Interleukin 1 Receptor1 signaling in Platelet Inflammatory responses
Interleukin-1β processing and secretion

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This thesis is dedicated to my Dad, who always believed in me,

My husband, Raghuram and my family for their unwavering support and encouragement,

and

My Acharya, Srimath Srimushnum Andavan.
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Abstract

Padmini Narayanan

Anucleate platelets alter their transcriptome through stimulated splicing of intron and exon containing hnRNA e.g. Interleukin-1β (IL-1β), BCL-3. Platelet TLR4 activation upon Lipopolysaccharide (LPS) binding induces accumulation of spliced IL-1β mRNA and IL-1β laden microparticle shedding. Interleukin-1 Receptor 1 (IL-1R1) shares downstream signaling molecules with TLR-4, and its presence and possible role in platelet responses is unknown. Here I show that, platelets do express surface IL-R1 and is functional upon binding IL-1β. Platelet IL-1R1 could recognize endogenous ligands as well observed through the in vivo accumulation of IL-1β during clot formation in mouse models. Platelets are the primary source of this IL-1β accumulation, dependent on post transcriptional processing events and translation of platelet IL-1β mRNA.

Proinflammatory IL-1β is produced as a leader less proprotein and its mode of secretion remains opaque. This study demonstrates that IL-1β is released in soluble form and also in association with plasma membrane shed microparticles and endo-lysosomal originated exosomes. The distribution of the newly synthesized IL-1β between these
compartments where agonist dependent. Direct visualization of unpermeabilized microparticles with Total Internal Reflection Microscopy and flowcytometry showed IL-1β surface display. Both microparticles and exosomes contain IL-1R1 and are essential for the IL-1β display on the particle surface. Blocking IL-1R1 in human platelets with IL-1Ra reduced IL-1β display on the microparticle surface and complete loss of IL-1R1 in IL-1R1−/− platelet shed microparticles showed no surface IL-1β. The shed microvesicles contain biologically active IL-1 that activates Nf-κB driven E-selectin expression in endothelial cells in an IL-1R1 dependent fashion.

Efficient IL-1 secretion is bi-parte signaling event, involving the surface receptor ligand stimulation that is closely followed by activation of ATP - induced purinergic P2X7 receptor and NLRP3 inflammasome which facilitate Caspase-1 dependent IL-1β activation and secretion. Platelets express P2X7 receptors and Pannxiein-1 channels, process IL-1β via NLRP-3 inflammasome complex formation. Inhibition of caspase-1 activity with cell permeable peptide inhibitors, or indirect inhibition of inflammasome assembly through inhibition of P2X7 receptor and Panx-1 activation resulted in decreased IL-1β laden microparticle release.

In summary, this study has identified novel functional platelet receptors and protein complexes and the signaling pathways involved in platelet IL-1 response. This extends our understanding of the role of platelets in both septic and sterile inflammatory responses.
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ADP  Adenosine diphosphate
ATP  Adenosine triphosphate
Akt  AK-8 thymoma (or transformed)
AIM-2  Absent in melanoma 2
ASC  Apoptosis associated speck like protein containing a CARD
CARDs  caspase recruitment domains
DAMPs  damage-associated molecular patterns
ERK  extracellular signal regulated kinase
IκB  inhibitory kappa beta
IL-1  Interleukin-1
IL-1R1  Interleukin-1 Receptor-1
IL-1Ra  Interleukin-1 Receptor antagonist
IL-1β  Interleukin-1 β
IRAK  interleukin-1 receptor associated kinase
IRF  interferon responsive factor
JAKs  janus kinases
JNK  c-Jun-n-terminal kinase
LPS  lipopolysaccharide
LRR  leucine-rich repeat
LSS  leaderless secretory protein
MAP  mitogen-activated protein
MyD88  myeloid differentiation primary response gene factor 88
NAIP  Neuronal apoptosis inhibitor protein
NF-κB  nuclear factor kappa B
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<tr>
<td>NOD</td>
<td>nucleotide oligomerization domain</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like receptor family, pyrin domain containing 3</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet-activating factor</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>P_2X_7</td>
<td>P_2X purinoceptor 7</td>
</tr>
<tr>
<td>PYD</td>
<td>pyrin domain</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>TAK</td>
<td>TGF-β activated kinase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TIR</td>
<td>toll-interleukin 1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>toll-interleukin 1 receptor (TIR) domain containing adaptor protein</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associate factor</td>
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<tr>
<td>TRIF</td>
<td>TIR domain containing adapter-inducing IFN-β</td>
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Figure 34 – Platelet IL-1 Response – Role of platelet IL-1R1 in TLR-4 mediated septic response and Thrombin mediated sterile inflammation. ........................................................................................................... 125
1.1 - AN OVERVIEW OF PLATELET FUNCTION

Platelets are small cellular fragments released from megakaryocyte precursors. They form the major cell type in blood, and exponentially outnumber other blood cells in circulation. Their primary function is to maintain hemostasis and in turn uphold the integrity of the circulatory system (Hartwig 2006). Endothelial cells line the vessel wall and help maintain an inert environment that restricts the interaction between circulating blood cells as much as possible. External trauma or injury, atherosclerosis and other conditions strips the endothelial cells from the vessel wall exposing the sub endothelial matrix proteins such as collagen, laminin, fibronectin, von-Willebrand factor (vWF), and others. Platelets are the primary responders under these conditions and interact with the exposed matrix proteins through their specific surface receptors leading to platelet

1.2 - PLATELET ADHESION, ACTIVATION, SECRETION:

Platelet activation is a stepwise process. This process commences upon binding of respective ligands with their receptors on the platelet surface. This binding leads to initiation of a signaling cascade followed by platelet shape changes. Platelets bind to the vWF in the vessel wall through the glycoprotein surface receptor GP1b-IX-V complex. This interaction leads to activation of α2β3 integrin leading to platelet tethering to the vessel wall and initiates platelet adhesion (Spiel, Gilbert et al. 2008). This process is further facilitated by the subsequent interaction of sub-endothelial collagen with GPVI and α2β1 receptors on the platelet surface (Clemetson and Clemetson 2001). With these interactions a monolayer of activated platelets are firmly adhered to the damaged vessel wall (Hankey and Eikelboom 2003). Adherent activated platelets undergo shape change. The discoid platelets transform from circular discs to speculated spears due to spreading
following cytoskeletal rearrangement. The increase in intercellular calcium drives this rearrangement through augmented actin filament and microtubule complexes. This is followed by extension of lamellipodia and the spreading of platelets (Hartwig 2006, Maxwell, Dopheide et al. 2006, Spiel, Gilbert et al. 2008).

The initial thrombus is called a platelet plug, which undergoes extension as adherent platelets recruit naïve circulating platelets. All along this process of adhesion, platelet granules concentrate to the center of the cell and are shed upon increased intracellular calcium ion influx. This process concentrates activators and coagulation mediators at the site of vessel injury aiding in the thrombus formation (Maxwell, Dopheide et al. 2006, Varga-Szabo, Pleines et al. 2008, Jennings 2009, Gasparyan 2010, Rumbaut and Thiagarajan 2010, Margetic 2012).

Platelets contain three types of granules and contain around 60 granules per cell on average (Morrell, Aggrey et al. 2014). The majority of the population is made up of the alpha granules. These are large structures that contain a plethora of proteins with varied function (mainly immune and platelet functions) (Rumbaut and Thiagarajan 2010, Morrell, Aggrey et al. 2014). Recent studies have identified over 300 different proteins in these granules including PF4, β thromboglobulin, P-Selectin, CD40L, CD63, VWF, thrombospondin, MMP-2, MMP-9 to name a few (Lemons, Chen et al. 1997, Maynard, Heijnen et al. 2007, Maynard, Heijnen et al. 2010, Koseoglu and Flaumenhaft 2013). The smaller dense granules are present in much lesser number than the alpha granules, but are the major source of platelet activators that are essential in thrombus formation process. They contain molecules including Serotonin, ADP, ATP, polyphosphates and glutamate.
These molecules aid in platelet aggregation and thrombosis. The third group of granules is of lysosomal origin and they are very sparse in distribution and contain mainly glycohydrolase and digestive enzymes (Lemons, Chen et al. 1997, Italiano and Battinelli 2009, Koseoglu and Flaumenhaft 2013).

Thromboxane (TxA\textsubscript{2}) is one of the important lipids made only upon stimulation and is rapidly secreted. TxA\textsubscript{2} is synthesized in platelets from arachodonic acid through the cyclooxygenase pathway. TxA\textsubscript{2} is a potent platelet agonist and signals through the thromboxane receptor (TR) and leads to aggregation (Clemetson and Clemetson 2001, Hankey and Eikelboom 2003). P-Selectin (CD62P) is another alpha granule protein of significance. In activated platelets CD62P is trafficked to the surface with alpha granule. The increased surface expression of CD62P facilitates the interaction between platelets and nucleated cells like neutrophils and monocytes (Furie, Furie et al. 2001, Singbartl, Forlow et al. 2001). ADP shed from dense granules aids subsequent platelet aggregation. ADP signals through two classes of receptors – transmembrane receptors- P2Y\textsubscript{1} and P2Y\textsubscript{12} and pyrogenic calcium channel family receptor P2X\textsubscript{1} (Sun, Li et al. 1998, Fabre, Nguyen et al. 1999, Gachet 2001). These interactions modulate intercellular calcium and facilitate the transition from transient aggregation to sustained irreversible platelet aggregates (Fig-1). ADP and TxA\textsubscript{2} act in concert with other platelet activators like thrombin to modulate thrombus formation at the site of vascular damage.
1.3 - PLATELETS AS IMMUNE CELLS

Dysregulation of the microvasculature is one of the prominent cause of inflammation (Stokes and Granger 2012). Roles of endothelium and leukocytes in this process are well studied, with platelets as well known mediators of thrombosis and hemostasis. Increasing numbers of immunologic vascular studies illuminate the role of platelets in the manifestation of inflammatory diseases. Platelets have in recent years emerged as major cell type participating in both innate and adaptive immune responses (Elzey, Tian et al. 2003, Soga, Katoh et al. 2007). The chemokines and cytokines secreted and shed by platelets shape the state of the innate and adaptive immune defense response mounted by the host organism against intruding pathogens (Elzey, Tian et al. 2003).

Platelets contain preformed immune mediators and inflammatory cytokines, pre-RNAs and are involved in varied aspects of vascular biology. They are vital in the pathogenesis and progression of inflammatory diseases such as atherosclerosis, arthritis, and transplant rejection, and infectious diseases including cerebral malaria, dengue, HIV and many more (von Hundelshausen and Weber 2007, Srivastava, Cockburn et al. 2008, Jennings 2009, Boilard, Nigrovic et al. 2010, Swaim, Field et al. 2010, Hottz, Lopes et al. 2013, Nascimento, Hottz et al. 2014). Platelet depletion studies have shown their involvement in activating local innate immune cells and showing that platelets are indispensable in mounting an active innate response. These effects are aided by granule secretion. Thus, exocytosis of platelet alpha and dense granules shape the immunological events at the site of thrombosis. Platelets collectively have an enormous surface area decorated with many receptors that allow platelets to function as immune surveillance
cells (von Hundelshausen and Weber 2007). Platelet surface receptors and their secretory molecules provide an interface between thrombosis and innate and acquired immune responses. (Figure 1)

**Figure 1** - Representation of Platelet activation and release of range of inflammatory molecules with varied functions in the both innate and adaptive immune response. Figure adapted from (Margetic 2012)

### 1.4 - PLATELET TOLL LIKE RECEPTORS IN IMMUNE RESPONSE:

Inflammation requires the interaction between surface receptors of inflammatory cells and various soluble factors. Immune cells employ Toll like receptors (TLRs) to mount proinflammatory responses against microorganisms. Research shows the association between these receptors and acute immune responses is indispensable (Akira
2003, Kaisho and Akira 2006). Stimulation of TLRs orchestrates the innate immune response mounted by the host against a microbial insult by recognizing their pathogen–associated molecular patterns (PAMPs) via their extracellular domain (Iwasaki and Medzhitov 2004, Kaisho and Akira 2006, Blair, Rex et al. 2009). Around 11 different TLRs have been identified, with different extracellular domains and they recognize a wide range of microbial products and other endogenous ligands (Akira 2003, Iwasaki and Medzhitov 2004).

Platelets are shown to express almost all members of this family of receptors (Cognasse, Hamzeh et al. 2005, Aslam, Speck et al. 2006); although not all of them function and their effects during platelet activation are not yet understood. Identifying just a few of these receptors has raised platelets to the status of important mediators of innate immune responses. The expression pattern of the TLRs is altered between resting and activated platelets. TLR-4, TLR1/2 and TLR-6 are shown to be present on the surface of platelets (Warkentin, Aird et al. 2003, Shiraki, Inoue et al. 2004, Andonegui, Kerfoot et al. 2005, Aslam, Speck et al. 2006). The level of expression between these receptors is different, but 1/2, 4, 6 are not altered upon activation (Warkentin, Aird et al. 2003, Aslam, Speck et al. 2006). TLR–9 is sparsely expressed on the surface of resting platelets and upon activation by thrombin there is a significant increase in the surface expression of this receptor after trafficking from the endosomal compartments (Cognasse, Hamzeh et al. 2005, Aslam, Speck et al. 2006, He, Franchi et al. 2013). Platelets release TLR-5 upon thrombin stimulation suggesting platelets store TLRs in the alpha granules, formed during megakaryocyte maturation, for release after activation (Coppinger, Cagney et al. 2004, Aslam, Speck et al. 2006). Activation of these TLR receptors in platelets not only
leads to alteration of the immune response, but also affects their thrombotic activity. Activation of platelet TLR-2 receptors induces platelet aggregation and increases adhesion to collagen, P-selectin expression, and generation of Reactive Oxygen Species (ROS) (Blair, Rex et al. 2009).

Activation of platelet TLR-2 receptor upon binding its specific ligand Pam$_3$CSK$_4$, results in the AKT kinase cascade activation and the resultant activation of PI3K and other downstream molecules. These activated platelets adhere to neutrophils and this leads to formation of increased platelet neutrophil aggregates in vivo. Administration of TLR-2 antibodies reduces these events and the TLR-2 knockout mouse did not develop these complications (Blair, Rex et al. 2009) thus, emphasizing the importance of TLR-2 in platelets and hints at the possibility direct platelet activation by microorganisms through these surface receptors.

1.5 PLATELET TLR-4/ LPS SIGNALING

Platelet TLR-4 signaling is an essential component in endotoxin mediated sepsis. Studies from many groups including ours, have contributed to the understanding of TLR-4/ lipopolysaccharide (LPS) mediated signaling and activation of platelets relevant to sepsis (Zhang, Han et al. 2009, Brown and McIntyre 2011, Srikanthan, Li et al. 2014). TLR-4 recognizes LPS, a Gram negative bacterial cell wall component, and requires a CD14 co-receptor to complete the TLR-4 signaling (Andonegui, Kerfoot et al. 2005, Cognasse, Hamzeh et al. 2005). Lipopolysaccharide binding protein (LBP) acts as a
carrier protein that facilitates LPS binding to its TKR-4 receptor, and requires exogenous circulating CD14 for optimal signaling in platelets (Palsson-McDermott and O'Neill 2004). We have shown that LPS is a non-classic platelet agonist, as it does not cause, but rather potentiates, platelet aggregation. Later in the result section I show that LPS stimulation of platelets leads neither to robust ROS production nor calcium ion release, and causes no significant increase in P-selectin expression. LPS does however, increase platelet adhesion to fibrinogen under shear flow, but not on par with classical platelet agonists like thrombin. Platelet TLR-4 stimulation, however, is very effective in increasing interleukin-1 β (IL-1β) intron exon containing heteronuclear mRNA (hnRNA) splicing and microparticle shedding. The newly synthesized IL-1β is associated with shed

![Figure 2 - TLR-4 LPS signaling in platelets. Model adapted from our previous publications (Shashkin, Brown et al. 2008, Brown and McIntyre 2011)
microparticles. Activation of established kinase cascades are essential for this signaling in platelets. IL-1β mRNA splicing in platelets requires MyD88, TIRAP and IRAK1/4 secondary signaling molecules, followed by AKT and JNK phosphorylation (Akira 2003, Brown and McIntyre 2011).

The activation of pro-IL-1β to the mature form was caspase-1 dependent. Thus we have shown that the platelet employs canonical receptors for proximal signaling and then in downstream signaling uses non-canonical kinases (Shashkin, Brown et al. 2008, Brown and McIntyre 2011, Brown, Narayanan et al. 2013). In in-vivo studies, platelets show increased functionality following TLR-4 stimulation, and also expand inflammatory stimuli by augmenting TNF-α expression. TLR-4 - LPS interaction increases platelet adhesion to fibrinogen via activation of outside-in signaling and expression of primary adhesion receptors GPIIb/GPIIia and CD40L (Clark, Ma et al. 2007). Platelet TLR-4 is essential for the innate immune response against bacterial infection (Vincent, Yagushi et al. 2002, Kirschenbaum, McKevitt et al. 2004). During severe sepsis an increase in platelet TLR-4 expression results which leads to increased activation of platelets that causes thrombocytopenia. Severe thrombocytopenia denotes poor prognosis of the condition (Bone, Francis et al. 1976, Andonegui, Kerfoot et al. 2005).
1-6 PLATELET SOLUBLE MEDIATORS IN IMMUNE RESPONSES:

Alpha granules are the major source of platelet derived chemokines and cytokines. Thrombospondin-1 (TSP), TGF-β, VEGF, Platelet factor-4 (PF4), RANTES, CCL5, CD40L, proplatelet basic protein (PPBP) are few of the potent inflammatory mediators present in these granules (Rendu and Brohard-Bohn 2001, Maynard, Heijnen et al. 2007, Maynard, Heijnen et al. 2010, Morrell, Aggrey et al. 2014). These molecules are capable of shaping both the innate and adaptive immune response. TSP causes endothelial cell activation, facilitates macrophage – platelet aggregate formation, and apoptosis, thus mediating an innate immune response during endothelial inflammation upon vessel injury (Silverstein and Nachman 1987, Lopez-Dee, Pidcock et al. 2011). Other platelet derived chemokines are involved in T-cell trafficking and expansion. PF4 levels in the circulation are used as markers for the severity of many infectious and inflammatory diseases involving dysregulation in both innate and adaptive immune response (Pitsilos, Hunt et al. 2003, Srivastava, Cockburn et al. 2008). This chemokine is the major mediator of platelet immune function with platelet depletion in a mouse malarial model showing decreased PF4 in the circulation that in turn affects the severity of the disease. Notably there was an increase in the pathogen burden after post infection platelet depletion, indicating time dependent role of platelets and antimicrobial effect of PF4 (Aggrey, Srivastava et al. 2013). PF-4 is one of the ligands for CXCR3, aiding in T-cell trafficking towards the site of vascular inflammation and expansion as well as differentiation of TH17 cells (Aziz, Cawley et al. 1995, Srivastava, Cockburn et al. 2008, Gleissner, Shaked et al. 2010).
ADP, shed upon platelet activation from the dense granule, is a well-known mediator of platelet aggregation. This ADP also activates other immune cells like monocytes, neutrophils, dendritic cells through their P2Y12 surface receptors. In dendritic cells ADP activation leads to increased antigen endocytosis and processing and differentiation of CD4+ T-helper cells into TH-17 cells. (Vanderstocken, Bondue et al. 2010, Younas, Hue et al. 2013). Polyphosphate and dense granule proteins are capable of NFκB activation and increase adhesion molecule expression in the endothelium (Bae, Lee et al. 2012). Other dense granule contents like glutamate, serotonin and others, increase monocyte differentiation into dendritic cells, and T-cell migration and differentiation as well (Ganor, Besser et al. 2003, Katoh, Soga et al. 2006, Leon-Ponte, Ahern et al. 2007, Soga, Katoh et al. 2007, Ganor, Grinberg et al. 2009). These reports establish the importance of platelets as a mediator of immune response.

1-7 AUTOINFLAMMATORY DISEASES

Autoinflammatory diseases are described as innate immune response complications that include disarray of cytokine signaling defects in innate immune cells like monocytes and macrophages. These diseases have both local and systemic manifestations, but seldom include the involvement of MHC-II, HLA or T-cell autoantibodies (adaptive immune response). The IL-1 family of cytokines and their receptors along with TNF-α is commonly linked to inflammatory conditions in general and are of utmost importance in these complications. Thus, targeting this signaling pathway has been the primary approach to alleviate these complications (Dinarello 2011).
IL-1β has been implicated in the pathogenesis of rheumatoid arthritis (Abramson and Amin 2002, Bresnihan and Cobby 2003), gout (Pope and Tschopp 2007, Terkeltaub 2010), periodic fever, pharyngitis, type2 diabetes (Dinarello, Donath et al. 2010), multiple myeloma (Costes, Portier et al. 1998, Anderson and Lust 1999, Lust and Donovan 1999), atherosclerosis (Rader 2012, Sheedy and Moore 2013), and many more inflammatory diseases. IL-1 possesses hematopoietic properties, and decreases thrombocytopenia and leukopenia upon administration after bone marrow transplantation, but often is accompanied with systemic inflammation. IL-1 is involved in both acute and chronic inflammatory condition (Dinarello 2011).

1-8 INTERLEUKIN-1 FAMILY

The IL-1 family is made up of 11 related ligands that bind their respective receptors (O’Neill 2008, Dinarello 2011). IL-1 receptors are immunoglobulin - like receptors that share similarities with TLR family of receptors and they contain TIR (Toll – Interleukin-1 receptor) domains in their cytoplasmic region. TIR is important for the function of the receptors that upon activation bind MyD88 and mediate a signaling cascade that is similar in fashion to TLR signaling (O’Neill 2008, Smith, Lipsky et al. 2009).

Just a few amino acid changes in the cytoplasmic region in Single Ig IL-1 Related Receptor (SIGIRR) and IL-1RAcP, (two receptors that belong to the IL-1 family) alter the charge in the TIR domain and inhibit the recruitment of MyD88 and halt the signaling. SIGIRR and IL-1RAcPb are inhibitory receptors that regulate the IL-1
inflammatory response (Dinarello 2009, Dinarello 2011). Unlike other cytokines, IL-1 controls inflammation at both the receptor and nuclear levels. There are multiple levels of regulation involving decoy receptors, e.g. IL-1Rll, and negative regulatory receptors. A co-receptor, IL-1RAcP is required to complete the signaling process upon ligand binding (Garlanda, Anders et al. 2009, Noris, Cassis et al. 2009, Dinarello 2011). IL-1α, IL-1β and IL-1Ra bind Interleukin-1Receptor1 (IL-1R1) and IL-1Rll. Ligand binding to the latter does not produce downstream signaling as it lacks a TIR domain. Upon ligand binding, IL-1R1 is rapidly endocytosed and the signaling takes place in the clathrin coated endosomes termed redoxosomes (Oakley, Abbott et al. 2009, Oakley, Smith et al. 2009, Spencer and Engelhardt 2014). The receptor endocytosis is a dynamin dependent process and is essential for successful IL-1R1 signaling. Soluble IL-1β concentrates within these redoxosomes and carries out IL-1 responses. The signaling mediators are recruited to these endosomes to carry out the cascade (Li, Harraz et al. 2006, Oakley, Smith et al. 2009). IL-1 shares downstream components with TLR signaling like MyD88, IRAK1/4, and TRAF-6 (Cao, Xiong et al. 1996, Kawagoe, Sato et al. 2008, O'Neill 2008, Weber, Wasiliew et al. 2010, Dinarello 2011). Targeted disruption or ablation of MyD88 shows decrease or loss of IL-1 and IL-18 secretion and function (Adachi, Kawai et al. 1998). IL-1R1 induces a sequence of phosphorylation, ubiquitination and degradation that culminates in the activation of Nuclear factor κ B (NFκB) and its nuclear translocation (Cao, Xiong et al. 1996, Walsh, Kim et al. 2008). Activation of NFκB along with JNK and p38 MAP kinases leads to expression of a cohort of IL-1β driven inflammatory proteins (Baud, Liu et al. 1999, Silverman, Zhou et al. 2003, Huang, Yang et al. 2004). IL-1β induces expression of itself along with other
interleukin ligands including IL-1α, IL-6, IL-8, and IL-18 (Silverman, Zhou et al. 2003, Walsh, Kim et al. 2008, Weber, Wasiliew et al. 2010).

1-9 INTERLEUKIN-1 β (IL-1β / IL-1F2):

IL-1β is the major regulator of the innate immune response affecting almost all cell types and organs. It is primary mediator of numerous autoinflammatory, autoimmune and infectious diseases (Donath, Storling et al. 2008, Dinarello 2011, Dinarello 2011). IL-1 dysregulation is quintessential for manifestation of many sterile inflammatory syndromes like type 2 diabetes (Dinarello, Donath et al. 2010), gout (Pope and Tschopp 2007), osteoarthritis (Fernandes, Martel-Pelletier et al. 2002, Daheshia and Yao 2008), myocardial infarctions (Hwang, Matsumori et al. 2001), etc. Monocytes donot necessarily show significantly increased IL-1β transcripts under some of these conditions, suggesting alternative IL-1β sources. IL-1β is a potent inflammatory cytokine as with its ability to affect varied types of IL-1R1 expressing cells at picogram levels in the circulation (van der Meer, Barza et al. 1988, Dinarello 2011). Inhibition of IL-1β signaling through naturally occurring IL-1R1 antagonist IL-1Ra (Anakinra) decreases disease progression in multiple myeloma and helps in increasing survival in these conditions (Anderson and Lust 1999, Lust and Donovan 1999). Blocking IL-1R1 signaling, especially IL-1β signaling, is the most widely used course of treatment for most autoinflammatory diseases (Hwang, Matsumori et al. 2001, So, De Smedt et al. 2007, Lachmann, Kone-Paut et al. 2009, Terkeltaub 2010)
TLR induced IL-1β synthesis in innate immune cells is the mechanism that drives endotoxin mediated sepsis. On the other hand, IL-1β synthesis is stimulated via many endogenous ligands generated upon thrombosis (Lindemann, Tolley et al. 2001), collagen deposition in fibrosis (Artlett 2012, Thacker, Balin et al. 2012), and ATP (Baroni, Pizzirani et al. 2007). IL-1β augments its own production through stimulation of its receptor IL-1R1. There exists dissociation between transcription and translation in IL-1β synthesis. In activated platelets the IL-1β accumulation observed is rapid, since mature IL-1β transcripts are found preloaded into the polysome fractions ready to be transcribed in resting cells (Lindemann, Tolley et al. 2001).

**1-10 IL-1β SYNTHESIS – PROCESSING – SECRETION:**

IL-1β is produced as a leaderless inactive pro-protein that needs further processing before being secreted in its active form. IL-1β along with another IL-1 family cytokine, IL-18 is cleaved by caspase-1, which itself is produced as an inactive precursor. Caspase-1 is also known as IL-1β converting Enzyme (ICE). Caspase-1 activation is the major event that shapes the IL-1β production in innate immune cells. Caspase -1 is activated through a multiprotein complex called the inflammasome that assembles upon activation of the pyrogenic P2X7 receptor – Pannexin ion channel after ATP binding (Eder 2009, Weber, Wasiliew et al. 2010, Dinarello 2011). IL-1β is also cleaved by non-caspase dependent manner through proteases such as Cathepsin G, Proteinase-3, Chymase, elastase, etc. however none of these cleavages results in the active 17kD protein (Fantuzzi, Ku et al. 1997, Joosten, Netea et al. 2009, Dinarello 2011).
1.11 INFLAMMASOME

The inflammasome is a multiprotein complex assembled upon activation of Nod-Like Receptors (NLRs). They provide a platform for autocatalytic activation of caspase-1. NLRs along with TLRs and C-type Leptin Receptors (CLRs) form the Pattern Recognition Receptors (PRR) that surveils the environment for Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Pattern (DAMPs) molecules. About 23 proteins are identified to be associated with the inflammasome family. They are divided into two groups depending on the domains they contain – NLRP – contains pyrin domains and NLRC- contain caspase recruitment domains (CARD). Assembly of seven different inflammasome complexes is possible depending on the signal recognized and the cytoplasmic receptor involved.

Figure 3: Assembly of different Inflammasomes upon stimulus leading to ICE activation and IL-1 cytokine cleavage. Figure adapted from (Turner, Arulkumaran et al. 2014)
The different types of inflammasome complexes include NLR family pyrin domain containing-1 (NLRP1/NALP1), NALP3/NLRP3 (Cyopyrin), NLRP6, NLRP12, Absent in Melanoma-2 (AIM-2), IPAF/NLRC-4 and retinoic acid inducible gene-1 (RIG-1) inflammasomes (Martinon, Mayor et al. 2009, Latz, Xiao et al. 2013). These formations of the complexes are dependent on cytoplasmic NLRs association with the adaptor protein ASC- Apoptosis associated Speck like protein. ASC contains a pyrin

**Figure 4** - Model of NLRP3 inflammasome activation and cytokine processing. The bipartite signaling events involved in the activation of IL-1 cytokine synthesis, processing and secretion. Figure adapted from (Turner, Arulkumaran et al. 2014)
domain (PYD) and a CARD domain. The interaction through the PYD domains between ASC and the NBLRs initiate the assembly of inflammasome complexes. ACS then recruits procaspase-1 via its CARD domain on to the inflammasome platform and leads to autocatalytic activation of caspase-1 (Hogquist, Nett et al. 1991, Martinon, Mayor et al. 2009, Weber, Wasiliew et al. 2010, Lopez-Castejon and Brough 2011).

NLRs are activated by various DAMPs, of which ATP and ROS are the prominent mediators. Generation of DAMPs is a classic event involved in the pathogenesis of inflammation and malignancies. These DAMPs activate caspase-1/inflammasome - mediated processing and secretion of IL-1β and IL-18, cytokines involved in the initiation and complications of auto inflammatory conditions (Chen and Nunez 2010). Figure 1-8 shows the process of activation of NLRP-3, one of the most studied inflammasome in inflammatory conditions. For efficient IL-1 cytokine production two levels of signals are required (Chen and Nunez 2010, Dinarello 2011, Lopez-Castejon and Brough 2011, Turner, Arulkumaran et al. 2014). The first step is the activation of surface receptors e.g. TLRs and IL-1R1, by their ligands. This is followed by the downstream cascade leading to activation of NFκB and subsequent increases in IL-1β transcripts and IL-1β pro-protein translation. ROS are produced during these signaling processes, and upon binding to NLRP-3 activates inflammasome themselves. The second signal relayed through the binding of extracellular ATP to its receptor P2X7, opening of K+ ion channels and activation of the inflammasome leading to cleavage activation and secretion of IL-1β and IL-18 (North 2002, Faria, Defarias et al. 2005, Liang and Schwiebert 2005, Barbe, Monyer et al. 2006, Ferrari, Pizzirani et al. 2006, Petrilli, Papin et al. 2007). Almost all PAMPs and DAMPs cause release of ROS. ROS
activate non-canonical NALP-3 inflammasome formation (Krelin, Voronov et al. 2007, Zhou, Tardivel et al. 2010). ROS mediates inflammasome assembly and in turn increases IL-1β processing under inflammatory conditions (Tassi, Carta et al. 2010, Dinarello 2011, Dinarello 2011, Turner, Arulkumaran et al. 2014). Inhibition of ROS production affected IL-1β secretion in murine dendritic cells (DCs), but had no effect on IL-18 processing that suggests a distinction between the processing and secretion of IL-1β and IL-18 (Schmidt and Lenz 2012).

NLRP-3 gene point mutations are observed in some of the auto inflammatory diseases and metabolic diseases (Franchi, Eigenbrod et al. 2009, Bonar, Brydges et al. 2012), which leads to constant activation of this inflammasome and increased caspase-1 activated cytokine production (Agostini, Martinon et al. 2004, Lachmann, Kone-Paut et al. 2009). Caspase-4 and caspase-5 are also required during some conditions to activate IL-1β through non-canonical inflammasome assembly. These inflammasomes have been examined in the context of endotoxin – TLR-4 mediated stimulation. Studies have shown caspase 11 (Casapse-4/5 anthology of humans) deficient mice have decreased IL-1β release upon LPS stimulation (Casson, Copenhaver et al. 2013). Caspase 11 in itself could bring about the activation or facilitate caspase-1 mediated cleavage, by potentiating caspase-1 activation (Py, Jin et al. 2014).
1.12 P2X7 RECEPTOR - PANNEXIN ION CHANNEL

P2X7 receptors are non-selective extracellular ATP-gated cation channels and are expressed on immune cells including monocytes, macrophages and dendritic cells (DCs) (Ferrari, Pizzirani et al. 2006). All cell types produce ATP regardless of pathogen or host and the pool of ATP increases at the site of acute tissue damage and during the inflammatory state that follows. Immune cells express ATP binding P2 nucleotide receptors that bind ATP and other nucleotides secreted in the environments. These signaling events shape the immune response mounted by these cells. Seven P2X ionotropic receptors, along with eight G-protein coupled P2Y receptors make up the P2 nucleotide receptor family. P2X receptors bind ATP and P2X7 is indispensable for ATP mediated inflammasome activation and IL-1β secretion (Ferrari, Pizzirani et al. 2006). The factor that sets this receptor apart from the other purinergic receptors is its ability to open large pores, which allow dye uptake and movement of larger molecules up to 900 Da along, with the typical activity of opening ATP gated the cation channel to facilitate flux of ions like Ca^{++} and K^{+}. (North 2002, Faria, Defarias et al. 2005, Liang and Schwiebert 2005) This movement of ions is necessary for IL-1 cytokine secretion.(Bachmann, Horn et al. 2006, Ferrari, Pizzirani et al. 2006) Efflux of K^{+} is important for inflammasome assembly, IL-1β and IL-18 maturation, and release. This is followed by Ca^{++} ion influx, which modulates IL-1β release as well. Figures 1-8 and 1-9 show the role of P2X7 and Pannexin ion channels in the IL-1β secretory pathway.
**Pannexins** (PANX-1, PANX-2, PANX-3) are a recently identified group of hemi channel proteins that share similarities with innexins – the proteins involved in the gap junctions in invertebrates (Panchin 2005, Barbe, Monyer et al. 2006). The full extent of their functions is yet to be understood. Research shows the coupling of PANX-1 activation along-side of P2X7 activation (Pelegrin and Surprenant 2006). The large pore formed through PANX-1 activation is essential for IL-1β maturation and secretion in immune cells. Opening of this pore might help in entry of PAMPs and ATP directly into the cell and activate NLR cytoplasmic receptors and inflammasome activation (Faria, Defarias et al. 2005, Liang and Schwiebert 2005). Inhibition of P2X7 and PANX-1 activation through gene targeting or inhibitors in macrophages and dendritic cells showed diminished immune capacity which decreased or no IL-1β and IL-18 cytokine secretion. Monocytes did not show such effects upon PANX-1 inhibition (Pizzirani, Ferrari et al. 2007, Bhaskaracharya, Dao-Ung et al. 2014). IL-1α, unlike IL-1β is synthesized as an active form and does not need processing. PANX-1 inhibition hinders IL-1α release. This suggests PANX-1 pore inhibition not only affects inflammasome mediated cytokine activation, but also influences the release mechanism (Pelegrin 2008, Netea, Nold-Petry et al. 2009).

### 1.13 MECHANISMS OF IL-1β SECRETION

IL-1 cytokines are leaderless proteins and are excluded from the regular Endoplasmic reticulum - Golgi secretory pathway. They employ a non-conventional route of secretion involving secretory lysosomes and other modes of release like
microparticle shedding and direct release upon cell death. IL-1β and IL-18 require processing by caspase-1 inflammasomes and IL-1α does not. Regardless the route of secretion of these molecules is still yet unclear. Over 75 leaderless secreted proteins are shown to be dependent on caspase-1 activity (Becker, Creagh et al. 2009). Figure 1-9 and 1-10 represents steps involved in the processing and release of IL-1 cytokines.

**Figure 5** outlines the overall IL-1R1 signaling leading to the release of the activated cytokine. (1) IL-1 ligand binding and initiation of IL-1R1 signaling which requires the (2) association of co-receptor IL-1RAcP and the approximation of the intracellular TIR domains of the receptors (3) followed by the recruitment of MyD88 and IRAKs and then TRAF-6 activation and (4) in-turn NFκB activation, nuclear translocation then IL-1β gene transcription and (5) synthesis of new cytokine. The translation of IL-1β mRNA takes place in free polysomes and the newly synthesized proprotein accumulates in the cytosol (6). The ATP extracellular pool increases during tissue damage and activates other cells (7) triggers the activation of ATP gated ion channel – P2X7 and (8) cation channels are opened for the movement of ions to and from the cell. (9) A rapid fall in K+
ions follow (Petrilli, Papin et al. 2007). (10) this brings about the oligomerization of inflammasome components and procaspase-1 auto activation. (11) After translation of a pool of pro- IL-1β enter the secretory lysosome. The activated caspase-1(ICE) can then translocate to modified lysosome that are able to release of their contents upon stimulation that are called the secretory lysosomes (Blott and Griffiths 2002). The pro-IL-1β is cleaved by active ICE in these secretory lysosomes. (12) Increase in the Ca^{++} ion influx serves as the stimulus for the secretion of the lysosomal content. (13) The rise in intracellular Ca^{++} activates Ca^{++} dependent phospholipase-A and phosphatidylinositol - specific phospholipase-C (14). And the secretory lysosome fuses with the plasma membrane leading to the extracellular release of mature IL-1β. Alternatively (14) pro-IL-

**Figure 5** - Schematic representation of events involved in the synthesis, activation and release of IL-1 after IL-1R1 stimulation. (Dinarello 2011)
β can be processed by ICE in the cytosol and trafficked out with the involvement of ATPase family member Rab39a, which is cleaved and associate with caspase-1 Rab39a is essential for IL-1β release upon LPS stimulus. (Becker, Creagh et al. 2009). Panx-1 channels are important in this release as inhibition of these channels affects IL-1β release and not cathepsin –D release, which is dependent on secretory lysosome release. (Pelegrin and Surprenant 2006, Qu, Franchi et al. 2007). (15) The mature IL-1β is released through microparticles belbbing and exosome exocytosis.

It is proposed that above mentioned modes of release are not mutually exclusive. The release of IL-1β takes place a continuum and is dependent on the potential and the duration of the inflammatory stimulus and contingent on the host cell (Lopez-Castejon and Brough 2011). As discussed earlier. IL-1β release might take any number of routes and they are basically grouped in three ways (Figure 1-10) 1) Rescue and release form the lysosomal degradation, 2) protected release via microparticles and exosomes and 3) terminal release. The Intensity and the duration of the stimulus influences the rate of employment of these non-classic routes of IL-1β secretion (Lopez-Castejon and Brough 2011). Autophagy is a major mechanism through which proteins and damaged organelles are targeted for degradation. These vesicles are called autophagosomes and upon fusing with lysosomes form autolysosomes and leads to protein digestion. Sometimes proteins escape their fate and reenter the cytosol and are rescued. Pro - IL-1β is actively recruited in to the autophagosome (Andrei, Dazzi et al. 1999, Blott and Griffiths 2002, Harris, Hartman et al. 2011) upon LPS stimulation, and inhibition of autophagy leads to increased release of IL-β. The monocyte a prominent source of IL-1β secretes this cytokine via secretory lysosomes.
Figure 6 - Mechanism of IL-1β release three distinct modes of IL-1β release dependent on the intensity of the stimulus explained in nucleated cells – 1) Endosomal degradation rescue and redirect 2) protected release along with microparticle and exosome shedding, 3) terminal release upon inflammatory apoptosis of the target cell Figure adapted from (Lopez-Castejon and Brough 2011).

1.14 MICROPARTICLES:

Microparticles (Mps) are phospholipid bilayer membrane vesicles derived from plasma membrane shed upon stimulation (Figure-1-10). These particles measure around 100 – 1000 nm and make up a component of the healthy circulatory system and increase in their levels during inflammation. Increased levels of circulating Mps are present in almost all autoinflammatory diseases e.g. atherosclerosis, coronary artery disease, active
multiple sclerosis, type-2 diabetes, sickle cell anemia, collagen induced arthritis (Knijff-Dutmer, Koerts et al. 2002, Andoh, Tsujikawa et al. 2005, Nomura, Inami et al. 2008, Boilard, Nigrovic et al. 2010, Burger, Schock et al. 2013). Almost all cells are capable of shedding Mps and the extent of shedding is dependent on the agonist involved. Mps were first described as “Platelet Dust” and Platelet derived Mps (PMP) make up the bulk of the circulating microparticle population, followed by endothelial cell Mps (EMP) (Wolf 1967, Gyorgy, Szabo et al. 2011). Microparticles are an important tool for the generating cell to affect its surrounding environment locally and in systemically distinct areas, as the membrane enclosed molecules are protected from destruction. Thus Mps enter the circulation to act elsewhere. The content of the Mps vary immensely dependent on the parent cell and the agonist. Immune cells package their Mps with a range of inflammatory cytokines and chemokines.

PMPs are involved in the pathogenesis of many disorders and diseases e.g. acute coronary disease, (Mallat, Benamer et al. 2000, Nozaki, Sugiyama et al. 2009), atherosclerosis (Michelsen, Noto et al. 2009), immune thrombocytopenic purpura, sickle cell disease, renal fibrosis, Alzheimer’s disease, type-2 diabetes (Koga, Sugiyama et al. 2006), arthritis (Knijff-Dutmer, Koerts et al. 2002, Boilard, Nigrovic et al. 2010), systemic sclerosis (Nomura, Inami et al. 2008) and much more. The common theme in all of the above mentioned conditions is the involvement of IL-1β in their development and progression. Bioactive IL-1β released along with the Mps have been demonstrated in many cells like THP-1 monocytes (MacKenzie, Wilson et al. 2001, Wang, Williams et al. 2011), murine microglia (Bianco, Pravettoni et al. 2005) and dendritic cells (Baroni,
Pizzirani et al. 2007, Pizzirani, Ferrari et al. 2007) and in platelets as shown by McIntyre et al. (Brown and McIntyre 2011, Brown, Narayanan et al. 2013).

The association of IL-1β with shed microparticle is dependent on the stimulating agonist. Platelet stimulation with LPS and thrombin induced IL-1β inclusion in the PMPs which is not observed in ADP shed PMPs (Weyrich, Elstad et al. 1996, Brown, Narayanan et al. 2013). Monocyte derived IL-1β laden Mps activate Human Umbilical Vein Endothelial Cells (HUVECs) in an IL-1R1 dependent manner (Wang, Williams et al. 2011). The importance of MP bound IL-1β, especially PMPs is important and is recognized in the development of collagen mediated arthritis (Boilard, Nigrovic et al. 2010), inflammatory bowel disease (Andoh, Tsujikawa et al. 2005), Systemic Lupus Erythematosus (Sellam, Proulle et al. 2009) and dengue fever (Hottz, Lopes et al. 2013).

1.15 EXOSOMES:

Exosomes (Exos) were first identified as being released form antigen presenting cells and immune cells (B-cells, T-cells) and defined as specialized microparticles. They later were shown to be shed by platelets as well (Heijnen, Schiel et al. 1999). Exos have been isolated from body fluids like blood, urine, breast milk, saliva and seminal fluid (Thery, Zitvogel et al. 2002, Thery, Ostrowski et al. 2009). Recent development in the field of exosomes has identified their role in immune response. (Figure-1-11) Exos are different form Mps in many aspects – they rise from a different compartment, they range from 50-100 nm in size and are released both constitutively and also upon stimulation (Figure-1-10). They are released upon exocytosis of Multivesicular bodies (MVBs), an
intermediate in the endo-lysosome secretory pathway (Gyorgy, Szabo et al. 2011). Exos are formed through invagination of endosomal membrane in MVBs and are released into the endosome lumen. Similar to MP release, a rise in Ca$^{++}$ ion intracellular leads to MVB fusion with the plasma membrane and the release of its contents. Rab GTPases, rhoA effectors and Rab 27 are involved in the fusion process (Yu, Harris et al. 2006, Raposo and Stoorvogel 2013). All Exos, despite the source, contain endosomal protein and lipid raft associated proteins like Rab GTPases, SNAREs, glycosylphosphatidylinositol-anchored protein (GPI), flotilin, annexins and tetraspanins. Exosomes are surrounded by phospholipid bilayer and their contents, similar to Mps, vary depending on the secreting cell and agonist (Baj-Krzyworzeka, Majka et al. 2002). Annexin-V binding, CD63, CD81, CD9 and LAMP1 are common markers used for identification (Chaput and Thery 2011, Gyorgy, Szabo et al. 2011). Exos are smaller and can’t easily be detected using flow cytometry. Western blotting, mass spectroscopy and transmission electron microscopy are the standard methods of detection.

Exosomes participate in wide array of biological process, but are best characterized for their immunoregulatory role of antigen presentation, as well as immunostimulatory and inhibitory functions (Raposo, Nijman et al. 1996, Denzer, Kleijmeer et al. 2000, Thery, Ostrowski et al. 2009). The exosome interchange is an important pathway of intercellular communication as they transfer mRNA, miRNA, receptors, cytokines, HIV particles (Valadi, Ekstrom et al. 2007, Izquierdo-Useros, Naranjo-Gomez et al. 2009) and much more through direct interaction with the surface molecules, endocytic and cell membrane fusion proteins (Thery, Ostrowski et al. 2009, Silverman and Reiner 2011, Raposo and Stoorvogel 2013).
Exos along with Mps play an important role in the pathophysiology of sepsis development. Many studies have shown the association between increased Exos in circulation and vascular complications and regulation of cardiovascular events in sepsis. These Exos were primarily of platelet origin and contain membrane NADPH oxidases and are able to produce ROS (Janiszewski, Do Carmo et al. 2004, Gambim, do Carmo Ade et al. 2007). Through this ability, Exos induce apoptosis in vascular smooth muscle cells and endothelial cells via oxidative stress. They mediate contractile dysfunction in the heart through a NO-mediated mechanism upon LPS treatment in mouse models (Azevedo, Pedro et al. 2007). Exos are essential in the mechanism of leaderless protein (LLS) protein IL-1β release. The pool of IL-1 directed to digestion is contained in the MVBs and are secreted upon stimulus to achieve an increase in cytokine levels when needed. This is one of the tools to prolong the availability of IL-1β as the half-life is mere minutes in circulation (Qu, Franchi et al. 2007, O'Keefe, Shelton et al. 2008). Exos are shown to be shed by immune cells like dendritic cells, and monocytes upon stimulation of surface receptors like P2X7, TLR4, TNFR, PAR-1 and others (Heijnen, Schiel et al. 1999, Qu, Franchi et al. 2007, Lachenal, Pernet-Gallay et al. 2011). Dendritic cells have been shown to shed IL-1β associated exosomes upon stimulation of P2X7 receptor and NLRP-3 inflammasome activation (Qu, Franchi et al. 2007).

Terminal release is the final possible mechanism of IL-1 release. Caspase-1 activation has been shown to cause decreases in membrane potential, resulting in cell death upon NLRC-4 activation with pathogenic bacteria. IL-1 is released through this pro-inflammatory cell death called Pyroptosis. Caspase-1 activation leads to formation of pores and in turn facilitates IL-1β release (Hogquist, Nett et al. 1991). LPS and ATP
stimulus after LPS priming has shown to cause pyroptosis in T-cells and macrophages (Perregaux and Gabel 1994, Miao, Leaf et al. 2010).

The central hypothesis of this thesis is that platelets express the IL-1R1 and that IL-1β affects platelet function. The secondary hypothesis is that the mode of IL-1β release is contingent on the inducing agonist. In this study I have shown that platelets express functional IL-1R1 and upon endotoxin stimulation of TLR-4 or stimulated by complete agonists, like thrombin and collagen synthesize IL-1β protein. The biologically active cytokine is released along with shed particles. IL-1R1 is expressed in these particles and the presence of its receptor is essential for active IL-1β binding to the surface of the MPs. (Chapters 3 and 4). Platelets contain all the major inflammasome components and P2X7 and Pannexin-1 (Panx-1) ion channels to carry out the proposed model of IL-1β processing and secretion similar to nucleated cells. Thus Platelet IL-1R1 signaling links thrombosis to inflammation and proposes platelets as the integral part of this association. (Chapter 5)
CHAPTER II
RESEARCH METHODOLOGY

2.1 Cell isolation

Human blood was drawn from healthy donors through the Cleveland Clinic blood bank. One unit (~420ml) of whole blood was collected in to acid-citrate-dextrose (ACD, 38 mM citric acid, 107 mM sodium citrate, 136 mM dextrose), that serves as anticoagulant. Washed Platelets were isolated from platelet rich plasma (PRP) following procedures approved by the Cleveland Clinic IRB. PRP was separated from whole blood upon centrifugation (200 X g, 20 min). Nucleated cell contamination was removed through filtering the separated PRP through double layered 5-µm mesh (BioDesign) twice. Platelets were isolated upon centrifugation (500 X g, 30 mins) from the nucleated cell free filtered PRP. The pelleted platelets were re-suspended carefully in 50 ml of PSG buffer (5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 μM Na₂HPO₄, 1 mM MgCl₂, and 5.5 mM glucose) and washed platelets were obtained after centrifugation (500 X g, 30
mins). The platelets were re-suspended in PSG before once again and centrifuged to remove any plasma contamination. All these processes were performed at room temperature, in the presence of 100 nM of Prostaglandin E₁ (PGE1) to avoid platelet activation. Finally, the washed platelets were re-suspended in 0.5% human serum albumin in Hank’s balanced salt solution (HBSS) - HBSSA at the concentration of 8 x 10⁸ platelets/ml for experimental purposes. Platelet were activated either by LPS (100ng/ml) along with human recombinant LPS- binding protein - LBP (100 ng/ml) and CD14 (100 ng/ml), Thrombin (0.1U), collagen (2µg/ml), IL-1β (100 pg/ml). In the case of change in the concentration of the agonist, the dosage is mentioned in the legend.

Murine studies were approved by the Cleveland Clinic IACUC. Mouse whole blood was obtained with the assistance of Dr. Wei Li, via hepatic artery puncture and collected in anticoagulant ACD containing tubes and the PRP was centrifuged (100 X g, 10 mins) in the presence of PGE1 for platelet isolation. PRP was made up twice its volume with sterile Phosphate buffered saline (PBS) containing PGE1 and centrifuged at (500 X g, 10 mins) to separate the platelets and then re-suspended in 0.5% HBSSA and used for further treatments.

2.2 Microparticle and exosome isolation

Platelets were treated with said agonist for overnight (~14-18 hrs) at 37°C. Whole cells were pelleted after centrifugation (300 X g, 15 mins), twice. The supernatant is then utilized to isolate microparticles (MPs) and exosomes (Exos) through differential centrifugation at 17, 000 x g for 90 min, followed by 110,000 x g for 2hrs respectively.
MPs and Exos were re-suspended in sterile PBS prior to treating cells or lysed with 10 X cell lysis buffer (Cell Signaling). The MP and exo utilized in the treatments were normalized to their protein content - 125µg/ml of Mps and 100µg/ml of exos were used in experiments unless mentioned in the legends.

2.3 Microparticle quantification

Microparticles were isolated from 2 X 10^8 washed platelets after treatment. Platelet free supernatant was centrifuged at 17,000 g for 90 mins then fixed with 2% paraformaldehyde (10 mins) and then either permeabilized with 0.1% Triton X100 (10mins) or left unpermeabilized. The MPs were washed (20,000 X g, 1 hr) and then utilized for flow cytometry analysis. U-V flour tagged 2.5µM (Invitrogen) polystyrene latex beads were used as internal standard with each sample. Mp population was gated using forward and side scatter plots obtained from 1µm beads (Sigma-Aldrich) run at the same settings in the BD LSR-II flow cytometer.

2.4 Western blotting

Either washed platelets were treated with specified treatments or microparticles and exosomes isolated were lysed using 1X cell lysis buffer (Cell Signaling) and the protein concentration was obtained using bicinchoninic acid (BCA) reagent. 25 µg of protein were resolved through SDS-PAGE and immunoblotted for specific protein of
interest. PVDF blots were blocked with 5% nonfat milk (1hr) followed by primary detection antibody (1:1000, overnight, 4°C) and then blotted with appropriate secondary antibody (1:10,000, 90 mins). The blots were developed using ECL-prime western blotting system (GE healthcare).

2.5 Mouse thrombosis

Thrombosis was induced in C57Bl/6 mice in the left carotid artery through ectopic exposure to filter paper saturated with 7.5% FeCl3 for 1 min (Baumgartner and Born 1968). Platelets were fluorescently labeled with 100 μl rhodamine 6G (0.5 mg/ml) prior to injection into the right jugular vein. Mice were anesthetized with ketamine (100 mg/kg, 10 mg/kg xylazine) before the initiation of the thrombosis. Thrombus formation was observed in real-time under a water immersion objective at 10 X magnification by intra-vital microscopy. Upon occlusive thrombus formation determined by the complete cessation of blood flow on the other side of the thrombus, then the section of the artery along with the thrombus is excised and embedded in paraffin. 6 μm sections of thrombus were obtained and used for confocal microscopy. In some cases, prior to FeCl3 induced thrombosis mice were treated with actinomycin D (0.6 mg/kg) through intraperitoneal injection for 30 min (Schwartz, Sodergren et al. 1968) to inhibit transcription. Whole blood was drawn from actinomycin D treated and control mice, 90 min after injection. Before the whole blood sample was stimulated with LPS for 60 min before the leukocytes were isolated to determine their ability to synthesize IL-1β mRNA through RT-PCR.
2.6 CONFOCAL MICROSCOPY

2.6-1 Mouse Thrombus Staining:

Paraffin embedded murine arterial thrombus along with the vessel was provided by Dr. Wei Li. These sections were blocked with 1% Bovine serum albumin (BSA) and 10% goat serum and then stained for immufluorescence microscopy or immunohistochemistry. The endogenous peroxidase was inhibited (Thermo Scientific), and IL-1β stained with rabbit polyclonal (1:250) Bcl-3 (1:250) or isotype IgG1 nonimmune (Santa Cruz Biotechnology). The primary antibody was detected using a Pierce peroxidase immunohistochemistry detection kit with metal-enhanced diaminobenzidine generation (Thermo Scientific) and counter stained with hematoxylin. In this immunofluorescence technique IL-1β primary antibody was detected with Alexa 488–conjugated goat anti-rabbit antibody (1:1000; Invitrogen, CA11098). Platelet membranes were visualized with Alexa Fluor 594–conjugated wheat germ agglutinin (5 mg/ml) or anti–P-selectin. The sections were mounted in Vectashield (Vector Laboratories) mounting media containing DAPI for confocal microscopy and plain mounting medium for immunohistochemistry. A composite merged image was formed using Image-Pro plus 6.1.

2.6-2 NFκB p-65 translocation:

Human umbilical vein endothelial cells (HUVECs) were generously provided from Dr. Keith McCrea and Dr. Paul DiCorleto’s laboratories. The HUVECs were grown in 8 well chamber slides coated with fibronectin (1µg/ml). The cells were fixed with 4%
para formaldehyde after treatment. Permeabilized with 0.1% Triton X 100 (10 mins) and blocked with 5% BSA. The NFκB p-65 subunit was stained with rabbit polyclonal primary antibody (1:100) and detected using anti rabbit alexa flour -488 secondary antibody (1:1000). The slides were mounted in DNA staining DAPI containing mounting medium and analyzed using a LEICA TCS-SP upright confocal microscopes. Superimposition of images from the blue and the green channels provide the nuclear translocation of p-65 subunit.

2.7 Total internal reflection fluorescence microscopy (TIRF)

Platelet-derived microparticles were stained with cleaved IL-1β monoclonal antibody and detected with Alexa488-conjugated secondary antibody. The stained Mp suspension was incubated in a glass bottomed microwell (MatTek) dish for 15 min before imaging at 100X by Total Internal Reflection Microscopy with a 1.46 N.A. objective in a Leica AM TIRF MC System (Leica Microsystems, Wetzlar, Germany) equipped with an ImageEM C9100-13 EMCCD camera (Hamamatsu, Bridgewater, N.J). The 10-mW diode laser was used for excitation and the penetration depth was set to 90 nm.

2.8 IL-1β ELISA

Mps and exos were isolated from platelets treated with either LPS (100 ng/ml) or thrombin (0.1U) overnight and the fractions free supernatant was saved. The IL-1β content of the different fractions was quantified using human IL-1β/IL-F2 duoset (R&D
Systems). The samples were processed according to the kit instructions and the level of IL-1 cytokine was determined using micro plate readers. The IL-1β content was expressed as pg/ml.

2.9 HUVEC E-selectin Assay.

HUVECs at passage 1 were seeded into 96 well plate coated with 0.1% gelatin and grown overnight at 37°C until 75-80% confluence. Cells were serum starved in 1% M-199 medium for 2 hrs. The cells were treated with IL-Ra (200 ng/ml), AF12198 (2 µM) or dynasore (40 µM) prior to the agonist treatment. HUVECs were stimulated with IL-1β (1ng/ml), TNF-α (10 ng/ml), LPS (1 µg/ml) as controls. The cells were fixed with 4% paraformaldehyde after 6 hours of incubation. The surface expression of E-selectin was assessed using mouse primary antibody (1µg/ml) and followed by HRP conjugated anti mouse secondary antibody. The E-selectin levels were detected using TMB substrate and the O.D. was determined using a plate reader. The level of E-selectin expression is represented as fold increase over the treatments in buffer.

2.10 Chemicals and reagents

Chemicals and reagents were purchased from the following sources: sterile filtered HBSS and M199 (BioWhittaker); sterile tissue culture plates; (Falcon Labware); human serum albumin; (Baxter Healthcare); endotoxin-free PBS; phenol-extracted LPS
(Escherichia coli O111:B4) that is free of lipoprotein contamination (List Biological Laboratories); recombinant LBP, recombinant soluble CD14, IL-1Ra and IL-1β ELISA kit, (R&D Systems); The mouse anti-IL-1β antibody in Western blot analysis (Sc-52012) and cleaved IL-β human (H117- Sc-23460) and mouse specific (m118 – Sc 71017) utilized in flow cytometry analysis were purchased from Santa Cruz. Anti ASC (Sc-11234), P2X7 (Sc-15200), Panx-1 (Sc-49695) were purchased from Santa Cruz. NLRP-3 and IL-1R1 antibody were purchased from R&D Systems. Aim-2, IL-1Ra and caspase-5 antibodies were purchased from Abcam. Caspase-1 inhibitors Z-WEHD-FMK, Z-YVAD-FMK (FMK002) and pan caspase inhibitor –Z-VAD-FMK were obtained from R&D Systems. Inhibitors A438079 and Af12198 were obtained from Tocris Biosciences. Other chemicals were from Sigma-Aldrich or Biomol Research Laboratories.

2.11 Expression of data and statistics

Experiments were performed at least three times with cells from different donors, and all assays were performed in triplicate. The standard deviation or standard errors of the mean from all experiments are presented as error bars. Graphing of figures and statistical analyses were generated with Prism4 (GraphPad Software) using either Student’s T test or one way analysis of variance (ANOVA) when appropriate. A value of p < 0.05 was considered statistically significant.
CHAPTER III

Platelet IL-1R1 signaling – A link between Sterile Inflammation and Thrombosis.

Introduction and rationale

Interleukin -1 beta (IL-1β) is the predominant proinflammatory cytokine involved in the pathology of numerous acute and chronic inflammatory conditions (Qu, Franchi et al. 2007, Dinarello 2009). IL-1β propagates the inflammatory cycle via activating the innate immune cells and endothelium in these conditions, but also can contribute to major complications. Monocytes are the primary source of IL-1β where this cytokine is produced as a leaderless proprotein. Inactive pro-protein IL-1β is transformed into an active cytokine through caspase-1 - mediated proteolytic cleavage, where caspase-1 in itself must first be activated in a similar fashion through inflammasome complex mediated proteolytic cleavage (Dinarello 2009, Dinarello 2011). The mechanism of
release of this leaderless cytokine lacking any prominent secretory sequence is yet to be fully understood. The release of Pro-IL1β and activated IL-1β from activated nucleated cells is proposed to take place through different routes. Activated cells like monocytes and endothelial cells shed IL-1β laden microparticles (MacKenzie, Wilson et al. 2001, Brown and McIntyre 2011, Wang, Williams et al. 2011, Wang, Aikawa et al. 2013) and this cytokine is included in exosome (Qu, Franchi et al. 2007) cargo as well, along with direct release in to the extracellular milieu (Eder 2009, Lopez-Castejon and Brough 2011). Nucleated cells produce IL-1β upon TLR-4 activation with LPS (Dinarello 2009) stimulus, as well as responding with production of active IL-1β via activation of its own receptor IL-1R1 (Dinarello, Ikejima et al. 1987, Warner, Auger et al. 1987).

Platelets are anucleate simple cells, but are capable of performing far greater tasks than originally realized. They contain preformed heteronuclear mRNA (hnRNA) for IL-1β, Bcl-3, and tissue factor and others that are transferred from their megakaryocyte progenitors during platelet generation. Platelets possess functional spliceosome complexes that are capable of splicing hnRNA upon stimulation (Weyrich, Elstad et al. 1996, Weyrich, Dixon et al. 1998, Weyrich, Denis et al. 2007). Platelets are gaining recognition as an important cell type involved in inflammation as well as in innate immune system dysfunction mediated diseases. Many studies, along with previous publications from our group, have evaluated the involvement of platelets in endotoxin-mediated inflammation. Platelets are the abundant inflammatory cell in the circulation and their strong involvement in the pathology of many of the autoinflammatory conditions puts them in focus as primary targets to counteract these manifestations. TLR-4 expression and function in platelets is now well documented (Shashkin, Brown et al.
Stimulation of TLR-4 on the platelet surface with LPS results in increased IL-1β hnRNA splicing and translation accompanied by release of IL-1β laden microparticles. This process requires sequential activation of kinase cascade and active cytokine release that is dependent on caspase -1 mediated proteolytic cleavage of pro-IL-1β into its active form (Shashkin, Brown et al. 2008, Brown and McIntyre 2011). LPS causes a robust cytokine response in platelets but is an atypical platelet agonist since TLR-4 activation does not induce any classic platelet activation patterns of adhesion, aggregation, calcium release or P-selectin mobilization from the α-granules to the platelet surface (Ward, Bingle et al. 2005, Shashkin, Brown et al. 2008, Zhang, Han et al. 2009). Among classic platelet agonists thrombin alone has been shown to modulate IL-1β mRNA transcript splicing in platelets (Shashkin, Brown et al. 2008).

TLR-4 and IL-1R1 contain TIR domains that are essential for their function. They also share downstream signaling molecules like MyD88, IRAKs, Traf6, and various MAP kinases (Cao, Xiong et al. 1996, Weber, Wasiliew et al. 2010, Yang, Wang et al. 2010). Due to these commonalities, the biological responses through TLR-4 and IL-1R1 activation are indistinguishable (O'Neill 2008, Weber, Wasiliew et al. 2010, Yang, Wang et al. 2010). Interleukin-1 family members and IL-1β in particular, lay at the center of development of many sterile inflammatory diseases (Abramson and Amin 2002, Dinarello 2009, Dinarello 2011). Platelets express TLR-4 but the presence IL-1R1 in platelets is yet unknown. Platelets possess the necessary machinery to synthesize IL-1β in response to LPS - mediated TLR-4 activation. Therefore, they contain the necessary downstream signaling components. IL-1R1 and TLR-4 receptors display a very close relationship and the dynamic crosstalk between these receptors has been documented in
nucleated cells (Kawagoe, Sato et al. 2008, O'Neill 2008, Yang, Wang et al. 2010). Thus we hypothesized that should platelets express IL-R1 receptor then it would be functional.

Activated platelets are capable of producing IL-1β \textit{in vitro} (Weyrich, Elstad et al. 1996, Shashkin, Brown et al. 2008). But in \textit{in vivo} IL-1β secretion is yet to be established. IL-1β is an integral part of disease pathology in endotoxin - mediated inflammation as well as in sterile inflammation, where a source of bacterial infection is absent. Many studies (Stark, Aghakasiri et al. 2012, Zhao, Su et al. 2013) along with previous publications from our group, have evaluated the involvement of platelets in endotoxin mediated inflammation (Shashkin, Brown et al. 2008, Brown and McIntyre 2011). The platelet role in sterile inflammation is yet unknown. Activated platelets isolated from septic patients show an altered transcriptome (Rondina, Brewster et al. 2012), but are cleared rapidly from the circulation. Therefore, following activated platelets in circulation would be difficult. We therefore sought to explore the activated platelet presentation in a large mass. Occlusive thrombi are formed upon accumulation of activated platelets and the transcriptome of platelets in this mass would show any such activation.

This chapter shows the presence of IL-R1 on the platelet surface and demonstrates that this receptor is functional. As a co-author of the publication that resulted from this work, I showed that platelet IL-1R1 stimulation is essential for LPS induced IL-1 response in sepsis. This study establishes that platelets produce and respond to this IL-1β through IL-1R1 and thus display an auto-stimulatory loop. IL-1R1 provides a mode of amplification of the LPS - mediated TLR-4 signaling response. The role of platelet IL-
1R1 activity under sterile conditions *in vivo* was analyzed during occlusive thrombus formation in wild type mouse models. These observations connect sterile thrombosis with proinflammatory IL-1β production and establish platelets as the major source of this cytokine.

This chapter comprises of findings contributed by me extracted from the co-author publication published in the Journal of immunology along with Dr. G. Thomas Brown (Brown, Narayanan et al. 2013). The relevant works of the co-author are identified and the credits are reported as required.

**RESULTS**

3.1 – Platelets express Interleukin 1 Receptor - 1 (IL-1R1)

Platelets express functional TLR-4 and in response to LPS induce a MyD88-dependent TLR-4 signaling cascade via the TLR-4-TIR domain. TLR-4 and IL-1R1 share structural similarities, TIR domains, and downstream signaling molecules. Moreover, IL-1R1 is an important signaling pathway involved in inflammation. Thus, we were interested to investigate the presence of IL-1R1 on the platelet surface. The expression status of IL-1R1 on human platelets was explored through flow cytometry (*Figure 7A*) and the observation was further confirmed through immune (western) blotting where unstimulated platelet lysates were resolved in acrylamide gels and probed
for the presence of IL-1R1 and the non–signaling decoy receptor IL-1R2. Monocytes were used as the positive control. (Figure 7B).

Platelets showed significant surface expression of IL-1R1 by flow cytometric analysis and also possessed the protein product of correct size similar to monocytes, upon Western blot analysis. They lacked IL-1R2 expression, and CD14 monocyte marker expression used to detect contamination in the platelet preparation, but expressed the platelet specific marker CD62b. Monocytes expressed both IL-1R2 and CD-14, but were negative for CD42b. Hsp90 and β-Actin were used as loading control.

![Figure 7 - Platelets express IL-1R1 - A) flow cytometry analysis – unstimulated platelets were stained with unconjugated primary antibody against IL-1R1 or IL-1R2 and detected with Alexa flour 488 conjugated secondary antibody (II°). B) Platelet and monocyte lysates were loaded in increasing quantity, resolved by SDS-PAGE and Immunoblotted for the presence of IL-1R1 (64kD) and absence of IL-1R2 (48kD) in platelets. CD42b and CD14 are used as positive and negative control.](image)
3.2 - Platelets express functional IL-1R1. (Experiments performed by G. T. Brown Co-author)

In nucleated cells the IL-1β cytokine response is carried out through TLR4 ligand activation as well as through functional IL-1R1 activation (Dinarello, Ikejima et al. 1987, Warner, Auger et al. 1987). We analyzed whether the same was true in the case of human platelets. The splicing of IL-1β hnRNA, as observed upon successful LPS-TLR-4 signaling in platelets was used as a positive readout. Freshly isolated and purified washed platelets were stimulated with human recombinant active IL-1β protein for 3 hours. At t=0 no processed mRNA was observed while after 3 hours of stimulation an accumulation of processed IL-1β mRNA was observed showing the activity of the platelet IL-1R1 receptors (Figure 8). IL-1R1 binds both IL-1α and IL-1β and results in successful signaling, and this activation is lost upon competitive binding of the endogenous receptor antagonist IL-1Ra (Anrakinra) (O’Neill 2008, Weber, Wasiliew et al. 2010). Stimulation with both the recombinant ligands activates the platelet IL-1R1 resulting in increased IL-1β mRNA splicing recorded using quantitative RT-PCR reactions (Figure 3-2a). Pretreatment of the washed platelets with IL-1Ra negates the activation of IL-1 receptor by both the ligands, thus confirming the accumulation of IL-1β transcripts were indeed through IL-1R1 activation. Surprisingly, IL-1Ra treatment decreased both autoactivation of resting platelets and also affected negatively the LPS mediated TLR-4 response as well. This observation of IL-1Ra inhibition of TLR-4 mediated signaling shows the probable crosstalk between the IL-1 and TLR-4 receptors and presence of a positive feedback loop between these receptors. IL-1β - laden
microparticles are released upon LPS platelet stimulation (Brown and McIntyre 2011) and this resultant IL-1β activates its functional surface receptor IL-1R1 on platelets. Thus, IL-1R1 - mediated signaling expands the LPS stimulated TLR-4 signaling (Figure 8a). This IL-1R1 signaling is MyD88 dependent. Upon ligand binding, the TIR domain of activated IL-1R1 recruits MyD88 and commences the IRAK complex formation and downstream signaling activation (Cao, Xiong et al. 1996, Kawagoe, Sato et al. 2008, Weber, Wasiliew et al. 2010). Disruption of this recruitment with cell permeable MyD88 antenapedia peptide, results in loss of function of this receptor (Adachi, Kawai et al. 1998). Thus IL-1R1 mediated rIL-1β signaling is disrupted. The decrease in activation is comparable to the inhibition of IL-1R1 through its antagonist binding (IL-1Ra). Both Myd88 inhibitor and IL-1Ra pretreatments significantly diminish the rIL-1β induced hnRNA splicing. This proves the expected requirement of MyD88 to carry out both IL-1 and TLR-4 mediated signaling holds true in platelets as well. (Figure 8b) Data from Dr. Brown’s experiments show that the shedding of active IL-1β laden microparticles was caspase-1 activity dependent. Upon inhibition of caspase-1 activation in human platelets with cell permeable small molecule inhibitors like Z-WEHD-FMK, Z-YVED-FMK and total loss of gene function in caspase-1 knockout platelets failed to respond to soluble rIL-1β stimulation. Inhibition of protein synthesis with pretreatment with cyclohexamide resulted in stunted response to LPS stimulation, which was not observed in the presence of exogenous rIL-1β (Data not shown). Human platelets produced IL-1β laden microparticles and responded to freshly isolated microparticles from LPS stimulated platelets in an IL-1R1 dependent pattern. Thus cross talk between TLR-4 and IL-1R1 signaling pathways is essential for the optimal LPS mediated platelet response, and this
activity is contingent upon production of active translation of spliced IL-1β mRNA and activation of the translated procytokine in to its active form through caspase-1 dependent proteolytic cleavage.

**Figure 8** - Platelet IL-1R1 is functional – A) rIL-1α & rIL-1β activate platelet IL-1R1- Highly purified washed human platelets were treated with LPS(100 ng) along with LBP(lipid binding protein) and CD14(150 ng) or IL-1α or IL-1α (100 pg) respectively. All these reactions were carried out with (white bars) or without (gray bars) the presence of IL-1R-antagonist (IL-1Ra). Resultant hnRNA splicing was determined after 3 hours of treatment by quantitative – RT-PCR. N=5 B) IL-1β mediated IL-1R1 activation is MyD88 dependent. Purified platelets (autoMACs 2X) were treated with IL-1β (100 pg), IL-1Ra (150 ng), or with IL-1β after pretreatment with IL-1Ra or MyD88 inhibitor (30 mins) and the accumulation of spliced IL-1β mRNA was determined through quantitative –RT-PCR. N=3. Significance attained using one-way ANOVA and p value <0.05.
3.3 – Lipopolysaccharide (LPS) is an atypical agonist

Classic activation of platelets is characterized by key events like calcium mobilization, shape change, increased P-selectin mobilization from the alpha-granules to the surface and increased adhesion and aggregation. These events are observed upon activation with strong agonists like thrombin, ADP, collagen and other classic platelet agonists (Hartwig 2006, Passacquale and Ferro 2011, Shaturny, Shakhidzhanov et al. 2014). LPS fails to initiate these principle platelet functions. LPS activation does not cause an increase in platelet aggregation, but instead just potentiates the activation upon classic agonist treatments (Shashkin, Brown et al. 2008, Zhang, Han et al. 2009). Thus LPS could be termed as an atypical platelet agonist.

Calcium mobilization is the important event during platelet activation that occurs as early as few seconds after agonist treatments (Passacquale and Ferro 2011, Shaturny, Shakhidzhanov et al. 2014). Calcium mobilization is frequently determined using a cell permeable fluorescent dye, and Fura-2 is widely used to assay intracellular calcium release (Rink and Sage 1987, Sage and Rink 1987, Sage, Adams et al. 1989). Washed platelets were pre-stained with Fura-2 and the release of calcium ions were measured upon excitation and the ratio of 340/380 corresponds to the amount of Ca^{++} released over a period of time represented in minutes. Thrombin, as previously shown in numerous studies, was very efficient in increasing the intracellular Ca^{++} levels at concentrations as low as 0.05 U/ml. LPS either with or without the presence of exogenous CD14 failed to induce significant Ca^{++} mobilization from intracellular compartments (Figure 9a). Next
the rate of aggregation was measured using an aggregometer. Washed platelets stimulated with thrombin (0.5 U) displayed significant homotypic aggregation, but LPS treatment did not cause measurable platelet aggregation (Figure 9b). Thus LPS is an atypical platelet agonist that fails to induce classical platelet activation events like calcium mobilization and aggregation. LPS isolated from two different sources *E.coli* and *S. minneasota* are capable of activating TLR-4 signaling cascade (Kappelmayer, Beke Debreceni et al. 2013). Their ability to activate platelet TLR-4 was analyzed by looking at the rate of AKT phosphorylation, which is shown to be essential in the platelet TLR-4 signaling in our previous studies (Brown and McIntyre 2011). Both the LPS sources were able to increase AKT phosphorylation as early as 2 minutes upon stimulation, which is comparable to the thrombin treated positive control (figure 9c).

3.4 - IL-1β is an atypical platelet agonist

IL-1β is a well-known proinflammatory cytokine and binds its receptor IL-1R1 with high affinity (Pope and Tschopp 2007, Dinarello 2011) and is an efficient mediator of inflammation even at lower concentrations (van der Meer, Barza et al. 1988). The expression of IL-1R1 on the platelet surface is a novel finding and the effect of this cytokine in platelets is yet unexplored. IL-1R1 mediated platelet activation upon IL-1β binding was investigated. Increases in P-selection expression and the appearance of activated gpIIb/IIIa epitopes are significant early events, along with previously
mentioned calcium release, which together with aggregation defines the ability of agonist to induce platelet activation (Shashkin, Brown et al. 2008, Gupta, Li et al. 2014,
Shaturny, Shakhidzhanov et al. 2014) IL-1β stimulation, similar to LPS stimulation, fails to activate classic platelet response. Flow cytometry analyses of thrombin treated platelets showed marked increase in surface P-selectin expression while no notable increase in P-selectin expression is observed upon rIL-1β mediated IL-1R1 activation (Figure 10a).

Figure 10 - IL-1β an atypical Platelet Agonist. A) Platelets upon a brief agonist treatment were stained with FITC conjugated anti P-selectin antibody and the histogram expression pattern of different treatments are superimposed on each other to show the difference B) FITC conjugated PAC-1 and analyzed under flow cytometry. The data is represented as histogram.

Therefore IL-1β, similar to LPS, is an atypical agonist as it fails to drive P-selectin expression upon platelet stimulation (Shashkin, Brown et al. 2008). PAC-1 antibody binds an activated gpIIb/IIIa epitope and is employed to ascertain the activation status of platelets. IL-1β, similar to LPS, was unable to increase the PAC-1 antibody
binding to the platelet surface (Figure 10b). These histograms suggests that IL-1β is an atypical platelet agonist capable of activating platelets in pathways independent of the classical activation cascade involved in thrombosis.

3.5 Accumulation of IL-1β with in sterile thrombi.

Platelets store preformed cytokines and hnRNA ready to be spliced upon activation and immediate secretion (Shashkin, Brown et al. 2008, Brown and McIntyre 2011, Brown, Narayanan et al. 2013). Surprisingly, many of the mRNAs of these cytokines are preloaded on to the ribosomes leading to an immediate and rapid release mechanism in platelets. Activated platelets translate constitutive mRNA immediately for, IL-1β and Bcl-3 to carry out continued secretion of these proteins upon prolonged duration (Weyrich, Dixon et al. 1998, Pabla, Weyrich et al. 1999, Lindemann, Tolley et al. 2001). The role of platelet IL-1R1 in IL-1 response during TLR-4 stimulated in vitro in human platelets was unexplained untill now. But IL-1β is known to be involved in development of inflammatory conditions also under sterile conditions (Dinarello 2009, Dinarello, Donath et al. 2010, Dinarello 2011, Dinarello 2011). Thus the role of platelet IL-1R1 under sterile conditions was investigated.

Visualizing individual activated platelets in vivo would prove to be challenging and was not a prudent approach. Thus, I alternatively sought to analyze the IL-1β expression pattern within large concentrations of activated platelets such as those that accumulated upon in vivo thrombus formation. FeCl₃ induced oxidative insult is the
standard method employed to induce in vivo arterial thrombosis. Mouse carotid arteries were exposed to FeCl₃ leading to oxidative damage to vessel wall and subsequent thrombosis. The clot maturation was followed and paraffin embedded clot section were made at 10 minutes up to 6 hours after clot formation. The IL-1β accumulation in these clot sections upon clot maturation was assessed using immunohistochemistry (Fig-11). The clot sections stained positively for IL-1β (Alexa-488) as early as 1 hour after thrombus formation and showed a significant accumulation from 3 to 6 hours of clot maturation. IL-1β showed a dispersed pattern in the early time points with concentrated staining in the center of the maturing thrombus which tapered upon moving away towards the edges of the thrombi. DAPI DNA staining shows that not many nucleated cells are enmeshed in the maturing thrombi upon early time points and only appear much later around the 3 -6 hour mark. Further to show that platelets are the source of the cytokine. I visualized platelets by selectively staining them with wheat germ agglutinin (WGA-594) – Alexa fluor 594 conjugate since platelets extensively express its ligand. Clot sections stained robustly with WGA-594 throughout the time period and the DAPI stained nucleated cells appeared much later and did not align positively with the IL-1β staining. At the 3 hour time point after thrombus formation, many infiltrating nucleated cells were observed and they coalesced to the areas with IL-1β deposition. Later at 6 hours organization of leukocyte areas were observed and they stained strongly with IL-1β. The significant accumulation of mature IL-1β is represented as bar graphs (Fig-12A)
Figure 11 - Thrombus section IHC - Scoring Scale – The clot sections were stained with anti-IL-1β antibody and arranged from light staining intensity to the darkest and awarded a score. This scale of intensity was used to assess the rate of accumulation of IL-1β and Bcl-3 in maturing thrombi.
Figure 12 - Platelet formed IL-1β accumulates upon sterile thrombus maturation. A) IL-1β accumulates within arterial thrombi over time. Fixed arterial sections were stained with hematoxylin and probed with rabbit nonimmune or rabbit antimouse IL-1β antibody. Primary Abs were detected with HRP-conjugated anti-rabbit secondary antibody and visualized. The IL-1β staining intensity from N=5 mice arterial thrombus were scored with the scale provided earlier and represented as a bar graph. (B) Platelets are the source of thrombus IL-1β – Thrombus sections were generated as mentioned in the methods section. Platelet membranes were stained with Alexa Fluor 594–conjugated wheat germ agglutinin (red), IL-1β with rabbit anti-mouse IL-1β and Alexa Fluor 488–conjugated goat anti-rabbit (green), or nuclei with DAPI (blue). Sections were imaged by confocal microscopy, and the images were pseudo colored and overlaid. Original magnification 63X pValue < 0.05.
Bcl-3, B-cell lymphoma-3, is a member of Ikb-α related regulator of NFκB and is essential for clot retraction (Weyrich, Denis et al. 2007). Resting platelets show little to no Bcl-3 protein but upon activation with agonists like thrombin platelets synthesize new protein following hnRNA splicing. Bcl-3 synthesis in platelet is regulated by mTOR activation. Bcl-3 translation is regulated involving the mTOR downstream target protein like eukaryotic translation initiation factor 4E binding protein-1 (4E-BP1) (Schmitz, Heit et al. 2008). Inhibition of mTOR upon rampamycin treatment inhibited platelet clot retraction process similar to the inhibition observed in the absence of Bcl-3 in clot retraction. Bcl-3 is essential for fibrin condensation in platelets that brings about the clot retraction process (Weyrich, Denis et al. 2007).

Figure 13 - Bcl-3 accumulation in platelet thrombi - Bcl-3 accumulates within arterial thrombi over time. Fixed arterial sections were stained with hematoxylin and probed with rabbit nonimmune or rabbit antimouse IL-1β antibody. Primary antibody were detected with HRP-conjugated anti-rabbit secondary Ab and visualized. Original magnification 63X. The IL-1β staining intensity from N=5 mice arterial thrombus were scored with the scale provided earlier and represented as a bar graph. pValue < 0.05.
Bcl-3 accumulation during *in vivo* thrombus formation was examined with a similar approach as before using immunohistochemical analysis of the clot sections. Bcl-3 accumulated within the clot section similarly to IL-1β. Significant accumulation of both the proteins commences as early as one hour and continues as the clot matures (figure 13). The accumulation of these proteins is posttranslation dependent due to the complete absence of transcription in anucleate platelets.

Platelets splice IL-1β hnRNA and translate new cytokines upon stimulation (Shashkin, Brown et al. 2008, Brown and McIntyre 2011). Weyrich et. Al. observed platelets in the resting state have IL-1β mRNA pre-loaded on to the free polysome fraction in their cytosol for active translation upon stimulation (Lindemann, Tolley et al. 2001, Denis, Tolley et al. 2005). Thus, to determine whether the observed accumulation of IL-1β *in vivo* was driven by stimulated hnRNA splicing or active translation, a similar *in vivo* thrombosis approach was followed. These experiments were performed with the assistance from Dr. Wei Li. Mice were treated with actinomycin-D prior to thrombosis initiation to inhibit global DNA transcription. Upon FeCl₃ mediated thrombus formation (Figure-3-6) sections of the arterial thrombi were stained for mature IL-1β (Alexa fluor - 568). As platelets are the primary cell type in these thrombi, activation marker P-selectin was used to visualize the platelet population. The thrombus showed significant accumulation of IL-1β after 3 hours of maturation consistent with the earlier experiments. Actinomycin-D treatment inhibited the new mRNA synthesis in nucleated cells, but translation was left unaffected. The newly formed IL-1β was found positively associated with the P-selectin stained platelets (Figure – 14A). The inability of the nucleated cells to transcribe new mRNA under these conditions was determined by running QRT-PCR for
IL-1β transcripts in comparison to GAPDH. Nucleated cells were isolated from actinomycin-D treated mice and then stimulated with LPS to determine the increase in IL-1β transcription (Figure – 14B). These cells were unable to transcribe new transcripts upon TLR-4 stimulation, thus inhibiting their ability to contribute to the IL-1β accumulation during thrombus formation and maturation. Platelets during thrombosis are activated in a nontraditional way and this state is marked by IL-1β accumulation. These data positively show platelets as the primary source of IL-1β accumulation in vivo during sterile thrombus formation, thus confirming the link between the thrombosis and inflammation.

Figure 14 - IL-1β accumulation in thrombi is transcription independent. A) Clot section obtained from Actinomycin–D treated (FeCl₃ induced thrombosis explained in methods) mice were probed with anti–P-selectin detected with Alexa Fluor 488 secondary Ab that fluoresces in the green channel (top left) and anti–IL-1β detected with Alexa Fluor 568 secondary Ab that fluoresces in the red channel (top right) as nuclei were stained with DAPI (bottom left). These images were then merged (bottom right). B) Whole blood was drawn from mice after 90 mins of actinomycin-D treatment and treated with LPS (1µg/ml) for 1 hour. Total leukocyte RNA was extracted. cDNA was reverse transcribed and the level of IL-1β expression in comparison to GAPDH was assessed by Q-PCR.
Summary

In this chapter, I have established IL-1R1 as a novel platelet receptor. Upon binding either of its endogenous ligands, IL-1α or IL-1β, IL-R1 receptor initiates a MyD88 dependent signaling event that provides an alternative pathway of platelet activation. IL-1R1 expression in platelets presents a new understanding of the role of platelets in the context of inflammation. Platelet IL-1R1 is stimulated through IL-1β containing platelet - derived microparticles (data not shown), thus generating a positive feedback loop. This uncovers a new route of platelet response to an important inflammatory mediator IL-1. I and my collaborator have shown that IL-1R1 activation through IL-1β laden platelet - derived microparticles is essential for the LPS - mediated platelet TLR-4 response. As LPS is central in septic inflammation (Andonegui, Kerfoot et al. 2005, Aslam, Speck et al. 2006, Clark, Ma et al. 2007), this cross talk in platelets confirms platelet TLR-4 and IL-1R1 co-operation as an important aspect in IL-1β production and propagation of inflammation during sepsis. Here I have confirmed platelets generate significant amounts of IL-1β in-vivo in the thrombi. This production is independent of mononuclear cell contribution and is juxtaposed to the vascular wall and thus capable of influencing almost all the cell types involved in inflammation and vascular remodeling under sterile conditions as these thrombotic events occur in the absence of any bacterial endotoxins. This report on platelet IL-1R1 shows the involvement of this functional platelet receptor and the resultant auto stimulatory loop in the platelet IL-1β mediated immune response during sterile inflammation.
IL-1β is the central molecule capable of activating almost all the cell types involved in innate immune responses and this report identifies platelets as targets putting them in the group of cell types influenced by this cytokine during an IL-1β orchestrated inflammatory response. IL-1 promotes inflammation and is involved in many processes like myeloid progenitor cell differentiation, angiogenesis, vessel wall remodeling, and plaque stability (Dinarello 2009, Dinarello 2009, Dinarello 2011, Alexander, Moehle et al. 2012). Platelets produce IL-1β in the absence of transcription, upon stimulation through a platelet unique process of hnRNA splicing due to obvious lack of transcription (Weyrich, Denis et al. 2007, Shashkin, Brown et al. 2008, Brown and McIntyre 2011, Rowley, Oler et al. 2011). Thus platelet IL-1β induces its own production through activation of its receptors on platelet surfaces as reported in its nucleated counterparts (Dinarello, Ikejima et al. 1987, Warner, Auger et al. 1987, Dinarello 2009). Circulating cells in a resting state lack any IL-1β mRNA but upon activation the mRNA levels, but become detectable in coagulated blood cells. The source of these IL-1β mRNA has been unclear (Mileno, Margolis et al. 1995). Monocytes are attributed as the primary source of IL-1β but their IL-1β synthesis is a delayed process and requires several hours after initial activation (Schindler, Clark et al. 1990). Here we have shown that platelets are capable of IL-1β synthesis and also could amplify this response through rapid autocrine activation of its own IL-1R1 receptors. This, along with the presence of IL-1β accumulation in in-vivo thrombi as early as 1 hour in preparations that were devoid of any nucleated cell
participation, establishes platelets as primary cells type involved in this response. This provides a viable connection between inflammation and thrombosis.

IL-1β synthesis in platelets is dependent on post transcriptional events and is devoid of any de novo RNA synthesis. This is proven through the actinomycin-D mediated inhibition of transcription, which inhibited the IL-1β production in the vessel walls, but failed to decrease IL-1β production in the thrombi. Thus, the IL-1β accumulation observed is independent of transcription, but dependent on hnRNA splicing and translation events. In the closed setup of a thrombus, activated platelets respond by shedding IL-1β microparticles and these particles in turn activate naïve circulating platelets via their IL-1R1 surface receptor and also the adjacent vessel wall endothelial cells and other nucleated cells as well. The IL-1β bound to the microparticles is different from the free cytokine secreted in to the environment. Soluble IL-1β half-life is just few minutes (Reimers, Wogensen et al. 1991). Platelet shed microparticles are found in the circulation for much longer and have a half-life of 10 times longer (Flaumenhaft 2006). Thus, IL-1β bound to the microparticles circulates for a longer duration and are capable of activating other nucleated cells adjacent to the to the site of activation or after entering the circulation act at sites far from the initial response.

IL-1Ra binds IL-1R1 and inhibits the IL-1β mediated response in nucleated cells. Surprisingly, IL-1Ra mediated inhibition stunted the LPS induced TLR-4 responses in platelets along with the expected IL-1R1 stimulating activity. Thus platelet TLR-4 signaling is unexpectedly coupled with IL-1R1 activation. Platelet IL-1R1 expands LPS
induced responses by responding to the active IL-1β that they synthesize. Thus the LPS response is dependent on IL-1 signaling. In addition, flow cytometry of platelet surface shows IL-1R1 is more abundant than in the monocytes. Platelets outnumber other immune cells exponentially and this robust expression of IL-1R1 surface receptor, combined along with the lack of IL-1RII decoy receptors, sets platelet mediated IL-1 responses apart from other cells. Soluble and particle bound IL-1 cytokine acts as potential endogenous atypical platelet agonist that mimics TLR-4 activation under sterile conditions and facilitates platelet IL-1 dependent immune responses. Thus, platelet IL-1 unmistakably plays a vital role in innate immune response under both septic and sterile conditions.

**Figure 15 - Chapter 3 Summary** – Autocrine loop between platelet TLR-4 and IL-1R1 signaling. Platelets express functional IL-1R1 signaling. Platelets shed IL-1β laden microparticles upon LPS stimulation of the surface TLR-4. These active IL-1β containing microparticles auto stimulate self-surface IL-1R1 and augment the LPS/TLR-4 signaling.
CHAPTER IV

Platelet derived Interleukin -1 β Associates with shed microparticles and exosomes to stimulate IL-1R1 dependent endothelial cell activation.

Introduction and rationale

Platelets are simple anucleate immune cells that hold a critical position in the host immune response. Platelets, upon activation secrete many chemokines and cytokines that are packaged in the granules that they shed (Gasparyan 2010, Margetic 2012, Fuentes, Fuentes et al. 2013, Koseoglu and Flaumenhaft 2013). These inflammatory agents are protected from the external environment, so their longevity is prolonged and are capable of exerting effects in the local sites and sites far removed from the point of release for a considerably elongated time period (Wagner and Burger 2003, von Hundelshausen and Weber 2007, Stokes and Granger 2012, Aggrey, Srivastava et al. 2013). Platelets typically shed three different types of granules upon activation. They are alpha granules,
dense granules and the granules of lysosomal origin. The composition of these granules is distinct and the heterogeneity of the granules shed depends on the intensity of the agonist. The alpha granules are the most abundant of all and are contain a varied range of growth factors like platelet-derived growth factor, TGFβ and immunogenic proteins like Platelet factor 4, cytokines and P-selectin, platelet specific activator proteins like fibronectin, von Willebrand factor (VWF), factor V, thrombospordin and many more. These factors facilitate platelet activation during thrombus formation (Furie, Furie et al. 2001, Rendu and Brohard-Bohn 2001, Maynard, Heijnen et al. 2007, Italiano and Battinelli 2009).

Dense granules are less abundant than the alpha granules. They differ markedly in their cargo signature as they mainly contain ADP, ATP, calcium ions and most along with histamine and serotonin. The lysosomal granules are sparse with less obvious functions (Furie, Furie et al. 2001, Rendu and Brohard-Bohn 2001, Koseoglu and Flaumenhaft 2013). Recent studies have proclaimed platelets as a functional cell type involved in innate immune system in addition to their primary function of mediators of homeostasis and thrombosis (Semple, Italiano et al. 2011).

Platelets have been associated with IL-1 mediated responses for a long time (Hawrylowicz, Santoro et al. 1989, Mileno, Margolis et al. 1995, Gawaz, Brand et al. 2000), and platelet derived IL-1 in the circulation stimulates a varied range of cells from endothelial cells (Hawrylowicz, Howells et al. 1991) to smooth muscle cells (Loppnow, Bil et al. 1998) and other circulatory blood cells through the IL-1 receptors on their surface (Dinarello 2011). Irrespective of this known association of platelets to the IL-1 cytokine, it has been difficult to attribute platelets as a major source of IL-1β. The levels of this protein is hardly detected in resting or naïve quiescent platelets in circulation or in
*vitro* through quantitative spectrometry analysis but yet upon stimulation an increase in IL-1β mRNA is observed (Burkhart, Vaudel et al. 2012). More so, the platelets are positively associated with IL-1 induced pathology in various immune diseases like cerebral malaria (Aggrey, Srivastava et al. 2013) or rheumatoid arthritis (Boilard, Nigrovic et al. 2010).

Activated platelets are shown to actively produce IL-1β and thus the state of activation and stimulation is essential for platelet mediated IL-1β synthesis (Weyrich, Elstad et al. 1996, Weyrich, Dixon et al. 1998). Platelets store preformed mRNA loaded to free polysomes and hnRNA to splice upon stimulation (Lindemann, Tolley et al. 2001). For instance IL-1β hnRNA splicing upon LPS stimulation (Brown and McIntyre 2011, Brown, Narayanan et al. 2013) and Bcl-3 during thrombin are adequately documented (Weyrich, Denis et al. 2007, Brown, Narayanan et al. 2013). Platelets actively splice introns and exons containing IL-1β hnRNAs upon LPS (Shashkin, Brown et al. 2008, Brown and McIntyre 2011, Brown, Narayanan et al. 2013) and thrombin treatment (Lindemann, Tolley et al. 2001) and swiftly translate the active cytokine that accumulates in a few minutes. On the other hand, activated monocytes show a slower time course of IL-1β mRNA synthesis through TLR-4 and IL-1R1 receptors (Arend, Gordon et al. 1989, Herzyk, Allen et al. 1992). The state of activation does not negatively affect platelet lifespan (Michelson, Barnard et al. 1996, Berger, Hartwell et al. 1998) and thus remain in the circulation in the state of heightened splicing and secretion of IL-1β for a prolonged time. Thus, the platelet IL-1 response is efficient and reasonably different from that of its nucleated counterparts.
IL-1β is synthesized as a pro-protein in its inactive form and upon further proteolytic processing is released as 17kD active protein. The IL-1β response in nucleated cells can be approached as 4 distinct processes: first is the transcriptome including induction, transcription and processing of IL-1β mRNA; secondly translation of spliced mRNA into actual product; pro-IL-1β, followed by inflammasome and Caspase-1 dependent processing; and then finally release. IL-1β is a leaderless protein and lacks any obvious signaling sequence and is thus excluded from the regular secretory pathway (Eder 2009, Weber, Wasiliew et al. 2010, Weber, Wasiliew et al. 2010). In nucleated cells, IL-1β is secreted through a range of modes - IL-1β associates with shed extracellular vesicles like microparticles (MPs) (MacKenzie, Wilson et al. 2001) and exosomes (Exos) (Qu, Franchi et al. 2007), but also through direct release into the external environment (Hogquist, Nett et al. 1991, Andrei, Dazzi et al. 1999, Blott and Griffiths 2002, Eder 2009, Lopez-Castejon and Brough 2011). The secretion of this cytokine could involve either of these routes in combination and the extent of their involvement might depend on the stimulus. Thus IL-1β release from synthesizing cells is opaque. Platelets upon activation release plasma membrane derived MPs and the much smaller Exos through exocytosis (Heijnen, Schiel et al. 1999). IL-1β released from platelets upon stimulation is unexplored. This release might coordinate with the particle shedding. We have previously shown the association of newly synthesized IL-1β with the shed MPs upon LPS mediated TLR-4 stimulation in platelets (Brown and McIntyre 2011). Thus other modes of secretion could possibly be active in platelet mediated IL-1 release as well.
The newly synthesized IL-1β binds its functional receptor IL-1R1 in expressing cells to initiate IL-1 responses. Studies show soluble IL-1β is endocytosed into redox-active signaling endosomes (redoxosomes) that organize IL1R1 signaling (Oakley, Abbott et al. 2009, Oakley, Smith et al. 2009, Spencer and Engelhardt 2014). These are microenvironments where IL-1 and other tightly regulated cytokine signaling takes place under the influence of compartmentalized reactive oxygen species (ROS). IL-R1 is immediately endocytosed upon ligand binding in to these redoxosomes along with NOD2. This process is essential for IL-1 mediated NFκB activation and this endocytosis is coordinated by lipid rafts and caveolin-1 (Li, Harraz et al. 2006, Oakley, Smith et al. 2009). The ability of particle associated IL-1β to initiate similar IL-1 receptor internalization and signaling needs to be explored.

Here, I have determined whether platelets upon stimulation shed IL-1β laden particle in response to traditional agonist (thrombin stimulation) as seen in the presence of endotoxin (nontraditional stimulation). Further, do platelets shed both MPs and Exos after these two treatments, if so, which type of particles associate with the newly synthesized IL-1? The mode of association with the particles and the activity of the released IL-1β were analyzed as well. The results show that both MPs and Exos contain bioactive IL-1β. At least some of the active cytokine associates with their receptor IL-1R1, while the majority is enclosed inside the shed particles. Both microparticles and exosomes activate endothelial cells isolated from human umbilical vein endothelial cells (HUVECs) in an IL-1R1 dependent manner and require dynamin mediated IL-1R1 endocytosis.
RESULTS

4.1 – Platelets shed particles that stimulate NF-κB dependent endothelial cell activation.

Activated platelets release both large microparticles (MPs) (~ 0.1 - 1 µm) and small (<100 nm) exosomes (Exos) after activation (Heijnen, Schiel et al. 1999). These particles originate from different cellular compartments. MPs and Exos were isolated by differential centrifugation from platelets activated by the complete agonist thrombin or the atypical agonist LPS. This particle isolation technique has been standardized and regularly followed in our recent publications from our laboratory (Srikanthan, Li et al. 2014). We have tested the purity of the isolated fractions using immuno blotting for fraction specific markers LAMP1, CD62P for microparticles and tetraspannins for exosomes. This method of separation yields pure population microparticles and exosomes with less contamination. The IL-1β mediated bioactivity of the particles isolated from platelets after agonist treatments, to activate endothelial cell in an IL-1R1 dependent manner was studied. The traditional signaling cascade of IL-1R1 upon IL-1β binding is described in the introduction section (Figure 1-6 and 1-9). Upon ligand binding MyD88 is recruited to its TIR domain initiating the IRAK – TRAF-6 recruitment, following a series of phosphorylation and degradation events results in TAK-1 activation of IKKb, which in turn allows the nuclear translocation of the p65 subunit. This also leads to activation of the MAP kinase pathway, ERK and JNK phosphorylation and downstream events. IL-1 stimulates endothelial cell MAP kinase cascades (Gourlaouen, Welti et al. 2013), and we
find MPs as well as Exos derived from either thrombin- or LPS-stimulated platelets induced phosphorylation of endothelial cell p42 and p44 ERK (Figure 16) and also activated NF-κB signaling via nuclear translocation of p-65 NF-κB subunits (Figure 17).

**Figure 16 - Endothelial cell - ERK activation upon Mp and Exo treatment.** A) HUVECs after above mentioned treatment were lysed and the rate of ERK phosphorylation is determined by Western blotting. (B) The density of bands was measured using the ImageJ program and the ratio between p-ERK/ERK is represented as bar graphs. LPS and thrombin shed mps and exos activated HUVECs equally. Data representative of one individual experiment (N=2)

Washed human platelets were treated overnight with LPS (and LBP and CD14 100 ng each) or thrombin (0.1 U). Microparticles and exosomes were isolated through differential centrifugation and washed to remove any agonist carry over. The particle used to treat these cells was normalized using protein content so as to effectively compare their bioactivity. Human umbilical vein endothelial cells provided by Dr. Keith McCrae’s laboratory were used in these assays. HUVECs were utilized in passage - 2 throughout
this study. HUVECs grown to confluence and were treated with MPs and Exos isolated from activated platelets. Recombinant IL-1β (1ng/ml), TNF-α (10µg/ml) and LPS (1µg/ml) were used as positive controls. The cells were lysed after treatment and the levels of ERK phosphorylation were analyzed using western blotting (Figure 16A). The relative density of the bands is represented as a bar graph (Figure 16B). The Mps and Exos were equally efficient in inducing ERK-phosphorylation. Significant increases in ERK activation were observed in comparison to the untreated controls.

**Figure 17- Nuclear translocation of p-65 NFκB subunit.** HUVECs were treated with either IL-1β, TNF-α or platelet derived Mps and Exos. Cells were fixed and permeabilized after 4 hours of treatment Nuclear translocation of p-65 subunit was visualized upon fluorescent staining (Alexa flour 488). Co localization of green florescence with nuclear stain DAPI is taken as positive translocation of the protein. N=3
The inflammatory response of endothelial cells is promoted by NF-kB -induced genes, and both exosomes and microparticles from platelets stimulated by LPS or thrombin induced the translocation of NF-κB immobilized in the cytoplasm into the nucleus (Figure 17). The Nuclear translocation of p65 subunit of NFκB is the key event during IL-1R1 signaling. This leads to induction of NFκB driven proinflammatory genes like IL-1β, IL-1α, IL-6 and much more (Libermann and Baltimore 1990, Hiscott, Marois et al. 1993, Mori and Prager 1996, Son, Jeong et al. 2008). Thus the rate of p65 nuclear translocation was assessed using confocal microscopy. HUVECs grown in chamber slides were treated for 4 hours and then washed, fixed and permeabilized. Cells were stained for p65 subunit of NFκB (Alexa fluor-488) and the merge of nuclear DAPI and the Alexa fluor-488 is interpreted as positive nuclear translocation (Figure 17). TNF-α treated HUVECs showed more robust translocation of NFκB than IL-1β treated cells. Both particles isolated from the LPS and Thrombin treated platelets efficiently induced nuclear translocation of the p-65 NFκB subunit.

4-2 Platelet MPs and Exos both carry proteolyzed IL-1β

IL-1β is synthesized as an inactive pro-protein that needs to be proteolyticly processed into its active form. The pro form of the cytokine is 33kD in size and is processed in to much smaller 17 kD active form. This process is shown to be dependent on caspase-1 activity and can also be cleaved by other proteases. But cleavage by others proteins seldom yield biologically active proteins (Hogquist, Nett et al. 1991, Guma,
Ronacher et al. 2009, Weber, Wasiliew et al. 2010). Earlier it was shown that platelets shed both proinflammatory MPs and Exos upon LPS and thrombin activation that were capable of activating HUVECs. The presence of proteolyzed active IL-1β in these particles was analyzed (Figure 18) and distribution of this protein between the particles was determined (Figure 19).

Washed platelets were treated with traditional or nontraditional agonists and the MPs and Exos were isolated from the supernatant through differential centrifugation before these particles were lysed. The SDS-PAGE was followed by anti-IL-1β immunoblotting of these lysates revealed that both the particles MPs and Exos contained proteolyzed 17 kD IL-1β. The abundance of the proteolyzed IL-1β in the particles was roughly equivalent between particles and among particles derived from thrombin-, LPS-, or collagen-stimulated platelets but needed further quantification (Figure 18).

Figure 18 – Platelet MPs and Exos contain proteolytic processed IL-1β — washed platelets were treated with Thrombin (Thr -0.1U), LPS (plus 100 ng rLBP and CD14) or collagen (Col 2 µg/ml) for 18 hours. MPs and Exos where collected from the cell free supernatant and the presence of proteolyzed active IL-1β product was detected using western blotting. N=5

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IL-1β content of these particles was then determined using quantitative IL-1β ELISA. The ELISA was standardized using the active recombinant IL-1β provided along with the kit. Thus the total inclusion of active IL-1β within the different fractions of the platelet supernatant, microparticles and exosomes was determined. The presence of soluble IL-1β in the supernatant, if any would suggest direct release of IL-1β from platelets as another possible route of secretion. In the LPS stimulated platelets, the bulk of the newly synthesized IL-1β was soluble and released into the supernatant, whereas with thrombin treatment significantly less soluble IL-1β was released in the supernatant (Figure 19A).

Figure 19 - Distribution of IL-1β between the Fractions: A) Platelets release free IL-1β in to the medium upon LPS stimulation - Platelet pellet, microparticles and exosomes fractions were lysed and the levels of IL-1β were detected using IL-1β ELISA (R&D - DLB50). The levels are measured in pg/ml in reference to the internal standard rIL-1β provided with the kit. (B) The comparison of IL-1β levels in Mps and Exos between LPS and Thrombin treatments. (N=6) Error bars +/- SEM pvalue obtained by one way ANOVA.
Whereas, both MPs and Exos isolated from thrombin treated platelets contained significantly higher concentration of IL-1β in comparison to the LPS derived particles. The particle mass was normalized to the protein content of the lysate rather than the number of the particles used, as thrombin - stimulated platelets shed exponentially more particles than LPS activation. As LPS particle associated IL-1β was far less than the soluble IL-1β released, the comparison of particles within the treatments is expressed in a separate graph. Significant amounts of IL-1β are released as particle bound cytokines upon thrombin treatment, whereas upon LPS treatment the majority is released as soluble form, although considerable amounts of the cytokine is shed associated with the particles as well. Thus both the agonist elicited more or less similar levels of response in activated platelets but the distribution within the fractions is varied between the agonists (Figure 19B).

4-3 IL-1β is contained on and within platelet microparticles.

The presence of IL-1β in the platelet shed particles has been established by the above experiments, but the pattern of expression of this cytokine within or on the particles is essential to ascertain the accessibility of the cytokine to activate target cells expressing IL-1R1. The IL-1β location in the MPs was analyzed by utilizing flow cytometry and TIRF – Total Internal Refractive Fluorescence microscopy. The flow cytometry analysis of the permeabilized MPs isolated from the agonist activated platelets
represented total active cytokine. MPs were stained with anti-IL-1β antibody that specifically identifies the cleaved 17 kD active form of the cytokine. Thrombin treated MPs contained far greater amount of active IL-1β than the LPS and collagen derived MPs (Figure 20A). This data suggests that significant amount of the cytokine is sequestered within the particles since the particles had to be permeabilized. But these MPs were capable of activating HUVECs so at least a part of the synthesized IL-1β must be surface bound and be readily available to activate the target cells. The microparticle and exosome mode of entry and activation of the target cells is still unclear. They could be directly internalized or a pool of the newly synthesized active IL-1β could be present on the surface of the particle, which could be available to interact with the IL-1R1 on the target cell. Thus, the display of IL-1β on the microparticle surface was determined. TIRF examination of intact MPs stained with anti-IL-1β (Cleaved form) revealed the display of IL-1β on their surface. Thrombin and LPS treated platelets release newly synthesized and processed IL-1β, at least in parts bound to the surface of the shed MPs (Figure 20B). Thus platelets shed IL-1β laden MPs where the significant amount of the active cytokine is sequestered with the particles, but also some amount of the cytokine is found bound to the surface of the MPs thus ready to activate the IL-1R1 expressing target cells.

4-4 - Platelet Derived particles contain IL-1R1

Activated platelets shed both microparticles and exosomes. Here, I have shown particles with different points of origins, microparticles budding from the plasma membrane and exosomes released from the endo-lysosomal compartment, both contain
processed IL-1β in their cargo. The mechanism of inclusion of the soluble IL-1β with in and on the surface of these particles with varied topology was investigated. The association of the IL-1β to the surface of the particle could be achieved through protein-protein interaction. IL-1β binds to its own receptor with a high affinity, and so can bind even to low concentrations. Platelet expresses functional IL-1R1. Thus, I hypothesized, that IL-1R1 is involved in the IL-1β inclusion along with the shed particles.

**Figure 20 - IL-1β is contained within and on the shed MPs** – A) MPs isolated after overnight treatment with respective agonist were permeabilized and stained with anti-IL-1β that only recognized cleaved cytokine. Flow cytometry analysis of the overnight MPs show the presence of mature IL-1β (N=3) B) IL-1β is present bound to the Microparticle surface - The Microparticles isolated as explained in methodology were stained with antibody against mature IL-1β and detected with Alexa fluor 488 conjugated secondary antibody and washed. The expression of IL-1β was visualized using TIRF. LPS and Thr derived MPs show positive association of surface bound active IL-1β than the control MPs.
Washed human platelets were treated with LPS or thrombin overnight and the MPs and Exos fractions where obtained as described in the methods section. IL-1R1 expression in these particle fractions was determined using immunoblotting (Figure 21). Microparticles and exosomes isolated from both the treatments contained IL-1R1. The levels of expression between the particles were significantly different, where exosomes contained more IL-1R1 than the corresponding microparticles. This was true for both LPS and thrombin-derived particles. The difference in the levels of IL-1R1 between the control and activated platelets is notable. This suggests regulation of IL-1R1 expression at the post transcriptional levels either with the stability or translation of the mRNA. Induction of IL-1R1 expression upon TLR-4 activation with LPS is reported in the nucleated cells like monocytes and dendritic cell (Gabellec, Griffais et al. 1996, Nagano, Takao et al. 2000).

Figure 21 - Platelet Mps and Exos express IL-1R1. A) Platelet pellet, microparticles and exosomes isolated after overnight treatment of LPS or thrombin or none were lysed and analyzed for IL-1R1 expression by Western blotting. B) Platelets lack endogenous IL-1Ra - IL-1Ra expression was detected in pellet and microparticle fraction of platelets and monocytes and the IL-1R1 presence was determined as well. N=5
IL-1Ra is the endogenous antagonist of IL1R1 and binds this receptor with equal affinity as IL-1β. Upon ligand binding, IL-1R1 is rapidly endocytosed and either degraded or else recycled to the surface (Dinarello 2009, Weber, Wasiliew et al. 2010). IL-1Ra does not stimulate the receptor but decreases the surface expression of the receptor. IL-1Ra association hinders IL-1β binding and obstructs IL-1R1 signaling. IL-1ra expression in platelets and the shed particles was assessed using western blotting (Figure – 21B). Platelet and monocyte lysates and LPS stimulated platelet derived microparticle lysates showed the presence of IL-1R1. Monocytes showed a band of slight higher molecular weight, which could due to post translational modification. The blot was striped and reprobed with anti – IL-1Ra antibody. Human platelets do not express IL-1Ra, were as monocytes serving as a positive control showed a significant expression of this protein. Thus endogenous IL-1Ra mediated control is not present in platelets, at least during in-vitro treatments. The IL-1R1 present in the particles could possibly help in IL-1β association with the shed MPs and Exos.

Microparticles shed from LPS and thrombin treated platelets retained IL-1β on their surface (figure 20B). So, I looked for the presence of its high affinity receptor IL-1R1 on MPs surface. Mps were isolated from activated platelets after specific periods of time and the surface expression and total amount of IL-1R1 and active IL-1β cytokine was determined using flow cytometry (Figure-22). The Microparticles were permeabilized with 0.1% Triton – X 100 to evaluate the amount of total IL-1β and IL-1R1 levels and compared to non-permeabilized samples. Both LPS and Thrombin induced MPs showed IL-1R1 on their surface, where thrombin microparticles expressed significantly more receptors than the LPS shed microparticles. The amount of IL-1β on
the surface of the microparticles was different between the treatments, similar to the IL-1R1, where thrombin- treated Mps showed more membrane bound IL-1β. But, the important inference from this data is of IL-1β on the surface closely follows the presence of IL-R1. The majority of the IL-1β and IL-1R1 in the LPS shed microparticles are sequestered inside the microparticles as the permeabilized microparticles displayed higher mean fluorescence intensity than the non-permeabilized samples (Figure 22A).

Thrombin is a complete platelet agonist, and upon platelet activation results in a large amounts of microparticles shedding than LPS, an atypical agonist stimulation. To avoid discrepancies in the number of microparticles, the results of the IL-1β inclusion are expressed as mean fluorescence intensity of the particles. Both thrombin and LPS stimulated platelet derived microparticles contained the IL-1β inclusion and IL-1R1 to a comparable extent. The inclusion of IL-1R1 closely followed the presence of its ligand, IL-1β. There was no significant difference in the levels of IL-1R1 within the permeabilized and the non-permeabilized thrombin microparticles, and IL-1β inclusion within the microparticles were greater than the surface bound cytokine but the results did not achieve significance (p=0.06) (Figure 22B). Platelet microparticles express IL-R1 on their surface irrespective of the stimulating agonist. The presence of IL-R1 that binds active IL-1β with greater affinity might influence the inclusion of the IL-1β within the particles and also help tether the newly synthesized bio active IL-1β on to the microparticle surface.
Figure 22- Difference between surface and internal inclusion of IL-1R1 and IL-1β in platelet shed Microparticles: Platelet was treated with LPS or Thrombin overnight and microparticles were isolated. Microparticles were stained for IL-1R1 and IL-1β with and without permeabilization with 0.1% Triton X100. Protein expression was analyzed through flow cytometry and the expression levels are expressed as mean fluorescence intensity of the particles. (A) LPS treated microparticles showed significant deference between surface and internal inclusion. (B) thrombin derived microparticles did show moderately significant difference between surface and internal content but not with IL-1R1. N=4 error bar= +/- SEM. pValue obtained through student unpaired t test.
4-5 IL-1R1 is essential for Membrane bound IL-1β laden microparticle shedding.

IL-1R1 is included in the stimulated platelet derived microparticles, but its exact role in the release of IL-1β microparticles is yet to be established. I asked whether the IL-1R1 was required for the surface expression of IL-1β, through inhibition and deletion of IL-1R1 in platelets. Firstly, IL-1R1 in human platelets was inhibited by rIL-1Ra.
pretreatment. IL-1Ra is an endogenous IL-1R1 antagonist and is shown to be absent in platelets (Figure 21B). Thus addition of external source of rIL-1Ra in excess will saturate the IL-1R1 on the platelet surface and would be unavailable for the active IL-1β binding.

Microparticles isolated from human platelets, either treated with LPS or thrombin for were analyzed using flowcytometry for the presence of cleaved IL-1β. Intact microparticles expressed active IL-1β on their surface. Microparticles shed after IL-1R1 stimulation through rIL-1β stimulation yielded highly active IL-1β positive microparticles, which serves as a positive control (Figure 23A). LPS stimulated platelets shed significant population of membrane bound IL-1β positive microparticles. Pre-incubation with IL-1Ra competitively binds the surface IL-1R1. Thus IL-1R1 is in turn made unavailable to bind the newly synthesized processed IL-1β. The histogram plot represents the negative shift in the IL-1β positive population (shaded in orange) in comparison to the original treatments (in green) (Figure 23-B, C). The reduction in IL-1 response in LPS treated cells following IL-1Ra pretreatment was expected as TLR-4 mediated LPS response is dependent on IL-R1 signaling (Brown, Narayanan et al. 2013). IL-1Ra treatment greatly decreased the LPS induced surface bound IL-1β positive microparticles shedding (Figure 23B).

Thrombin, a classic platelet agonist causes immediate changes in activated platelets. From our previous studies its known that thrombin is active, even at lower concentrations. Platelet treatment with 0.1U of thrombin induces shape change, significant microparticle shedding and aggregation in minutes (Gupta, Li et al. 2014).
Whereas, LPS mediated microparticle shedding is delayed but is stretched over hours (Weyrich, Elstad et al. 1996, Shashkin, Brown et al. 2008, Brown and McIntyre 2011). Thrombin treatment yielded an appreciable number of active IL-1β positive microparticles. This response was moderately decreased upon IL-1Ra pretreatment (Figure 23C). This result shows that the IL-1R1 signaling has limited role in the thrombin mediated IL-1 response.

The essential role of IL-1R1 for the surface expression of IL-1β on intact shed microparticles was investigated using TIRF microscopy. This technique eliminates excessive background fluorescence and facilitates visualizing of single cells at the glass and water interface. Surface expression of IL-1β on intact microparticles were visualized by staining the microparticles with Alexa Flour488 tagged anti cleaved IL-1β antibody (Figure 24). Both LPS and thrombin treated cells shed processed IL-1β bound microparticles in comparison to the control microparticles. rIL-1β treated platelets shed microparticles that serves as positive control. IL-1β treatment showed a clear increase in number of IL-1β positive microparticle population over the microparticles isolated from control platelets. Any IL-1β positive microparticle population isolated from control platelets are through autoactivation of the surface IL-1R1 signaling in resting platelets. IL-1Ra treatment decreased the autoactivation of IL-1R1 in the control cells. IL-1Ra pretreatment leads to saturation of IL-1 receptor. Thus, a significant decrease in the active IL-1β bound particles was observed, as seen in the earlier flow cytometry analysis (Figure 24). The TIRF analysis showed IL-1Ra affected LPS induced microparticles shedding, but no such decrease in MP shedding was evident in thrombin treated cells.
These data suggest that IL-1R1 inclusion in the shed microparticles is essential for IL-1β surface expression. But this claim need to further verified using total IL-1R1 knockout mouse models to show with absolute certainty.

Figure 24 - IL-1R1 INHIBITION DECREASES SURFACE BOUND IL-1β: TIRF Images – Cleaved IL-1β - Washed Platelets were pretreated with IL-1Ra or none for 30 mins. Cells were treated with 1 ng/ml IL-1β, 100 ng/ml LPS+CD14+LBP or 0.1 U Thrombin for overnight. IL-1β surface expression in the Microparticles was examined with unconjugated anti-cleaved IL-1β antibody, and detected with Alexa fluor-488 secondary antibody. Microparticles were visualized under TIRF microscopy.
To determine, whether the IL-1R1 receptor was required for surface IL-1β expression I utilized platelets from IL-1R1 global knock out mice. IL-1β surface expression on microparticles derived from platelets of wild-type (WT) and IL-1R1<sup>−/−</sup> (KO) mice were analyzed using flowcytometric techniques (Figure 25). Platelets were isolated from WT and KO mice and treated with LPS or thrombin or mouse rIL-1β (positive control). The active IL-1β bound microparticles were detected using flow cytometry. IL-1β surface expression on the intact microparticles was identified with the binding of anti-cleaved IL-1β antibody detected with Alexa fluor-488 secondary antibody and expressed as histograms, where green tinted histograms show WT platelets and orange histograms corresponds to KO platelets. Alexa-488 IL-1β expression was increased with LPS, thrombin and rIL-1β treated in WT platelet derived microparticles. Exogenous rIL-1β mediated IL-1R1 stimulation resulted in a far greater IL-1 cytokine positive microparticles than the others, similar to the observation with human platelets.

The lack of IL-1R1 in the KO mice platelets affects the association of active IL-1β to the MP surface. This was most evident from the observation from the KO mice platelet response to exogenous mice rIL-1β treatment, which showed dramatic decrease in IL-1β positive microparticles. The amount of IL-1β surface bound particle shedding from KO mice platelets was also significantly decreased after LPS and thrombin treatments. This decrease in surface bound IL-1β on shed microparticles was more profound in the complete absence of IL-1R1 than in the IL-1Ra mediated inhibition observed in the previous experiments (Figure 25A). The IL-1ra inhibition could quite
possibly be incomplete. Whereas, the complete absence of the receptor; resulted in markedly decrease in IL-1β positive microparticle shedding (Figure 25B).

The decrease in proinflammatory IL-1 cytokine laden microparticles in KO platelets could be possibly through a diminished IL-1 response for LPS, but thrombin does not stimulate platelets through IL1R1 so for these particles the lack of IL-1R1 fully accounts for the inability to bind the active IL-1β. The negative control shows the antibody fails to KO platelet derived microparticles (Figure 25C).

Figure 25 - IL-1R1 expression is essential for surface expression of Mature IL-1β – Platelets isolated from wildtype and IL-1R1 null mice and treated with (A) LPS(1 ng), (B)thrombin(0.1 U), (C) IL-1β (1 ng) overnight and stained for IL-1β surface expression and analyzed with flow cytometry. Wildtype population represented in green tinted and kockout population in orange histogram.
4-6 Microparticles provide IL-1β to internal endothelial cell signaling complexes to stimulate E-selectin expression.

IL-1β induced IL-1R1 signaling takes place in clathrin coated endosomes. The receptor endocytosis followed by H₂O₂ production is essential for IL-1 signaling facilitating the downstream signaling within the ROS contained endosomes termed redoxsosomes (Oakley, Smith et al. 2009). IL-1β signaling to downstream kinases in HEK293 cells over-expressing IL-1R1 (Hansen, Dittrich-Breiholz et al. 2013) and to H₂O₂ production in endothelial cells occurs within dynamin containing clathrin-coated endosomes (Spencer and Engelhardt 2014). Dynamin is a GTPase involved in the process of receptor endocytosis from the plasma membrane. Treatment with cell permeable small molecule dynamin inhibitors like Dynasore efficiently restricts the process of endocytosis (Macia, Ehrlich et al. 2006, Kirchhausen, Macia et al. 2008). Dynasore inhibition of dynamin GTPase activity blocks endocytosis of growth factor receptors in endothelial cells (Gourlaouen, Welti et al. 2013) and IL-1R1 endocytosis in target cells resulting in dis-regulation of NF-κB induced gene expression (Hansen, Dittrich-Breiholz et al. 2013).

LPS and thrombin - derived platelet MPs activated HUVECs and initiated kinase cascades and downstream NF-κB translocation (Figure 16, 17). Thus it is essential to establish the involvement of IL-1R1 in the particle mediated responses. Inhibition of IL-1R1 signaling with IL-1Ra or its endocytosis with Dynasore would provide a new understanding of this activation process. Platelets were prestained with WGA-Alexafluor-594 and then stimulated with LPS. The prestained microparticles shed by these platelets were isolated and utilized in this experiment. The HUVECs were stained with calceinAM
and the rate of accumulation of WGA stained LPS derived platelet microparticles with in the cytosol of the target cell, was visualized under fluorescence microscopy. The particles accumulate in endothelial cells as punctate inclusions and were distinct from cytosolic calcein. This punctuate inclusion were visibly decreased in the presence of dynasore (Figure 26).

Finally, the ability of platelet derived microparticles and exosomes to activate endothelial cell adhesion molecule surface expression was determined using E-selectin cell based ELISA. The role of IL-1R1 in the activation if any was explored with the help of IL-1R1 selective inhibitors like IL-1Ra and AF12198 (a small molecule peptidic inhibitor of IL-1R1). Soluble IL-1β, signals through internal receptor rather than the surface IL-1R1. As mentioned earlier, upon binding its ligand IL-1R1 is rapidly internalized in a dynamin dependent process and signals in the clatrin coated endosomal compartments called redoxosomes (Li, Harraz et al. 2006, Oakley, Smith et al. 2009). The requirement of internalization of the IL-1R1 in platelet microparticle and exosome mediated IL-1 response was investigated as well, using dynasore inhibition.

Microvesicles isolated from LPS and thrombin stimulated platelets were used to activate HUVECs and the increase in the E-selectin surface expression was obtained using fixed intact cell based ELISA. The increase in expression is represented as fold change over the control HUVEC cells. Both Microparticles (left) and exosomes (right) released from platelets stimulated by either LPS or thrombin contained bio-active IL-1β and were successful in activating the endothelial cells. Particle treatment induced increase in E-selectin on HUVEC cell surface. (Figure 27). Inhibition of IL-1R1 signaling with
IL-1Ra, the endogenous receptor antagonist or IL-1R1 specific cell permeable small molecule inhibitor AF12198, prevented activation of HUVECs. IL-1R1 signaling blockade in the presence of IL-1Ra and Af12198 significantly decreased stimulation of E-selectin expression by both microparticles and exosomes irrespective of the stimulating agonist. The particle bound IL-1β signals in similar fashion as soluble IL-1β, since inhibition of the IL-1R1 internalization with the dynamin inhibitor; Dynasore reduced E-selectin expression comparable to IL-1R1 inhibition (Figure 27).

Thus stimulation of endothelial cells is highly dependent on IL-1β transported by particles released from two distinct compartments of activated platelets. The particles possibly bind the IL-1R1 receptor on the target cells through the surface bound IL-1β that they express and initialize receptor endocytosis. Neither type of particle themselves interferes with IL-1R1 internalization and inflammatory signaling.
Figure 26- Dynasore treatment Inhibited microparticle uptake.

HUVECs were pretreated with 40µM dynasore for 30 mins and then Alexa fluor 594 WGA stained Mps shed microparticles are added to HUVECs and the uptake of the particles was observed. Cells were stained with Calcein AM and then fixed. Microparticle (in red) uptake by was visualized under fluorescence microscopy.

Figure 27- Platelet derived particles activate Endothelial cells via IL-1R1 signaling.

HUVECs were treated with either Mps or Exos isolated from LPS (A,B) or Thrombin (C,D) in the presence of buffer or IL-1Ra (200 ng), AF12198 (2 µM) or Dynasore (40 µM) for 6 hours. The increase in Eselectin expressions is taken as positive endothelial cell activation and is determined via cell based E-selectin ELISA assay and the results in O.D. are represented as fold increase over the buffer containing MP/Exo treatment with the inhibitors. N=5, error bar= +/- SEM and pvalue derived from one way ANOVA.
Summary:

Platelets upon activation release both microparticles originating from the plasma membrane and the exosomes of endo-lysosomal origin (Heijnen, Schiel et al. 1999). Platelet derived microparticles and exosomes are present in the circulation of normal individuals at nearly equivalent levels (Srikanthan, Li et al. 2014). The majority of the microparticles in the circulation is of platelet origin and expresses platelet specific surface markers like CD41a, CD62P and others. Expression of certain population of microparticles serves as physiological markers of many inflammatory diseases (Thery, Ostrowski et al. 2009, Gyorgy, Szabo et al. 2011, Burger, Schock et al. 2013). Elevated levels of platelet derived MPs are reported in IL-1 driven autoinflammatory conditions like rheumatoid arthritis (Knijff-Dutmer, Koerts et al. 2002, Boilard, Nigrovic et al. 2010) Sjogren's syndrome, systemic lupus erythematosus (Sellam, Proulle et al. 2009) and these predict the severity of the disease. Elevation in microparticle levels in patients coincides with the presence of IL-1β in the pathogenesis of the inflammatory condition. IL-1β is presented as major cause of disease pathology along with other cytokines like TNF-α, IL-18, IL-6 and others. These cytokines in different combination dictates the course and severity of the inflammatory diseases – such as inflammatory bowel disease (Andoh, Tsujikawa et al. 2005), type 2 diabetes (Koga, Sugiyama et al. 2006), and atherosclerosis (Michelsen, Noto et al. 2009). Platelet shed MPs isolated from the synovial fluid of arthritic joints contain inflammatory IL-1β and IL-1α (Boilard, Nigrovic et al. 2010).
Exosomes play a role in inflammatory disease development and progression as well. The much smaller size of the exosomes (<100 nm) severely restrict the ability to identify the origin of these particles (Chaput and Thery 2011, Gyorgy, Szabo et al. 2011), but platelets do however release both particles after stimulation (Heijnen, Schiel et al. 1999), so platelet-derived microparticles and exosomes most likely appear in the circulation. Here we have shown that the newly synthesized IL-1β cytokine can be released bound or sequestered within either of two types of particles. The two distinct population of platelet particles shed from different cellular origin contain this synthesized active cytokine.

Microparticles and exosomes released from the same cell are unique in nearly all aspects. They arise from different compartment of the platelets where MPs are shed through outward blebbing and pinching of the plasma membrane while exosomes are formed in the multivesicular bodies (MVBs) and released in to the environment following the endosomal pathway and exocytosis. The cargo proteome of these particles differs significantly and is unique to the cellular origin. Part of cytosol enters the MPs during the process of budding, and the plasma membrane protein form the cargo of the shed microparticles. Whereas exosomal proteome is lysosomal in origin, and is quite varied and is dependent on the proteins entering the endo-lysosome degradation pathway. Exosomes are enriched in ubiquitinated proteins (Thery, Zitvogel et al. 2002, Silverman and Reiner 2011, Burger, Schock et al. 2013, Raposo and Stoorvogel 2013, Srikanthan, Li et al. 2014). The extensive characterization of the proteome of hepatocyte exosomes (Milstone, Fukumura et al. 1998) and platelet microparticles (Garcia, Smalley et al. 2005, Little, Smalley et al. 2010) show the proteins of these two classes of particles differ.
Though varied in their proteome Mps and Exos shed from activated platelets both expressed IL-1β and its receptor IL-1R1. The cytokine contained in these particles is a proteolytically cleaved product that’s biologically active capable of activating endothelial cells.

The role of platelet derived microparticles has been described in varied pathological conditions. The activating agonist and intensity of the agonist provides an opportunity for heterogeneity in the platelet derived microparticle cargos (Rendu and Brohard-Bohn 2001, Garcia, Smalley et al. 2005). A wide range of literature points to the importance of platelet derived MPS in inflammation (Thery, Ostrowski et al. 2009, Gyorgy, Szabo et al. 2011, Burger, Schock et al. 2013). The platelet exosome functions are yet to be explored to this extent. Platelet derived exosomes along with microparticles as whole are termed as platelet derived microvesicles (PMVs). PMVs are involved in varied homeostatic multi-cellular processes including hemostasis, immune response, maintaining vascular health and others. They participate in thrombotic events, pathogenesis of inflammatory diseases and in cancer metastasis (Aatonen, Gronholm et al. 2012). Recently, studies from our laboratory have explained a novel role of platelet derived exosomes in platelet activation through their unique ubiquitin signature. Platelet derived Exos inhibit platelet activation *ex-vivo* and extends its inhibitory effects to FeCl₃ induced *in-vivo* thrombosis. MPs on the other hand showed no such enrichment of ubiquitinated proteins and conversely facilitated platelet activation (Srikanthan, Li et al. 2014). In contrast to the effect of Exos on platelets themselves, Exos as well as MPs shed from LPS and thrombin treated platelets successfully stimulated ERK phosphorylation and induced NF-κB nuclear translocation with resultant increase in E-selectin expression.
on the surface of the activated endothelial cells. These processes are the hallmark of endothelial cell activation and both particles were capable of activating endothelial cells. This process was IL-1R1 signaling dependent and thus is attributed to the active IL-1β present in these shed particles.

The leaderless IL-1β protein can be released in its soluble form or bound to the microparticles and exosomes shed by the activated cells (Andrei, Dazzi et al. 1999, Eder 2009, Lopez-Castejon and Brough 2011). A significant portion of IL-1β synthesized upon LPS stimulation were released in to the supernatant in its free soluble form. Platelets upon both LPS and thrombin mediated surface receptor stimulation released particle bound newly synthesized IL-1β. These platelet-derived MPs and Exos contained biologically active IL-1β that stimulates endothelial cell inflammatory responses. The presence of surface bound IL-1β along with the pool of sequestered intraluminal active IL-1β suggests a much more complicated mode of action of these proinflammatory particles. The principle behind the binding of active IL-1β to the particle surface and the occlusion of major portion of the IL-1 cytokine within the particles was investigated. Platelet derived particles expressed IL-1R1 on their surface, but this was also included within the particles. The surface IL-1R1 binds active IL-1β, but its association with its ligand within the particles is speculative, although likely - the unknown factor is the process of activation carried out by the IL-1β laden particles, whether the IL-1β needs to be surface bound or the internal pool of active IL-1β activates the target cells, or potentially they also may act in concert to bring about the activation of the treated cells.
Nonetheless, IL-1R1 expression and activity is essential for IL-1 response observed upon LPS, Thrombin and IL-1β treated platelets. Inhibition of IL-1β association with its receptor IL-1R1 through the antagonist– IL-1Ra treatment proved to adversely affect the IL-1 response. IL-1Ra treatment saturated the surface IL-1R1 and either rendered it unavailable for the exogenous IL-1β or newly synthesized IL-1 to bind its partner. Ultimately, this diminishes the presence of active IL-1β positive MPs. The complete deletion of IL-1R1 in the IL-1R1^−/− mice platelets showed far more profound decline in the IL-1 response upon agonist activation. Thus establishes the integral role of IL-1R1 in the IL-1 response elicited by the activating agonists in platelets and also in anchoring the active IL-1β to the surface of the shed MPs. Surface expression of IL-1R1 is essential for the display of its ligand IL-1β on the surface of the platelet derived particles.

IL-1R1 signaling takes place in specialized clathrin coated endosomal compartments termed as Redoxosomes. The receptor internalization after ligand binding is the rate limiting step in this process (Oakley, Smith et al. 2009). I determined whether IL-1β association with exosomes or microparticles suppresses receptor-cytokine internalization and interferes with signaling. Upon addition of platelet derived WGA-594 stained Mps were internalized by HUVECs and was present as punctuate inclusions with their cytoplasm. Dynamin inhibition by the presence of Dynasore decreased this uptake. Dynasore treatment would affect the IL-1R1 internalization, which is also a dynamin dependent process (Oakley, Smith et al. 2009). Accordingly, IL-1R1 dependent MPs and Exos activation of HUVECs was hindered in the presence of Dynasore. Thus dynamin dependent internalization of IL-1R1 in to the endosomal compartment is essential for the
particle mediated cellular activation. The particle addition did not hinder the internalization of the receptors, but in turn requires this process to fully exert its effect on the target cell. The element that was recognized to initiate the internalization, whether the surface displayed cytokine or the internal pool of IL-1β is yet unclear and requires further investigation. But the essential event is the concentration of the cytokine within the endosome to carry out successful IL-1R1 signaling.

Lastly, the association of a soluble protein to a particle would significantly alter the rate of clearance of the protein from circulation. The half-life of the soluble form of IL-1β is just few minutes before it is cleared from the circulation (Kudo, Mizuno et al. 1990, Reimers, Wogensen et al. 1991, Di Santo, Benigni et al. 1999). Whereas the kinetics of microparticle clearance from circulation is much longer ranging from 10 mins in rabbits (Rand, Wang et al. 2006) to as long as 30 min in mouse models (Flaumenhaft 2006). Exosomes have even longer clearance rate seen in melanoma derived exosomes with half-life of more than an hour (Takahashi, Nishikawa et al. 2013). Thus the particle bound IL-1β is present in the circulation for considerably longer period time than its soluble counterpart. Data from this study shows that activated platelets shed two distinct particles, MPs and Exos that contain and express biologically active IL-1β. These particles activate endothelial cells in an IL-1R1 signaling dependent manner. Although, the majority of the active cytokine is sequestered within the particle and is inaccessible to the target cells immediately, a part of active IL-1β is displayed bound to the IL-1R1 expressed on the particle surface. The successful inclusion of otherwise soluble cytokine increases its activity duration in the circulation and significantly augmenting the inflammatory response exerted by this cytokine.
**Figure 28 – Chapter 4 summary** – Platelet IL-1β response under TLR-4–LPS stimulation (sepsis) and thrombin-PAR-1/4 stimulation (sterile Inflammation). Release of active IL-1β laden microparticles and exosomes capable of activating other IL-1R1 expressing target cells like endothelial cells, monocytes and naïve platelets in circulation.
CHAPTER V

PLATELET MEDIATED IL-1β PRODUCTION IS DEPENDANT ON INFLAMMASOME ACTIVATION

Introduction and rationale

Platelets in circulation maintain hemostasis. With their new found role as innate immune cells, platelets form a prominent link between pathological thrombosis and inflammation (von Hundelshausen and Weber 2007, Phipps 2011, Semple, Italiano et al. 2011). Platelets express TLR-4 and translate and secrete the pro inflammatory cytokine IL-1β upon LPS stimulation (Shashkin, Brown et al. 2008, Brown and McIntyre 2011). The presence of the IL-1β receptor IL-1R1 on platelets has raised the importance of platelets in the immune response process (Brown, Narayanan et al. 2013). Platelets retain the ability to alter their proteome upon stimulation through induced mRNA splicing and translation. The TLR-4 and IL-R1 receptors are functional in platelets and upon binding
their ligands they immediately initialize a MyD88 dependent downstream signaling leading to the splicing of intron and exon containing heteronuclear (hn) IL-1β hnRNA. The newly spliced IL-1β mRNA is swiftly translated and released in its active form along with the shed microparticles depending on the agonist (Shashkin, Brown et al. 2008, Brown and McIntyre 2011). This TLR-4 mediated IL-1 response in platelets is highly dependent on the positive feedback loop with the IL-1β stimulation of platelet IL-1R1 surface receptors (Brown, Narayanan et al. 2013).

In nucleated cells, IL-1β is synthesized as a pro – protein and is cleaved by active caspase-1 to a 17kD biologically active cytokine. Caspase -1 is the canonical activator of IL-1β, which in itself is a produced as in an inactive preform that is proteolytically cleaved in to its active product by inflammasomes. The enzymatic cleavage of IL-1β by caspase-1 is essential for its activity (Eder 2009, Guma, Ronacher et al. 2009, Weber, Wasiliew et al. 2010). The same holds true in platelets, where inhibition of capase-1 activity, either through small molecule inhibition or deletion of caspase-1 proved to affect IL-1β response by decreasing in mRNA splicing (Brown, Narayanan et al. 2013). Thus caspase-1 activity is detrimental for the platelet active IL-1β response and secretion.

Inflammasomes, are large protein complexes that provide a platform for caspase -1 activation and in turn facilitate activation of caspase-1 - dependent proteins e.g. IL-1β, IL-18 (Franchi, Eigenbrod et al. 2009, Martinon, Mayor et al. 2009). The NOD-like receptors are important component of this intracellular multiprotein complex and the activating agent determines the type of NLR activation and thus the activated inflammasome are identified as NLRP1, NLRP-3, and AIM2 inflammasomes. ASC an
adaptor protein with CARD- caspase binding domain that recruits pro-caspase-1 and through a poorly understood process leads to caspase-1 autoactivation. Caspase-1 is termed IL-1 Converting Enzyme (ICE) as this active caspase-1 is responsible for the maturation of IL-1 and IL-18 (Martinon, Mayor et al. 2009, Schroder and Tschopp 2010, Latz, Xiao et al. 2013). NLRP3 is one of the widely studied inflammasome complex associated with IL-1 and innate immunity. Constitutive activation NLRP3 or lack thereof is associated with numerous autoinflammatory diseases where IL-1β levels and activity are responsible (Artlett 2012, Bonar, Brydges et al. 2012).

NLRs along with TLRs are termed pattern recognition receptors (PPR). These PPRs are employed by the innate immune system to effectively recognize exogenous and endogenous insults against the host and to wage a prompt and efficient immune response. Any disruption of these members results in dysregulation of inflammasome function and is associated with cause inflammatory disease pathogenesis (Becker and O'Neill 2007). There remains a significant cross talk between the TLRs and NLRs, which makes them integral in the response mounted by the other (Kawai and Akira 2011, Hanamsagar, Hanke et al. 2012, Fuentes-Antras, Ioan et al. 2014). The TLR-4 mediated response is achieved in a combination of two waves of stimulus, an initial LPS induced TLR-4 mediated induction of IL-1β. This is closely followed by activation of NLRP3 inflammasomes via Damage Associated Molecular Patterns (DAMPs) or Pathogen Associated Molecular Patterns (PAMPs) and subsequent activation of IL-1 cytokines (Franchi, Eigenbrod et al. 2009, Hanamsagar, Hanke et al. 2012, Heid, Keyel et al. 2013, Fuentes-Antras, Ioan et al. 2014). The presence of NLRs in platelets is yet unknown. Thus the presence of inflammasome system in platelets was studied. The identification of
platelet NLRs would signify that platelets IL-1 response is similar to its nucleated counterparts.

Inflammasomes are activated by, changes in intracellular K$^+$ ion levels, Reactive Oxygen Species (ROS), and also through P2X7– purinergic receptors (Tassi, Carta et al. 2010, Tschopp and Schroder 2010, Heid, Keyel et al. 2013). Purinergic receptors are divided into classes in respect to the ligands they bind such as adenosine binding P1 receptors and P2 receptors recognize ADP and ATP. P2 receptors are further sub divided into metabotropic – P2Ys, P2Us, etc. and ionotropically receptors – P2Xs and P2Zs (Brown, Townsend-Nicholson et al. 2002, Kaczmarek-Hajek, Lorinczi et al. 2012). Platelets are known to express ADP sensing P2Y1 and P2Y12 receptors and the P2X1 ion channel that recognize ATP that is involved in platelet activation (Wang, Ostberg et al. 2003, Oury, Toth-Zsamboki et al. 2006). P2X7 gated cation channels and are activated by extracellular ATP. Upon ligand binding, P2X7 increases intracellular Ca$^{++}$ ions and allows K$^+$ ion efflux, which is important for NLRP3 inflammasome activation (Ferrari, Pizzirani et al. 2006). P2X7 pore formation is dependent on secondary signaling molecules like Ca$^{++}$ ions (Faria, Defarias et al. 2005), Pannexin-1 (Panx-1), a large hemichannel junction protein forms a protein interact with P2X7 receptors and facilities the release of ATP in to the environment (Pelegrin and Surprenant 2006). P2X7 is intertwined with the host immune response to a significant extent and is proven to be a viable target to counteract the inflammation in many chronic inflammatory conditions and neurodegenerative diseases (Romagnoli, Baraldi et al. 2008, Takenouchi, Sekiyama et al. 2010, Arulkumaran, Unwin et al. 2011).
P2X7 ATP gated ion channel receptor and its accompanying Panx-1 ion channels dependent IL-1β secretion have been observed in other immune cells. The presence of these receptors and components in platelets are yet unknown. Thus I sought to investigate to find that platelets do express P2X7 receptor and Panx-1 hemichannels and the necessary components to form a functional NLRP-3 inflammasome complex. Cell permeable inhibitors and mimic peptides against these proteins were utilized to analyze the role of these proteins in the IL-1 response mounted by platelets upon stimulation with thrombin, complete agonist and the atypical agonist LPS.

RESULTS

5.1 Platelets express P2X7 receptors and inflammasome components

Platelets are known to express P2X1 and other P2Y receptors but it was not found that they could express other purinergic receptors. Quiescent washed platelets were lysed and the presence of P2X7 receptor and its associated partner Panx-1 pore junction protein was investigated using western blotting. The presence of inflammasome components like NLRs, ASC adaptor protein and inflammasome associated Caspases 1, 4 and 5 were also verified. Untreated peripheral blood mononuclear cells (PBMCs) were used as positive control (Figure 29). Platelets expressed the P2X7 receptor (68 kDa) and Pannexin-1 (48 kDa) in ample amount whereas monocytes contained P2X7R but did not show Pannexin-1 expression. Pannexin-1 in monocytes is not known but is present in dendritic cells (DCs) and macrophages. Inhibition of Panx-1 through mimetic peptide did
not affect ATP induced dye uptake in monocytes, suggesting PBMCs might not express this ion channel (Bhaskaracharya, Dao-Ung et al. 2014). So it’s quite possible monocytes lack Pannexin-1 but could possess either Panx-2/3 ion channels to carry out larger molecule uptake and rapid ion flux.

**Figure 29 – Platelet contain essential components to form an activate inflammasome**
Untreated Platelets and PMBCs were lysed and the resolved in SDS page and western blotted for the presence of inflammasome components with respective antibodies listed in the materials section.

The surface receptor and the stimulus determine the kind of inflammasome assembled. Around nine different types of inflammasome complex have been identified depending on the NLR involved with each activated through different stimulus. Figure 1-7 shows these inflammasome assemblies. *Bacillus anthraces* toxin activates NLRP-1, microbial PAMPs and endogenous DAMPs like ROS activates NLRP-3 inflammasome, one of the best characterized inflammasome complex in immune cells. NALP-2/5 and NLRP4 are activated by bacterial flagillin and the activators of NLRP6 and NLRP-12 are
still unknown. Regardless of the NLR involved their activation, these complexes catalyzes caspase-1 activation. Untreated platelet lysate analysis by western blotting showed the presence of two NLRs - NLRP3 and AIM-2 and the adaptor protein –ASC along with Caspase 1 and 5. Other NLRs were absent, at least in resting platelets (Figure 29).

Thus, platelets are capable of forming functional NALP-3/NLRP-3 and AIM-2 inflammasome upon ATP activation of P2X7 receptor. Platelet IL-1 response could follow the similar sequence of activation in P2X7 signaling followed with opening of PANX-1 ion channels allowing uptake of larger molecules as explained in nucleated cells. We have already shown that platelet express caspase - 1 and its activity is essential for LPS mediated IL-1β secretion (Brown, Narayanan et al. 2013). Here I show

**Figure 30 - Thrombin induces ROS production:** A) DCFH-DA assay – ROS generated upon agonist treatments was measured using DCFH DA florescent dye and the fluorescence intensity is represented as arbitrary units against time. Washed platelets were treated with LPS, THR or left unstimulated and the ROS generation over 2 hours was measured. (N=3)
that, platelets also express caspase-5, a caspase that mediate non-canonical IL-1 cleavage. Thus both canonical and non-canonical inflammasome complexes could be active in platelets

ROS is one of the DAMPs capable of NLRP-3 inflammasome activation. ROS are essential for IL-1R1 signaling as well. Thus the intracellular ROS production upon platelet stimulation with agonist was measured as well. The levels of ROS along with the release of secondary messenger molecules like Ca\(^{++}\) ions and ATP release sets the course of an effective IL-1 response. The intercellular ROS generation was assessed using 2', 7'-dichlorodihydrofluorescein diacetate (H\(_2\)DCFDA) assay. Washed platelets were stained with cell permeable H2DCFH-DA (10 µM) for 30 minutes and then the intracellular ROS production upon agonist treatment was measured. The nonfluorescent H\(_2\)DCFDA is converted into, the highly fluorescent compound, upon cleavage of acetate groups and oxidation during ROS generation. Platelets were treated either with LPS (100 ng/ml) along Lipopolysaccharide Binding Protein (LBP) (100 ng/ml) and CD14 (100 ng/ml) or thrombin (0.1 U). Thrombin treated platelets generated far more ROS than LPS stimulated cells. The increase in fluorescence intensity is plotted as graph against the time of release. Thrombin treatment resulted in a significant induction of ROS production in platelets relative to LPS treated or control cells (Figure 30). Thrombin as a complete agonist is capable of inducing all classic platelet activation events like intracellular Ca\(^{++}\) release, ATP release and significant ROS production than the other incomplete platelet agonists. Thus the significant increase in the DAMPs upon thrombin treatment could result in the robust activation of inflammasome complex in platelets.
5-2 Thrombin stimulates robust platelet IL-1β response.

LPS or thrombin stimulation of platelet surface receptors TLR-4 and IL-1R1 respectively leads to MyD88 dependent signaling events and IL-1β mRNA splicing (Shashkin, Brown et al. 2008, Brown and McIntyre 2011, Brown, Narayanan et al. 2013) and active IL-1β cytokine laden microparticle and exosome shedding (chapters 3 & 4). Here I show that this stimulated release of mature active IL-1β microparticles (MPs) occur as early as 1 hour after agonist treatment with thrombin treatment yielding a significant amount of MP shedding in comparison to the LPS. Previous studies reveled the kinetics of IL-1β mRNA splicing where LPS induced a slow event with peak in activity after 3 hours of induction followed by a prolonged accumulation of transcripts untill 18 hrs. Whereas, thrombin induced splicing rapid significant accumulation of mature transcripts at 1 hour and then soon tapered off (Shashkin, Brown et al. 2008).

![Figure 31- Platelets shed mature IL-1β laden microparticles.](image)

Flow cytometry analysis of microparticles isolated from LPS and Thrombin activation as early as 1hr show the presence of mature IL-1β. The mean fluorescence intensity of the particles are represented as bar graph. Error bar = +/- SEM, p value obtained with using one way ANOVA. (N=5)
2008). The rate of release of secondary signaling molecules and other DAMPs that activate inflammasomes coupled to the kinetics of IL-1β mRNA splicing could explain the swift IL-1 response observed with thrombin stimulation (Figure 31).

5.3 – Caspase-1 activation is essential for platelet IL-1 response.

Caspase-1 requirement in IL-1β processing is well established. We in earlier studies have shown that inhibition of caspase -1 either with small molecule inhibitors or upon total knock out of protein expression significantly diminished the LPS induced TLR-4 mediated IL-1β platelet response (Brown, Narayanan et al. 2013). Thrombin induced IL-1 responses might as well be dependent on inflammasome mediated Caspase -1 activation. The identification of inflammasome associated components in platelets further solidifies the requirement of caspase -1 in IL-1β secretion process. Two different approaches were taken to inhibit caspase-1 activity in platelets, to further understand the IL-1β processing and secretion in platelets. Firstly, inflammasome mediated caspase-1 activation was inhibited indirectly by targeting the P2X7 and Panx-1 channel activation and lastly caspase -1 was inhibited directly with small molecule peptide inhibitors. This pretreatment was followed by agonist stimulation and the microparticles were isolated. Permeabilized Mps were stained for mature –IL-1β using unconjugated primary antibody followed by Alexa fluor-594 conjugated secondary antibody. The microparticles were analyzed using LSR II flow cytometer and the fluorescence was recorded and mature IL-
1β content is represented as mean fluorescence intensity of the microparticles to normalize the microparticle count between treatments.

**5-4 P2X7 and PANX-1 activity are required for stimulated IL-1β laden microparticle shedding.**

Upon activation P2X7, along with the involvement of Panx-1, augments the IL-1 response in nucleated cells, increasing proinflammatory microparticle and exosome shedding (Ferrari, Pizzirani et al. 2006, Pizzirani, Ferrari et al. 2007, Qu, Franchi et al. 2007, Pelegrin 2008). Platelets express P2X7 as well as Panx-1 and were hypothesized to

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**Figure 32 - P2X7 activation is required for IL-1β Microparticle shedding.** (A) Platelets were treated with LPS, rib, rCD14 (100 ng/ml) (B) thrombin (0.1 U) for 1 hour with / without the presence of P2X7 and Panx-1 inhibitors. Microparticles were isolated as described in the methodology and the expression of mature IL-1β was assessed using Flow cytometry. Data is represented as mean fluorescence intensity. N= 4 error bar = +/- SEM, p value obtained with using one way ANOVA.
participate in the platelet IL-1β response. Their effect on IL-1β release was then assessed by utilizing cell permeable inhibitors. Washed platelets were pre-treated with either A-438079 (100 nM) or a pannexin-1 mimic peptide PANX-1 (400 µM) to inhibit P2X7 or Panx-1 activation respectively. This was then followed by stimulation with LPS or thrombin and the amount of active IL-1β laden microparticle shed were quantified using flow cytometry (Figure 32). As hypothesized IL-1β laden microparticle shedding was stunted with the inhibition of P2X7 and Panx-1 activation. This inhibition in turn leads to the decrease in activation of inflammasome complexes and ultimately the caspase-1 dependent cleavage of newly synthesized pro-IL-1 cytokine.

5-5 Direct inhibition of caspase activity hampers IL-1 response.

Caspase-1 activity in platelets was inhibited directly with selective caspase -1 inhibitors or with inhibitors that affected the function of all caspases. Washed platelets were treated with either pan-caspase inhibitor – Z-VAD-FMK (100 µM) or Caspase-1 inhibitor Z-WHED-FMK (10 µM) or caspase-1/5 inhibitor Z-YVAD-FMK (10 µM) for 30 minutes prior to agonist treatment. The further stimulation of these platelets with LPS or thrombin took place in the presence of the inhibitors. Inhibition of caspase -1 significantly impacted IL-1 processing, which was inferred from the severe decrease in the IL-1β laden MP shedding irrespective of the stimulating agonist (Figure 33). The highly selective inhibitor Z-WHED-FMK also effectively inhibited the MP shedding. Whereas Z-YVAD-FMK that inhibits caspase 4/5 activation to a greater extent than
caspase-1 failed to inhibit LPS induced IL-1β MP shedding effectively (Figure 33A). On the other hand, both selective inhibitors of caspase-1 and pan caspases decreased thrombin induced IL-1β laden MP shedding (Figure 33B). This data along with the presence of caspase -5 (Figure 29) in platelets presents with a possibility of activation of non-canonical caspase-5 associated inflammasome upon thrombin stimulation.

**Figure 33 - Caspase -1 activation is essential for IL-1β Microparticle shedding.** (A) Platelets were treated with LPS, rLBP, rCD14 (100ng/ml) (B) thrombin (0.1U) for 1 hour -with / without the presence of pan caspase or specific caspase-1 inhibitors. Microparticles were isolated as described in the methodology and the expression of mature IL-1β was assessed using flow cytometry. Data is represented as mean fluorescence intensity. N= 4 error bar = +/- SEM, p value obtained with using one way ANOVA.
Summary

The platelet inflammatory response upon LPS- endotoxin stimulation and thrombin activation presented in the previous chapters, explains the role of platelet IL-1β in both septic and sterile inflammation, respectively. These platelet IL-1 responses are mediated by biologically active IL-1β laden Mps and Exos shed upon stimulation. IL-1β is synthesized as 37 kDa proprotein that then is proteolytically cleaved in to its 17 kDa active form through capsase-1, IL-1 converting enzyme (ICE). This activation is facilitated by multi protein inflammasome complexes (Franchi, Eigenbrod et al. 2009, Martinon, Mayor et al. 2009). P2X7 receptors are key players involved in the IL-1β processing and release by activating NLRP-3 inflammasome complex and ICE (Ferrari, Pizzirani et al. 2006). Understanding P2X7 and Inflammasome activation in platelet would provide new of targets to effectively counteract IL-1β response at different stages of IL-1 processing and release.

IL-1β response mounted by TLR-4 signaling is a bi-phasic system that involves initial TLR-4 induction of IL-1β that needs to be followed by the secondary ATP mediated stimulation of P2X7. The resultant P2X7 associated inflammasome activation is critical for IL-1β processing and release (Fuentes-Antras, Ioan et al. 2014). Platelets express ATP activated purinergic receptor P2X7 and the dye uptake channels Panx-1 is present as well, which aids in the release of molecules over 700-900 kDa but also small molecules like ATP and K⁺. Components necessary to form a functional NLRP3
inflammasome complex identified in platelets. Thus, these findings present the possibility of the involvement of bi-phasic signals in platelet TLR-4 response.

Caspase -1 activity is indispensable for active IL-1β secretion (Hogquist, Nett et al. 1991, Eder 2009, Weber, Wasiliew et al. 2010, Lopez-Castejon and Brough 2011). Our previous studies in LPS mediated platelet IL-1β synthesis have also shown the importance of Caspase-1 (ICE) in the processing of this cytokine. Pharmacological inhibition or genetic ablation of ICE resulted in a profound decrease in IL-1β mRNA splicing, thus stunting the platelet TLR-4 inflammatory response (Brown, Narayanan et al. 2013). With the advantage of small molecule inhibitors, the importance of ICE activation in platelet IL-1 response in both sterile (thrombin) and septic (LPS endotoxin) conditions was analyzed. ICE activation was directly affected using cell permeable peptide inhibitors and indirectly by targeting the P2X7 and Panx-1 activation. Inhibition of ATP gated P2X7 receptor and Panx-1 prevents the activation of NLRP-3 inflammasome activation and ultimately inhibit ICE activation (Ferrari, Pizzirani et al. 2006, Latz, Xiao et al. 2013). Platelet P2X7 and Panx-1 inhibition prior to agonist stimulus affected IL-1β processing and MP shedding. This inhibition of IL-1 processing and release hampers the platelet IL-1R1 positive feedback loop that augment TLR-4 mediated IL-1 response (Chapter 3) (Brown, Narayanan et al. 2013). The direct inhibition of ICE affected LPS response as expected. Inhibition of ICE activation via disrupting P2X7 or Panx-1 activation or selective or broad inhibitors of caspase-1 affected active IL-1β MP shedding in thrombin stimulated platelets. This shows the requirement for activation of NLRP-3 caspase-1 inflammasome in the processing and release of platelet derived active IL-1β irrespective of the stimulating agonist.
Non-canonical Caspase-4/5 inflammasomes could mediate IL-1β activation. In murine macrophages, Caspase-11 mouse ortholog of Caspases -4/5 interacts with caspase-1 and potentiates its activation (Lin, Choi et al. 2000, Aachoui, Leaf et al. 2013, Py, Jin et al. 2014). Pretreatment with ICE specific Z-WHED-FMK effectively inhibited both LPS and thrombin induced IL-1β MP shedding. Z-YVAD-FMK that inhibits caspase4/5 effectively than caspase -1 presence inhibited thrombin derived MP shedding significantly, but failed to decrease LPS response (Figure 33). Thus platelets could active both canonical and non-canonical inflammasome complexes depending on the stimulus.

Nonetheless, anucleate platelets express the necessary components to initiate, synthesize process and release pro inflammatory IL-1β cytokine. Platelet mediated IL-1 response employ all the important hallmark processes seen in nucleated immune cells, such as translation, P2X7 activation, NLRP3 inflammasome mediated ICE processing of pro-IL-1β and active IL-1β release along with microparticle and exosome shedding.
CHAPTER VI

Discussion, Conclusion and Future Direction

Discussion

Platelets, the principle mediators of cellular thrombosis, also form the major cell type in innate immune system (Clemetson and Clemetson 2001, Varga-Szabo, Pleines et al. 2008, Gasparyan 2010, Fuentes, Fuentes et al. 2013). Platelets are abundant and make up the bulk of the blood cells in the circulation and are the primary responders at the site of injury. Thus, they exert a major command over the immune responses during inflammation. Platelets secrete a range of cytokines and chemokines that affect the immediate local environment of secretion and also systemically at site far removed as the inflammatory molecules and microparticles are released (Jennings 2009, Phipps 2011, Stokes and Granger 2012, Fuentes, Fuentes et al. 2013).
Platelets play an integral part in the immune response, a process different from their role in well documented thrombosis and hemostasis. The finding from this study would extend this role of platelets and extends the platelet function into inflammation. The presence of novel receptors and the signaling mechanisms have been postulated, thus expanding the scope of platelet mediated responses in the context of thrombosis and sterile inflammation. Platelets upon activation release IL-1β (Lindemann, Tolley et al. 2001, Zimmerman and Weyrich 2008). In activated nucleated cells this soluble protein is released in association with the microparticles and exosomes shed from different compartments of the activated cells (Andrei, Dazzi et al. 1999, MacKenzie, Wilson et al. 2001, Qu, Franchi et al. 2007, Wang, Williams et al. 2011). The newly synthesized leaderless cytokine could be engulfed with in the microvesicles upon their formation thus providing a mode of release of IL-1β in platelets. The association of the active IL-1β with both microparticles exosomes arising from different cellular location is noteworthy. The cytokine contained within the particle would be confined and sequestered from the target cells but the intact microvesicles were capable of activating the target cells. This could be through particle surface bound active cytokine, but such display of IL-1β on shed microvesicles and the mode of its association are yet to be explained. Here I have shown that platelets upon receptor stimulation synthesize and release IL-1β in its soluble form and also in association with the shed microvesicles.
IL-1R1 binds its ligand IL-1β with high affinity and is included in the shed microvesicles and provides a possible means for the anchorage of the IL-1 cytokine to the surface of the microparticles. This interaction does not hinder the bioactivity of the IL-1 bound to the microparticles and exosomes as they retain the ability to activate the target endothelial cells.

IL-1β is a chief proinflammatory cytokine that mediates numerous acute and chronic inflammatory conditions (Clemetson and Clemetson 2001, Spiel, Gilbert et al. 2008, Varga-Szabo, Pleines et al. 2008). It is produced as 37 kDa pro-form and is activated by proteolytic cleavage via caspase-1 (Hankey and Eikelboom 2003, Hartwig 2006). Platelets processes a functional TLR-4 signaling pathway and upon LPS stimulation they splice IL-1β hnRNA and translate IL-1β mRNA and the freshly synthesized active cytokine released along with the shed microparticles (Maxwell, Dopheide et al. 2006, Spiel, Gilbert et al. 2008). Interleukin-1 Receptor-1 binds IL-1β and shares downstream signaling components with TLR-4 receptors. Through this study the presence of functional IL-1R1 signaling pathway in platelets have been established (Varga-Szabo, Pleines et al. 2008). Platelet IL-1R1 is essential for optimal IL-1 response following TLR-4 stimulation with LPS. Where platelets produce and respond to the IL-1β synthesized through show stimulated splicing of IL-1β mRNA and active translation. Platelet IL-1R1 augments, TLR-4 mediated IL-1response through a positive auto-stimulatory loop.

Functional IL-1R1 signaling in platelets establishes the role of platelets in IL-1response during both septic and sterile inflammation. In this study I have shown that
platelets release active IL-1β in its soluble form and in association with the shed microvesicles upon surface receptor stimulation with both endotoxin (LPS) and complete agonists like thrombin and collagen. Thus platelet derived IL-1β could play an essential role in the pathogenies of inflammation driven by IL-1β under both sterile and endotoxin mediated inflammatory conditions.

The accumulation of active IL-1β in the in vivo thrombus was unique to platelets as its independent of transcription. IL-1β accumulated in the platelet amassed in occlusive thrombi within few minutes in comparison to the IL-1β accumulation in stimulated monocytes commences after few hours (Rumbaut and Thiagarajan 2010). This striking contrast in the kinetics along with the appearance of nucleated cells much later appearance within the thrombus shows that, platelets are the primary source of this IL-1 accumulation in the sterile thrombus (Maxwell, Dopheide et al. 2006). Upon vessel wall injury the collagen from the subendothelium is exposed which in turn activates the platelets, the first responders at the site of injury. Activated platelets release thrombin, ATP and ADP and other platelet activating factors upon degranulation. These agonists orchestrate platelet adhesion and the thrombotic plug formation (Rendu and Brohard-Bohn 2001, Varga-Szabo, Pleines et al. 2008, Koseoglu and Flaumenhaft 2013, Shaturny, Shakhidzhanov et al. 2014). From this study it is shown that the classical platelet activators, thrombin and collagen also activate the platelet IL-1R1 mediated IL-1 response, forming an inflammatory condition under the sterile thrombus formation through IL-1β laden microparticle and exosome shedding. Significant accumulation of IL-1β was observed in a maturing sterile thrombus (Gasparyan 2010). Platelet IL-1R1
pathway could inflammatory response under sterile conditions over long duration of time well past their initial activation.

IL-1β is synthesized as a leaderless proprotein that requires inflammasome dependent caspase-1 cleavage to achieve its active 17kD form (Jennings 2009, Margetic 2012). IL-1β lacks a definitive secretory signal and thus excluded from the classical endoplasmic reticulum/Golgi secretory pathway. Activated nucleated cells are shown to release this cytokine through non-classic routes (Rumbaut and Thiagarajan 2010, Koseoglu and Flaumenhaft 2013, Morrell, Aggrey et al. 2014). Monocytes and dendritic cells actively secrete majority of the active IL-1β in its soluble form and a significant amount of the protein associated with the microparticles and the exosomes released (Qu, Franchi et al. 2007, Eder 2009, Weber, Wasiliew et al. 2010, Lopez-Castejon and Brough 2011, Wang, Williams et al. 2011). Platelets release both particles exosomes originating from the endo-lysosomal compartment and the microparticles budding from the plasma-membrane. Platelets derived microparticles from the major population of the circulation and increase in these levels have been observed under inflammatory conditions (Baj-Krzyworzeka, Majka et al. 2002, Thery, Ostrowski et al. 2009, Gyorgy, Szabo et al. 2011, Aatonen, Gronholm et al. 2012, Burger, Schock et al. 2013). Platelet derived exosomes and microparticles showed the presence of active IL-1β upon both quantitative and qualitative analysis using western blotting, quantitative ELISA and flowcytometry. Identification of the inclusion of high affinity IL-1β binding partner – IL-1R1 in the microvesicles has contributed to the understanding of inclusion of cytokines through protein – protein interaction within the particles during protected release of inflammatory molecules upon cellular activation.
Platelets activated with LPS in particular released active IL-1β in its soluble form. Both LPs and thrombin stimulated the release of particle bound IL-1, with thrombin resulting in much rapid and increased release of cytokine contained microparticles. The inclusion of active IL-1β with particles with different topology and point of origins is intriguing. There has to be binding partner present to facilitate such association. Interestingly, platelet IL-1R1 was also found to be included in the shed particles from different sites origination. TIRF analysis of the intact microparticles isolated from both LPS and thrombin treated platelets displayed active IL-1β on their surface. Competitive inhibition of surface IL-1R1 in platelets in the presence of endogenous IL-1Ra or pharmacological small molecule inhibitor AF12198, disrupted the display active IL-1β on microparticle surface. Microparticle studies performed with IL-1R1−/− mice derived platelets revealed the absolute necessity of the IL-1R1 for the display and inclusion of IL-1β with the microparticles. The IL-1R1−/− platelets response to LPS and IL-1β stimulation was stunted as signaling by these two agents is dependent on IL-1R1. But the decrease in IL-1 display in IL-1R1−/− derived platelets upon thrombin treatment identifies the role of IL-1R1 in the IL-1β association to the shed microvesicles. IL-1R1 is present on the surface and also included with in the microparticles, which was observed upon gently permeabilization of the intact microparticles. The inclusion of this receptor on the surface and within the microparticles was completely in concert with its ligand IL-1β display on the surface and sequestered with in the particle.

The presence of IL-1R1, a membrane bound receptor in the microparticles is expected, but its presence in exosomes released from the endo-lysosomal compartment needs further investigation. IL-1R1 is rapidly endocytosed in a dynamin dependent
process and is targeted for lysosomal degradation or recycled back to the cell surface (Li, Harraz et al. 2006, Oakley, Smith et al. 2009, Weber, Wasiliew et al. 2010). It could be possible the IL-1RI included in the exosomes could be as a result of escaping the degradation. Platelet derived IL-1β laden microparticles are significantly different from the soluble form of the cytokine released from other activated innate immune cells like monocytes. Where the release of platelet IL-1β microparticle was rapid in hours rather than overnight, but ultimately produced quantitatively less IL-1β than the monocytes as observed by quantitative IL-1β ELISA. The IL-1β containing microparticle and exosomes release upon platelet surface receptor activation could also have qualitative difference between the other cellular sources. Multiple active IL-1β cytokine molecules could be expressed on their surface and contained within a single platelet derived microparticle or exosome dependent on the stimulating agonist. The association of the active cytokine with the microparticles and exosomes might alter the rate of clearance of these particles from circulation. In general, soluble IL-1β is cleared rapidly from circulation with a half-life of just a few minutes (Kudo, Mizuno et al. 1990, Reimers, Wogensen et al. 1991, Di Santo, Benigni et al. 1999). Microparticle bound IL-1, is dependent on the rate of clearance of the particles and significantly extended ranging from 10 mins (Rand, Wang et al. 2006) to 30 mins (Flaumenhaft 2006). Exosomes are cleared at a different phase than the microparticle, where melanoma derived exosomes are shown to degraded at two different compartments with half-life of few mins to 72 mins (Takahashi, Nishikawa et al. 2013). Thus the particle bound IL-1β is present in the circulation for extended periods of time are capable of activating naïve cells and propagating the initial inflammatory stimulus.
In endothelial cells, soluble IL-1β signals through the internal IL-1R1 receptors; that are internalized following ligand binding to the lipid rafts (Oakley, Smith et al. 2009). This dynamin dependent internalization of the receptor is essential for its signaling with in the endosomal compartments termed as Redoxosomes (Spencer and Engelhardt 2014). Dynasore, a small molecule inhibitor of dynamin treatment diminishes the soluble IL-1β signaling by inhibiting the internalization of the surface receptors in transformed kidney cells (HEK293) and in IL-1R1 expressing endothelial cells. Both microparticle and exosome associated processed IL-1β, activated NFκB signaling in endothelial cells. As a result this lead to an in increased expression of adhesion molecules (E-Selectin) on the cell surface. This activity was IL-1R1 dependent as inhibition of IL-1R1 signaling with IL-1Ra or AF12198 resulted in decreased E-selectin expression. Particle bound IL-1β also signals through the internal IL-1R1 receptor as dynasore mediated inhibition of receptor endocytosis decreased endothelial cell activation to the as effective as IL-1R1 inhibition. The exact mechanism of signaling of the particle bound IL-1β is yet unclear. As to whether the surface bound IL-1β or the sequestered pool of active cytokine is responsible for the activation of the endothelial cells needs further investigation.

IL-1β synthesis and release is dual signal dependent process (Figure -1-10) (Lemons, Chen et al. 1997, Maynard, Heijnen et al. 2010). Initial signal through a surface receptor like TLR-4 that initiate IL-1 induction, and closely followed by a secondary activation of NLRP3 Inflammasomes through ATP gated P2X7 ion channels. Inflammasomes provide the platform for caspase-1 pro-form in to its active form, which
in turn activate newly synthesized pro-IL-1β. NLRs are intracellular DAMPs sensors that are essential for IL-1β response in the nucleated cells. NLRP-3 (Cyropyrin) is essential for IL-1β activation and mutation in this gene is the cause for many IL-1β mediated inflammatory conditions (Lemons, Chen et al. 1997, Maynard, Heijnen et al. 2007, Koseoglu and Flaumenhaft 2013). Endogenous DAMP - ROS is reported to activate the NLRP-3 in nucleated cells (Clemetson and Clemetson 2001, Hankey and Eikelboom 2003, Italiano and Battinelli 2009). Here I have shown that irrespective of the activating agonists, platelets do follow this bi-phasic signaling event during IL-1 response. Platelet surface TLR-4 or IL-1R1 receptor activation provides the initial signal, which leads to increased mRNA splicing, and translation and accumulation of pro-IL-1β. The second signal is initiated upon extracellular ATP binding its receptor P2X7 and inflammasome activation that leads to cleavage of IL-1β through active- Caspase-1. Both LPS and Thrombin mediated IL-1β production was dependent P2X7 activation and in turn Caspase-1 activation.

Thrombin stimulation of platelets resulted in rapid release of IL-1β positive microparticles in larger quantity than the LPS treated cells. This could be through increased activation of P2X7 receptor signaling upon ATP generation and also through exponential increase in ROS production. The ROS release could affect platelet IL-1 response at different levels. TRAF-6, (TNF receptor associated factor-6) is essential for TLR-4 signaling and could be activated by intracellular ROS binding (Stokes and Granger 2012). This mode of activation is reported with IL-1R1 signaling, where endosomal NOX-2 generated H$_2$O$_2$ dependent activation of TRAF-6 potentiates its binding active IL-1R1-MyD88 complex.
in the endosome compartment termed Redoxosomes (Elzey, Tian et al. 2003, Soga, Katoh et al. 2007). Reports from our previous studies show that platelets activated with TRAF6 decoy peptides have significant accumulation of spliced IL-1β mRNA far greater than after LPS stimulus. Thus, the robust release of secondary mediators like ATP and ROS drives the thrombin mediated platelet IL-1β response. Previously observed thrombin induced rapid accumulation of IL-1β transcripts could be explained through increased activation of TRAF-6 through ROS (von Hundelshausen and Weber 2007, Margetic 2012). The rapid rate of splicing coupled with the inclusion of IL-1β mRNA preloaded in the free poly ribosomes in the activated platelets facilitates the swift translation and accumulation of IL-1β in the cytosol (Jennings 2009). The ATP surge after thrombin treatment activates the P2X7 receptors, providing the secondary wave in the IL-1 response. This could be achieved also through thrombin generated ROS induced NLRP-3 activation. This compounded activation leads to increased caspase-1 activation and results in swift accumulation of mature - IL-1β. Increase in intracellular Ca^{++} levels drives microparticle shedding, this along with the availability of mature-IL-1β in the cytosol results in amplified shedding of IL-1β laden microparticles. This is a proposed mechanism of thrombin induced IL-1response in platelets, though plausible needs further investigation.
Figure 34 – Platelet IL-1 Response – Role of platelet IL-1R1 in TLR-4 mediated septic response and Thrombin mediated sterile inflammation.
Conclusion:

Platelets have long been implicated in the thrombosis associated with inflammation (Srivastava, Cockburn et al. 2008, Gasparyan 2010, Swaim, Field et al. 2010, Hottz, Lopes et al. 2013). Platelets function as immune cells connecting thrombosis to inflammation in cardiovascular diseases (Akira 2003, von Hundelshausen and Weber 2007, Stokes and Granger 2012). This study reveals previously unrecognized pathways in platelets and postulates new modes of activation of platelets and furthers its role as an immune cell. Platelet derived IL-1β plays a critical role in the pathogenesis of inflammatory diseases conditions like collagen-induced rheumatoid arthritis (Kaisho and Akira 2006) or, dengue fever (Iwasaki and Medzhitov 2004, Blair, Rex et al. 2009). Platelets induce acute phase responses in murine experimental cerebral malaria (Kaisho and Akira 2006). This study contributes to deeper understanding of platelet IL-1 response at multiple levels starting from initiation to synthesis, to processing and release of active IL-1β. Important pathways involved in the IL-1β response, IL-R1 signaling pathway, P2X7–Panx-1 activated NLRP-3 inflammasome pathway, and caspase-1 dependent IL-1β processing pathways, have been identified in platelets. For the first time, the role of IL-1R1 in the platelet immune responses and the release of IL-1β have been established. While, the clinical role of platelet P2X7 receptors needs investigation, here I have provided evidence for its role in platelet IL-1 response under sterile and septic conditions. These novel receptor signaling pathways might not be important anti-thrombotic targets, but are essential for platelet inflammatory response. Hence, this study has generated valuable therapeutic targets for diseases like atherosclerosis and other disease conditions,
with a major inflammatory component and involvement of platelet inflammatory response.

The major observations from this study is, adhesion of the platelets is not required for the release IL-1β laden microparticles and exosomes. Thus activated circulating platelets contain the inflammatory potential to activate endothelial cell function far from the initial site of activation. Moreover, the platelet derived particle bound IL-1 is different from its soluble form. The microparticles and exosomes are released to remain in circulation for longer times than the soluble forms, so activated platelets thus could remain active for a prolonged duration of time. This study establishes a prominent role of platelet derived IL-1 in both endotoxin mediated sepsis and in endogenous IL-1R1 driven sterile inflammation. From this study it is evident that activated and not necessarily aggregated, platelets are capable of independently influencing endothelial activation. Endothelial cells play prominent role in cardiovascular disease, thus providing a new relation between platelets and inflammation.
**Future directions:**

This study provides understanding in to the role of platelets in IL-1β response and the proinflammatory microparticles and exosomes in the context of sterile and septic response. The data from study also leads to many interesting avenues of investigations. Particle bound IL-1β is shown to activate endothelial cells in an IL-1R1 dependent manner. Yet the mechanism through which the particles exert such effect is unknown. The target cells might respond to the particle surface bound IL-1β or the sequestered internal pool of the cytokine. The mechanism of this particle uptake and processing with in the target cell needs investigation. Quite possibly, the intact microparticles and exosomes could be endocytosed and then through changes in the endosomal compartments, the internal content of the particles could be released and made available for binding free IL-1R1 in the target cell. This could be investigated using pharmacological inhibitors of endocytosis and degradation pathways. The other aspect is to address the pool of IL-1β seen by the target cell. Digestion or cleavage of surface bound IL-1β using proteases would aid to differentiate between the effect of surface bound and internal particle IL-1β.

The next avenue of investigation is to analyze the in vivo effects of the platelet derived IL-1β laden microvesicles isolated upon both LPS and thrombin treatment. They are capable of activating endothelial cells under *in vitro* conditions, but the identification of rate of clearance, efficiency in –vivo would provide the clinical relevance for these observations. Platelet derived pro-inflammatory microparticles and exosomes quite
possibly contain other cytokines along with IL-1β. Platelets are shown to express Homeo Box protein-1 (HMGB-1), IL-6, IL-18, and other inflammatory cytokines that are involved in many disease conditions along with IL-1β. Thus it would be prudent to investigate the presence and the role of these cytokines in the platelet mediated inflammatory responses.

Platelets possesses functional spliceosome and a stock of heteronuclear mRNA from their megakaryocyte ancestors. It’s well established that activated platelets alter their transcriptome, through surface receptor stimulated hnRNA splicing. Previous studies have identified AKT and Jnk kinase activations are essential for TLR-4 signaling induced IL-1β mRNA splicing. There remains a gap between the receptor stimulation and kinases cascade and the resultant increase in IL-1β mRNA splicing. The naïve platelets do not possess spliced IL-1β mRNA and only accumulate upon surface receptor stimulation. So there has to be a possible inhibitory mechanism that prevents hnRNA splicing under normal conditions. This control could be through the activation of MAP kinase pathways. Platelets lack nucleus and the splicing reactions are extranuclear and takes place in the cytoplasm. This process of extra-nuclear splicing is essential in the nucleated cells like hematopoietic cells. Platelets would provide the perfect and simple system to study the cytoplasmic splicing events without the influence of nuclear-co-transcription events.

Effects of platelet IL-1R1 activation on platelet function other than immunological aspects are yet to be explained. Recent studies have shown that lack of IL-1R1 in platelets affect aggregation. Platelets isolated from IL-1R1−/− knockout mice
should decreased aggregation and adhesion upon collagen stimulation, but this effect was not observed upon strong stimulation of thrombin (Beaulieu, Lin et al. 2014). Thus IL-1R1 effect on classic platelet function is subtle but could not be disregarded. Exploring the role of platelet IL-R1 function in both human and mouse derived platelets with IL-1R1 selective inhibitors and KO would be another avenue of promising research. The identification of the mechanism of IL-R1 involvement in platelet activation, adhesion and aggregation in vitro and in vivo would help understand the role of platelets in inflammatory conditions involving thrombosis as a whole. This study would help comprehend the extent of platelet involvement as a mediator of thrombosis and as an immune cell as well.
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