fractionated from the top down with 20-25 drops per fraction (approximately 500µl) using Auto-Densi Flow and Automated Drop Counter into 2mL collection tubes. GFP expression was measured using a fluorimeter (Diana Lopez Lab) and the filter set for GFP (485/535). The two fractions containing the highest amount of GFP were pooled and centrifuged at 150,000 rpm for 20min, 4°C. The pellet was resuspended in 100µl of Hanks buffered salt solution (HBSS) and kept at 4°C until ready to use.
Electron Microscopy

Membrane preparations from 293 P-2-GFP were resuspended in a small volume of neutral Tris-buffered saline, treated with 100 µg/ml trypsin for 1 hour at 37°C, washed and negatively stained with 5% neutral Na-phosphotungstate for 30 seconds. Images were taken at 52,000-fold initial magnification on a Phillips CM10 transmission electron microscope.

Hemolytic assay

This assay was adapted from the CH$_{50}$ assay for total classical pathway hemolytic activity (Current Protocols in Immunology). Sheep erythrocytes (Rockland Immunochemicals) were washed and resuspended to a final concentration of 2x10$^8$/ml in cold GVB++ buffer (gelatin veronal (barbitol) buffer containing Ca$^{2+}$ and Mg$^{2+}$). 100µl of erythrocyte solution was then added to a 12x75cm borosilicate glass tube in a pan of slushy ice. P-2-GFP enriched fractions were then added to the tubes in 4 serial dilutions starting with 50µl and diluting 2 fold. The final volume in each tube was completed to 200µl with GVB++. For controls, tubes were supplemented with 100µl of GVB++ buffer alone. The tubes were covered with parafilm and incubated for 1 hour in a 37°C waterbath, with intermittent shaking. After the incubation period, 1.3 ml GVB++ was added to each tube except in total lysis controls which received 1.3 ml of water instead. The tubes were centrifuged at 1,250 x g, 5 min, 4°C and the supernatant was immediately read on a spectrophotometer (OD$_{412}$). Percent hemolysis of fractionated lysates was determined by dividing the OD$_{412}$ of the test sample by OD$_{412}$ of the total lysis tube (x 100).
**Immunoblotting**

Cells were harvested for western blot analysis by washing twice in ice-cold PBS and then lysed using RIPA buffer (150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0) containing 1X protease inhibitor cocktail (Roche) at a ratio of 100ul of RIPA per 1x10^6 cells. The lysate was kept on ice for 30 minutes and then centrifuged at 14,000 rpm for 15 minutes at 4°C. The protein concentration in the supernatant was measured using a colorimetric DC protein assay (Biorad). 10-50µg of protein was mixed with 5X loading buffer (10% SDS, 50% glycerol, 0.01% bromophenol blue, 0.3125M Tris HCl, 25% 2-mercaptoethanol) and boiled for 5 minutes before loading onto 4-15% Tris HCl gradient Ready Gels (Biorad) and run in a mini format 1D electrophoresis system (Biorad). Precision Plus Protein Prestained Standard (Biorad) was used as a molecular weight standard. The gel was transferred onto nitrocellulose (Millipore) membrane overnight at 38mA. The membrane was blocked in 5% milk in Tween TBS buffer (w/v) for 2 hours at room temperature and probed with primary antibodies: polyclonal rabbit anti-mouse *Mpeg1* (Abcam), polyclonal rabbit anti-mouse P-2 cyto (21st Century), or monoclonal mouse anti-mouse Actin (Sigma) at 1:1000 dilution in 5% milk in TTBS, and secondary antibodies: goat anti-rabbit horseradish peroxidase and donkey anti-mouse horseradish peroxidase (Santa Cruz) at 1:5000 dilution in 5% milk in TTBS.

**Quantitative RT-PCR**

RNA was extracted from cells following RNeasy (Qiagen) instructions. 1µg of RNA was converted to cDNA using QuantiTect Reverse Transcription kit
(Qiagen) following supplier’s protocol. qRT-PCR was performed using Taqman® Gene Expression Assays (Applied Biosystems) for murine *Mpeg1* and GAPDH, with the latter serving as a housekeeping control gene. All assays were performed on the Applied Biosystems 7300 PCR platform.

**siRNA Constructs and RNA interference**

Three P2-specific, chemically synthesized 19-nucleotide siRNA duplexes were obtained from Sigma. The sense sequences were as follows:

- ccaccucacuuucaauca targeting the coding region of P2,
- gagauuuccaggaacuuu and caauacgcucuugcac targeting the 3'UTR of P2.

A scramble siRNA of the second P2-specific sequence was used as a control for all experiments. The sense sequence of the scramble siRNA was as follows:

- guuggaauuuaugauaccua. Transfection of siRNA into macrophages was carried out using Amaxa Nucleofector System (Lonza) according to manufacturer’s instructions. All transfections were carried out using 2-4x10⁶ cells and a final concentration of 1µM siRNA (P2-specific siRNAs were pooled). Immediately after transfection cells were plated in antibiotic-free IMDM containing 10% FBS.

**Gentamicin Protection Assay**

*Cultivation of Bacteria*

*S. typhimurium* LTZ-2 was obtained from Greg Plano, University of Miami. ETEC and *S. flexneri* 24572 were obtained from George Munson, University of Miami. *M. smegmatis* was obtained from ATCC. *S. typhimurium*, *ETEC* and *S. flexneri* were cultivated in Luria-Bertani broth (LB) and *M. smegmatis* in Middlebrook 7H9 broth at 37°C with shaking.
Infection of Macrophages

Macrophages were plated at approximately 5x10^5 cells per well of a 12 well plate and stimulated overnight with LPS (1ng/ml) and IFN-γ (100U/ml). The following day bacteria were added directly to wells at MOI of 10 or 100 (as indicated for specific experiments). Salmonella were grown for 16-18 hrs at 37°C with shaking in LB and then diluted 1:33 and grown for another 3-3.5 hrs at 37°C with shaking to induce invasive phenotype (protocol from Ken Fields via Olivia Steele-Mortimer). Viable Salmonella numbers for MOI calculation were based on CFU assays indicating approximately 10^9 bacteria per ml following growth conditions. Salmonella were allowed to invade cells for 20-30 min and then cells were washed twice in PBS and fresh media (IMDM supplemented with 10% FBS) containing 50µg/ml gentamicin (Invitrogen). The same protocol was followed for infection with E.coli, ETEC, and S. flexneri. M. smegmatis were grown for 16-18 hrs and then washed in PBS. The bacterial pellet was resuspended in complete medium and placed in a water bath sonicator for 15 minutes to obtain a single cell suspension. The OD$_{600}$ was calculated to obtain the concentration of viable M.smegmatis with and OD600 0.1=10^7/ml. M.smegmatis were co-incubated with cells for 1 hour prior to washing and addition of gentamicin. After 30 min in the presence of gentamicin, some cells were harvested for the “0” timepoint. After 2 hrs in culture the gentamicin concentration was reduced to 5µg/ml for the remainder of the experiment. Cells were harvested for CFU assays by washing once in PBS to remove antibiotics and then adding 0.5ml of 0.1% Triton-X 100 (Sigma) in H$_2$O. The cell lysate was diluted 1:200, 1:2,000, and 1:20,000 in
duplicate or triplicate and plated on LB agar for overnight growth at 37°C. Colonies were counted by eye, plates with colony numbers <30 and >300 were determined uncountable.

**Immunofluorescence**

For localization of P-2-fusion protein, stably or transiently transfected RAW264.7 cells were stimulated overnight with LPS (1 ng/ml) and IFN-γ (100 U/ml) in glass bottom dishes with No. 1.5 coverglass (MatTek Corp). Cells were washed once with PBS and organelles were labeled. ER-Tracker™ Blue-White DPX and LysoTracker™ Red (Invitrogen) were used to label cells at a working concentration of 1 µM for 30 minutes at 37°C. For all other stains, transfected cells were fixed with 3% paraformaldehyde (PFA) for 15 minutes at room temperature, permeabilized with 0.5% saponin, blocked with 10% normal goat serum and incubated with primary and secondary antibodies. Anti-CD107a (LAMP-1) (BD Pharmingen), anti-CD11b (BD Pharmingen), anti-golgin97 (Invitrogen), anti-EEA1 (Calbiochem), anti-GM130 (BD biosciences), and Hoechst 33258 (Invitrogen) were used to identify cellular organelles. Secondary antibodies were all raised in goat. Specimens were kept in PBS and imaged at room temperature on a Leica SP5 inverted confocal microscope with a motorized stage and Leica DFC495 camera. Images were analyzed using Leica application suite advanced fluorescence software.

**Nitrite and ROS detection**

For nitrite detection, adherent PEM were stimulated overnight with IFN-γ (10ng/ml) and then stimulated with LPS (100ng/ml) in the presence of N-Acetyl
Cysteine (NAC) (Sigma) or NG-nitro-L-arginine methyl ester (NAME) (Sigma) for 48 hours. At indicated timepoints following LPS addition, 50µl of supernatant was collected for analysis of NO2- production using Griess reagents. 50µl of 1% sulfanilamide in 3% H₃PO₄ was added to 50µl of supernatant followed by 50µl of 0.1% napthylethylene dihydrochloride in 3% H₃PO₄ and the wells were read on a spectrophotometer at 550nM. Sodium nitrite was used a standard at concentrations ranging from 1µM to 125µM.

For ROS detection, adherent PEM were stimulated overnight with IFN-γ (10ng/ml) and then labeled with 10µM CM-H₂DCFDA (Life Technologies) in PBS for 30 minutes at 37°C, followed by washing and addition of complete media. Inhibitors were added 30 minutes prior to addition of LPS, PMA (1µM), or H2O2 (200µM). 30 minutes later, cells were scraped and immediately analyzed by flow cytometry.

**Mutagenesis of P-2 cytoplasmic domain**

The P-2 fusion protein expression plasmid, P2-RFP-N3-v5 was subjected to site-directed mutagenesis PCR to construct the following mutants of the P-2 cytoplasmic domain, 1) Mm P2 678-KYKK→QFQQ, 2) Mm P2 684-Y→F, 3) Mm P2 692-S→A, 4) Mm P2 692-S→D. PCR was carried out using the following program, 95°C 2min; 95°C 30sec, 58°C 30sec, 72°C 7min (16 cycles); 72°C 10min and PfuTurbo® Hotstart DNA polymerase (Agilent) according to manufacturers instructions. For mutant 1, primer constructs were as follows: Forward 5’-cagcagaaggaataccaggaaattgaggag and Reverse 5’-gaactgccgagtaccatagtgccaaggt. For mutant 2, primer constructs were as
follows: Forward 5′-cggaagtacaagaagaaggaatccaggaaattgaggagcag and Reverse 5′-ctgctcctcaatttctggaattcctctctgtactccg. For mutant 3, primer constructs were as follows: Forward 5′-gccttggtggaagttagcaaca and Reverse 5′-ctctgctcctcaattttctgtactccg. For mutant 4, primer constructs were as follows: Forward 5′-gattgtgggaagttagcaaca and Reverse 5′-ctctgctcctcaattttctgtactccgta. Mutant nucleic acids are underlined. The resulting PCR product was treated with DpnI overnight at 37°C to digest template DNA and PCR purified according to Qiagen kit instructions. The linear mutant plasmid was then treated with optikinase (USB) for 30min at 37°C, 15min at 65°C, and then T4 ligase (Roche) for 1 hour at room temperature before chemical transformation into Top10 competent cells (Invitrogen). Transformants were selected on LB agar containing Kanamycin (60µg/ml) and checked by restriction digest for presence of the P-2-RFP-N3-v5 plasmid and sequencing for the correct mutagenic sequence.
References

Allavena, P., A. Sica, G. Solinas, C. Porta and A. Mantovani

Auffray, C., M. H. Sieweke and F. Geissmann

Balaji, K. N., N. Schaschke, W. Machleidt, M. Catalfamo and P. A. Henkart

Banchereau, J. and R. M. Steinman


Bedard, K. and K. H. Krause

Celli, J. and B. B. Finlay

Chakravortty, D. and M. Hensel

Chua, J. and V. Deretic

Chudakov, D. M., S. Lukyanov and K. A. Lukyanov
Cirillo, D. M., R. H. Valdivia, D. M. Monack and S. Falkow  

D'Angelo, M. E., M. A. Dunstone, J. C. Whisstock, J. A. Trapani and P. I. Bird  


de Chastellier, C.  
2009  The many niches and strategies used by pathogenic mycobacteria for survival within host macrophages. *Immunobiology* 214(7):526-42.

Dennert, G. and E. R. Podack  

Ding, A. H., C. F. Nathan and D. J. Stuehr  


Fadeel, B., S. Orrenius and J. I. Henter  
Fallman, M., K. Andersson, S. Hakansson, K. E. Magnusson, O. Stendahl and H. Wolf-Watz
1995  Yersinia pseudotuberculosis inhibits Fc receptor-mediated

Figueroa, J. E. and P. Densen
1991  Infectious diseases associated with complement deficiencies. Clin

Fleetwood, A. J., H. Dinh, A. D. Cook, P. J. Hertzog and J. A. Hamilton
2009  GM-CSF- and M-CSF-dependent macrophage phenotypes display
differential dependence on type I interferon signaling. J Leukoc Biol

1993  An essential role for interferon gamma in resistance to

Gagnon, E., S. Duclos, C. Rondeau, E. Chevet, P. H. Cameron, O. Steele-
Mortimer, J. Paiement, J. J. Bergeron and M. Desjardins
2002  Endoplasmic reticulum-mediated phagocytosis is a mechanism of

Rondeau and M. Desjardins
2001  The phagosome proteome: insight into phagosome functions. J Cell

Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad and K. Ley
2010  Development of monocytes, macrophages, and dendritic cells.
Science 327(5966):656-61.

Glynn, A. A.
1969  The complement lysozyme sequence in immune bacteriolysis.
Immunology 16(4):463-71.

Goransdotter Ericson, K., B. Fadeel, S. Nilsson-Ardnor, C. Soderhall, A.
Samuelsson, G. Janka, M. Schneider, A. Gurgey, N. Yalman, T. Revesz, R.
Egeler, K. Jahnukainen, I. Storm-Mathieson, Å. Haraldsson, J. Poole, G. de Saint
Basile, M. Nordenskjold and J. Henter
2001  Spectrum of perforin gene mutations in familial hemophagocytic

Gordon, S.
35.
Gordon, S. and P. R. Taylor

Gulig, P. A., T. J. Doyle, M. J. Clare-Salzler, R. L. Maiese and H. Matsui
1997  Systemic infection of mice by wild-type but not Spv- Salmonella typhimurium is enhanced by neutralization of gamma interferon and tumor necrosis factor alpha. *Infect Immun* 65(12):5191-7.

Hadders, M. A., D. X. Beringer and P. Gros

He, X., Y. Zhang and Z. Yu


Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel and M. Aguet

Hume, D. A.

Hwang, J. Y., T. Ohira, I. Hirono and T. Aoki

Isberg, R. R., T. J. O’Connor and M. Heidtman  

Jackson, S. H., J. I. Gallin and S. M. Holland  


Jutras, I. and M. Desjardins  


Kaufman, R. J., M. V. Davies, V. K. Pathak and J. W. Hershey  

Kawano, H., T. Nakatani, T. Mori, S. Ueno, M. Fukaya, A. Abe, M. Kobayashi, F. Toda, M. Watanabe and I. Matsuoka  

Kemp, I. K. and V. E. Coyne  

Laroux, F. S., X. Romero, L. Wetzler, P. Engel and C. Terhorst  


Mah, S. A., G. W. Moy, W. J. Swanson and V. D. Vacquier

Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi and M. Locati

Manzanero, S.

Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche and G. Dougan

McCormack, R., L. R. de Armas, M. Shiratsuchi, J. E. Ramos and E. R. Podack

Meresse, S., O. Steele-Mortimer, E. Moreno, M. Desjardins, B. Finlay and J. P. Gorvel
Muhlebach, T. J., J. Gabay, C. F. Nathan, C. Erny, G. Dopfer, H. Schrote, V. Wahn and R. A. Seger
1992 Treatment of patients with chronic granulomatous disease with recombinant human interferon-gamma does not improve neutrophil oxidative metabolism, cytochrome b558 content or levels of four antimicrobial proteins. *Clin Exp Immunol* 88(2):203-6.

Nakajima, H. and P. A. Henkart

Nathan, C.

Pasternack, M. S., C. R. Verret, M. A. Liu and H. N. Eisen

Peitsch, M. C., P. Amiguet, R. Guy, J. Brunner, J. V. Maizel, Jr. and J. Tschopp

Pluddemann, A., S. Mukhopadhyay and S. Gordon

Podack, E. R., G. Biesecker and H. J. Muller-Eberhard
1979 Membrane attack complex of complement: generation of high-affinity phospholipid binding sites by fusion of five hydrophilic plasma proteins. *Proc Natl Acad Sci U S A* 76(2):897-901.

Podack, E. R. and G. Dennert

Podack, E. R. and P. J. Konigsberg

Podack, E. R. and H. J. Muller-Eberhard
Podack, E. R. and J. Tschopp

Pollock, J. D., D. A. Williams, M. A. Gifford, L. L. Li, X. Du, J. Fisherman, S. H. Orkin, C. M. Doerschuk and M. C. Dinauer

Preissner, K. T., E. R. Podack and H. J. Muller-Eberhard

Prost, L. R., S. Sanowar and S. I. Miller

Randow, F. and C. Munz

Reed, S. G.


Smith, S. M.  

Spilsbury, K., M. A. O'Mara, W. M. Wu, P. B. Rowe, G. Symonds and Y. Takayama  

Terashima, M., M. Kobayashi, M. Motomiya, N. Inoue, T. Yoshida, H. Okano, N. Iwasaki, A. Minami and I. Matsuoka  

Tschopp, J., H. J. Muller-Eberhard and E. R. Podack  

Tweten, R. K.  

Uellner, R., M. J. Zvelebil, J. Hopkins, J. Jones, L. K. MacDougall, B. P. Morgan, E. Podack, M. D. Waterfield and G. M. Griffiths  
1997 Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. EMBO J 16(24):7287-96.


Varin, A., S. Mukhopadhyay, G. Herbein and S. Gordon  
Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos and F. C. Fang

Voskoboinik, I. and J. A. Trapani


Wilson, P. M., R. H. Fryer, Y. Fang and M. E. Hatten

Wright, D. E., A. J. Wagers, A. P. Gulati, F. L. Johnson and I. L. Weissman


Young, J. D., Z. A. Cohn and E. R. Podack

Young, J. D., C. F. Nathan, E. R. Podack, M. A. Palladino and Z. A. Cohn
Zalman, L. S., L. M. Wood and H. J. Muller-Eberhard

Zhang, X., R. Goncalves and D. M. Mosser