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2.1 Protein tyrosine kinase as signaling molecules

2.1 Bruton’s tyrosine kinase (Btk)

In lymphoid and myeloid cells, several protein tyrosine kinases activated following receptor engagement are crucial components of receptor induced signal transduction cascades. Signaling pathways that are initiated by these tyrosine kinases can lead to a variety of outcomes, including cytokine production, cell differentiation and apoptosis. Tec family of non-receptor protein tyrosine kinases represent the second largest family of non-receptor tyrosine kinases and is composed of eight members, five of which are expressed in mammals: Bmx/Etk, Btk/Atk, Itk/Tsk/Emt, Tec and Rlk/Txk. Btk is expressed in all hematopoietic cells with the exception of T lymphocytes and plasma cells (Smith, C.L. et al 2001). Structurally, Btk has an amino terminal pleckstrin homology (PH) domain, which associates with phosphatidylinositol (3,4,5) triphosphate (PIP3) and plays a role in targeting the proteins to the membrane. PH domain is followed by a Tec homology (TH) domain, which contains a Zn$^{2+}$-binding Btk homology (BH) motif, followed by one or two proline-rich region(s) (PRR). Next to this are Src homology domains SH3 and SH2 and then the carboxy terminal kinase domain (Miller, A.T. et al 2002).

2.1.1 Btk in B cells

2.1.1.1 Signaling pathway in B cells

Btk exists either as an asymmetric or symmetric homodimer via intermolecular SH3-PRR interactions in the inactive state. In the inactive state, IBtk has been shown to bind the PH domain of Btk. Upon stimulation of B cell receptor (BCR), Src family kinases, such as Lyn, are activated, which function to phosphorylate ITAM motifs of the Igα and Igβ chains and IBtk becomes dissociated from Btk, via an unknown mechanism. Consequently, phosphatidyl inositol 3 kinase (PI3-K) product phosphatidyl inositol-3,4,5-triphosphate (PIP3), becomes available for binding of PH-domain containing proteins, such as Btk (Satterthwaite, A.B. et al 2000). Btk binds to PIP3 and Gβγ subunit through its PH domain. Concomitantly, the phosphorylation of Igα and Igβ ITAM leads to the recruitment of the Syk kinases to the Ig chains via the Syk SH2 domains (Miller, A.T. et al 2002). B-cell linker protein (BLNK) is recruited to a phosphorylated tyrosine
on Igα that is distinct from the ITAM motif. Syk then phosphorylates BLNK, thereby providing binding sites for Btk and PLCγ2. The binding of Btk to BLNK through its SH2 domain is followed by phosphorylation of Btk by a Src kinase (Rawlings, D.J. et al 1996). Subsequently, Btk undergoes autophosphorylation and carries out its functions by phosphorylating the tyrosine residues of PLC-γ2, leading to its activation (Rodriguez, R. et al 2001). PLC-γ2 leads to hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP2) into inositol (3,4,5)-triphosphate (IP3) and diacylglycerol (DAG), which leads to calcium mobilization and activation of the protein kinase C (PKC) pathway (Smith, C.L. et al 2001).

2.1.1.2 Btk in B cell function

The X-chromosome linked immunodeficiencies in CBA/N mice (xid) and X linked agammaglobulinemia in man (xla) are genetic diseases caused by a single conserved (as in xid) or multiple distinctive (as in xla) alterations of the structure of the protein tyrosine kinase Btk (Tsukada, S. et al 1994). xid mice have a missense mutation at a conserved arginine residue within the Btk pleckstrin homology domain (Thomas, J.D. et al 1993). Although Btk is affected in both xid and xla, B cell depletion in murine xid is less severe than that seen in human xla. Mice bearing xid have a half to one-third conventional follicular B cells (B2 cells) of normal mice, a severe reduction of B-1 cells, that predominate in the peritoneum (Hardy, R.R et al 1984) and low titres of circulating antibodies of either IgM and IgG3 (Smith, C.I.E. et al 1994). Peripheral B cells from xid mice are functionally impaired; they are unresponsive to activation by mitogenic anti-Ig and by thymus-independent-2 (TI-2) antigens, such as haptenated Ficoll (Sieckman, D.G. et al 1978). Moreover, xid B cells show a high rate of spontaneous apoptosis ex vivo and a significantly lower amount of endogenous Bcl-2, than found in B cells from normal donors (Woodland, R.T. et al 1996). The dysregulation of pathways that normally protect against apoptosis might thus contribute to the reduced pool of mature peripheral B cells in xid mice. Ectopic expression of anti-apoptotic genes, bcl-2 or bcl-xL, stabilizes and preferentially expands this subpopulation in xid mice (Woodland, R.T. et al 1996).
In bone marrow the number of pro-B cells is similar in xid and wild type mice, indicating an unimpaired commitment to the B cell lineage. However, the first manifestation of Btk deficiency in xid mice is evident during pro-B to pre-B cell transition, placing the initial effects of murine xid and human xla mutations at the same stage of B cell development (Michael, P.C. et al 2001). In xid mice there is a reduction in pre-B cell production, however this impairment is offset by increased survival of cells progressing from the pre- to immature B cell pool, suggesting that Btk-independent homeostatic mechanisms act to maintain this compartment (Michael, P.C. et al 2001).

Btk signaling defects also affect the late stages of B cell development. Peripheral B cells can be categorized into fractions I, II and III based on the relative levels of IgM and IgD (Hardy, R.R. et al 1983). Fraction I (IgD$^h$IgM$^l$) cells are considerably diminished in the xid mice. However, xid mice exhibit a higher than normal proportion of immature B cells [(fraction III) sIgM$^h$, sIgD$^l$, heat stable antigen (HSA)$^h$] in the spleen and reduced numbers of mature recirculating lymph node B cells, thus, xid restricts entry of immature peripheral B cells into the long lived mature B cell pool (Rollnk, A.G. et al 1999).

2.1.2 Btk in mast cells

2.1.2.1 Signaling pathway in mast cells

Mast cells play pivotal roles in the initiation of allergic reactions. Cross-linking of the high affinity receptor for IgE (FcεRI) on these cells activates intracellular signaling pathways that lead to degranulation, release of histamine and other preformed mediators, de novo synthesis and release of lipid mediators, and secretion of preformed and denovo synthesized cytokines (Beaven, M.A. et al 1993). These bioactive mediators lead to allergic inflammation. FcεRI is a heterotetrameric receptor composed of an IgE binding α-subunit, a β-subunit with four transmembrane domains, and two disulfide bonded γ-subunits (Ravetech, J.V. et al 1991). Several protein-tyrosine kinases are activated following receptor cross-linking; Lyn associates with FcεRI β-subunit and phosphorylates β- and γ-subunits (Eiseman, E. et al 1992). Phosphorylated immunoreceptor tyrosine based activation motifs on β- and γ-subunits recruits more Lyn and Syk (Jouvin, M.H. et al 1994). These activated receptor-bound protein tyrosine
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kinases, then phosphorylates target proteins such as phospholipase C-γ. Activated phospholipase C-γ results in hydrolysis of phosphotidylinositol 4,5- bisphosphate (PIP₂) into two second messengers; inositol 1,4,5-trisphosphate which mobilizes Ca²⁺ from intracellular storage sites, and diacylglycerol (DAG) which activates protein kinase C (Nishizuka, Y. 1984). Both protein kinase C and Ca²⁺ in turn are required for degranulation (Ozawa, K. et al 1993).

Three Tec family protein tyrosine kinases, Btk, Emt/Ttk/Tsk (Emt), and Tec are also expressed in mast cells (Kawakami, Y. et al 1994; Kawakami, Y. et al 1995). In contrast with Lyn and Syk, Btk and Emt do not physically associate with FcεRI. However, these Tec family protein kinases are activated by FcεRI crosslinking. Lyn and other Src family protein tyrosine kinases activate Btk through phosphorylation of Tyr-551. Activated Btk then autophosphorylates at Tyr-231 in mast cells (Wahl, M.I. et al 1997) and phosphorylates target proteins such as phospholipase C-γ (Takata, M. et al 1996). Recently, role of Btk in FcεRI signaling has been shown (Kawakami, Y. et al 1994).

2.1.2.2 Btk functions in mast cells

Unlike B cell development, mast cell development is apparently normal in Btk mutant mice (xid). However, mast cells derived from these mice exhibit significant abnormalities in FcεRI-dependent function. FcεRI cross-linking induces activation of three major mitogen activated protein (MAP) kinases: ERK1/2, JNK1/2, and p38 (Kawakami, Y. et al 1997). Phosphorylation targets of MAP kinases include transcription factors. Typically, JNK phosphorylates c-Jun, a component of the AP-1 transcription factor, at the critical serine residues in the activation domain of c-Jun and results in increased c-jun transcriptional activity. xid mutation affect JNK activities severely and to a lesser extent, p38 in FcεRI-stimulated mast cells, while ERK activities are not significantly changed (Kawakami, Y. et al 1997). Cultured mast cells derived from bone marrow cells of xid mice exhibit mild impairments in degranulation, and more profound defects in the production of cytokines such as IL-2, IL-6, GM-CSF and TNFα, upon FcεRI cross-linking (Hata, D. et al 1998a). In these Btk deficient cells there is reduced JNK activation which accounts for the lower cytokine production (Hata, D. et al 1998b). Also the transcriptional activities of these cytokine genes are severely reduced.
in FceRI stimulated xid mast cells. However, xid mutation had little or no detectable effect on the posttranslational regulation of TNFα expression. Also, xid mice primed with anti-dinitrophenyl monoclonal IgE antibody exhibit mildly diminished early-phase and severely blunted late-phase anaphylactic reactions in response to antigen challenge in vivo (Hata, D. et al 1998a). It has also been shown that Btk regulates apoptotic process upon growth deprivation in mast cells by regulating the activity of JNK/p38 (Kawakami, Y. et al 1997). Bone marrow mast cells (BMMC) die by apoptosis upon growth factor (recombinant mouse IL-3) withdrawal. However, in xid-BMMC apoptotic death is delayed by 48 h as compared to wild type-BMMC. Thus, pleckstrin homology domain of Btk is important for growth factor deprivation apoptosis since, expression of the Btk protein with xid mutation (R28C) in the pleckstrin homology domain failed to respond by rapid apoptotic cell death (Kawakami, Y. et al 1997).

Thus, Btk regulates two arms of the FceRI signaling process: the PLC/ Ca²⁺/ PKC, and JNK signaling pathway.

2.2 Effector functions of phagocytes
2.2.1 Reactive nitrogen intermediates production

Macrophages, neutrophils and other phagocytic cells acts as key components of the antimicrobial and tumoricidal immune responses, by their ability to generate large amounts of highly toxic molecules such as reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) (Bogdan, C. et al 2000).

RNIs refers to oxidation states and adducts of the nitrogenous products of nitric oxide synthases and includes nitric oxide (•NO), nitrite (NO₂⁻), nitrogen dioxide (•NO₂), nitrate (NO₃⁻) and peroxynitrite (OONO⁻) (Nathan, C. et al 2000). Nitric oxide (NO) is a simple gas with free radical chemical properties and plays an important role in diverse physiological processes such as smooth muscle relaxation, neurotransmission, immune responses and inflammation (Lowenstein, C.J. et al 1992; Knowles, R.G. et al 1992).

Production of nitric oxide (NO) from L-arginine and molecular oxygen is catalyzed by NO synthase (NOS) enzymes and P-450-type cytoplasmic hemeproteins (in active form are homodimers containing FAD and FMN and require tetrahydrobiopterin and NADPH for activity) (Marletta, M.A. 1993).
At least three different NOS isoforms exist, each characterized by a unique pattern of tissue specific expression and regulation. The neuronal (n-NOS) and endothelial (e-NOS) types are constitutively expressed in neuroectodermal and endothelial cells, respectively and are reversibly activated by Ca$^{2+}$/calmodulin in vitro (Lowenstein, C.J. et al 1992). In intact cells, NO synthesis by constitutive NOSs is upregulated mainly by Ca$^{2+}$ influxes in a rapid and transient fashion (Bredt, D.S. et al 1990). In contrast, the third NOS isoform, so-called inducible-type or i-NOS/NOS2, is expressed to a significant levels in macrophages and related cell types in response to stimuli such as lipopolysaccharide (LPS), (Lowenstein, C.J. et al 1993; Xie, Q. et al 1993). The main rate limiting step for NO production by macrophages, and all other cell types expressing only the inducible NOS isoform, is the cellular concentration of the enzyme and Ca$^{2+}$ influxes play only a marginal role. In fact both NO production and i-NOS enzymatic activity are very low or undetectable in unstimulated macrophages and hepatocytes, both in vivo and in vitro, and they are efficiently induced within a few hours after stimulation of these cells with *Eisherichia coli* lipopolysaccharide (LPS) or γ-interferon (IFN-γ), (Geller, D.A. et al 1993).

### 2.2.1.1 Regulation of iNOS expression

While iNOS is turned on rapidly to enable the organism to respond to a wide range of pathogens, the expression of iNOS must be tightly controlled because NO is potentially capable of injuring host tissue indiscriminately. In many cell types, the crucial checkpoint for production of NO is at the level of transcription of the inducible nitric oxide synthase (iNOS) gene.

The promoter of iNOS gene, present at the 5' end is about 1749bp (Xie, Q. et al 1993). The promoter sequence can be divided into two regions. A 400 bp region immediately upstream of the main transcription site for the murine iNOS gene is responsible for LPS stimulated transcription and contain consensus sequence for binding to number of transcription factors. These include three nuclear factor interleukin 6 (NFIL6) binding sites, one tumor necrosis factor response element (TNF-RE), one NFkB binding site and one Oct site. A region further upstream defined as the distal enhancer region contains numerous elements with the potential for trans-acting factors. These include
NFkB site and several interferon regulatory binding elements (IRF-E), (Goldring, C.E. et al 1996).

Studies involving cloning and sequencing of the promoter region and by using reporter gene, have shown that both these areas the proximal promoter (region I) and distal enhancer (region II) are required for maximal induction of iNOS transcription (Lowenstein, C.J. et al 1993; Xie, Q. et al 1993). Both regions I and II are necessary for LPS-activated expression and only region II mediates IFNγ regulation (Lowenstein, C.J. et al 1993). In accordance with these findings it's seen that LPS and IFNγ responsive elements are selectively concentrated in two distinct regulatory regions (Goldring, C.E. et al 1996) and both regions I and II contain potential binding sites for NFkB (Lowenstein, C.J. et al 1993; Xie, Q. et al 1994).

2.2.2 Reactive Oxygen Intermediates (ROI) Generation

ROI refers to intermediate reduction products of O2 en route to water, including superoxide (O2⁻), hydrogen peroxide (H2O2) and hydroxyl radical (OH•), and reactive products of these with halides and amines (Nathan, C. et al 2000). The production of these ROIs via activation of the NADPH oxidase of phagocytes including macrophages and neutrophils in response to phagocytic or membrane stimuli, is known as respiratory burst and these reactive oxygen species play an important role in the bactericidal activity of these cells.

In unstimulated cells the NADPH oxidase is unassembled and inactive, and its seven protein components are segregated in the membrane and cytosolic locations (DeLeo, F.R. et al 1996). The activation of NADPH oxidase is elicited by microbial products (LPS and lipoproteins), IFN-γ, IL-8 or by IgG binding to Fc receptors on phagocytes. The NADPH oxidase is a highly regulated enzyme complex composed of a number of proteins: in the cytosol, gp40phox, gp47phox, gp67phox, and rho family GTPase, Rac1/Rac2, and in the membrane gp91phox—gp22phox—Rap1a, which together comprises cytochrome C. On activation, the cytosolic components migrate to the membrane and associate with the membrane bound components to assemble catalytically active oxidase.
The primary product of the reaction catalyzed by the NADPH oxidase is superoxide (O$_2^-$), which can be converted to H$_2$O$_2$ by superoxide dismutase (SOD), or to hydroxyl radicals (OH•) and hydroxyl anions (OH$^-$) by the iron catalyzed Haber-Weiss reaction (Bogdan, C. et al 2000).

RNIs and ROIs are important in immunological host defense and play critical role in bactericidal, anti-viral and anti-tumor agents (Nathan, C. 1992).

Murine Th2 cells also regulates Th1 activity via modulation of the induction of nitric oxide synthase in macrophages. According to the pattern of lymphokines secretion, murine CD4$^+$ T cells can be divided into two categories (Th1 and Th2). Th1 cells produce primarily IL-2 and IFN$\gamma$ whereas Th2 cells secrete IL-4, IL-5 and IL-10. A novel pathway exists by which Th2 cells regulate the activity of Th1 cells via the macrophages. Th1 cells produce IFN$\gamma$ which activates macrophages to kill intracellular parasites through the induction of NO synthase and the Th2 cells inhibit the induction of this enzyme via the IL-4 they secrete (Liew, F.Y. et al 1991). Similar to above, another cytokine of the Th2 subset, IL-10, in a dose dependent manner inhibits the microbicidal activity of IFN$\gamma$ treated macrophages against intracellular Toxoplasma gondii (Gazzinelli, R.T. et al 1992). Therefore, these findings on the downregulation of Th1 lymphokine production and inhibition of nitric oxide by IL-4 and IL-10 indicate that induction of IL-4/IL-10 may be an important strategy by which parasites evade IFN$\gamma$ dependent cell mediated immune destruction. Recent data also show that iNOS is required for the effects of IL-12 in innate immunity such as Macrophage and NK cell activation (Diefenbach, A. et al 1999). There exist hierarchies of crosstalk because IL-12 induces and to some degree works through iNOS (Diefenbach, A. et al 1999), iNOS in turn helps in downregulating IL-12 (Diefenbach, A. et al 1999).

### 2.2.3 Cytokines

Soluble mediators called cytokines or interleukins regulate the immune response as well as the effector phase of immune reactions. These cytokines play a role in immune regulation, hematopoiesis, and inflammation (Akira, S. et al 1990). Most cytokines are multifunctional and more than one cytokine can act on the same target cell and mediate the same or similar function(s). One of the major outer membrane components of Gram-negative bacteria, LPS, is a potent activator of monocytes and macrophages and results
in the release of immune mediators such as IL-1β, TNF-α and IL-12 (Nathan, C.F. 1987).

### 2.2.3.1 Interleukin-1β (IL-1β)

Mammalian IL-1 is a proinflammatory cytokine, produced particularly by mononuclear phagocytes, and other cell types like vascular cells, lymphocytes, neutrophils, and dendritic cells, in response to injury and infection (di-Givoine, F.S. et al 1990). The biological activity of IL-1 are encoded by two distinct genes, termed IL-1α and IL-1β, which share approximately 25% primary amino acid sequence identity. These two gene products have similar three-dimensional structures, bind to the same target cells, and elicit identical biological responses (Sims, J.E. et al 1988). IL-1β is the major form of IL-1 released from the cells, while IL-1α is primarily cell associated (Brody, D.T. et al 1989). Both IL-1α and IL-1β are synthesized as 31.5 kDa precursor proteins, and are proteolytically cleaved to generate mature proteins of 17 kDa (Kostura, M.J. et al 1989). While IL-1α is active even as a precursor protein, IL-1β proform is biologically inactive (Brody, D.T. et al 1989). The maturation of precursor IL-1β to its mature form is mediated by endoproteinase termed IL-1β-converting enzyme (ICE) (Thromberry, N.A. et al 1992).

IL-1β, is produced from macrophages in response to stimulation by LPS. IL-1β (Dinarello, C.A. 1996), together with TNF-α (Tracey, K.J. et al 1994) and IL-6 (Akira, S. et al 1993), contributes to the patho-physiology of septic shock and bacterially mediated local tissue destruction.

The expression of IL-1 is under stringent control. IL-1 activity is usually undetectable in normal biological fluids, but can be transiently induced in response to infection, inflammatory stimuli, and tissue injury. The activity of IL-1 is regulated at the transcriptional, post transcriptional, and posttranslational levels (Fenton, M.J. 1992). p38 mitogen activated protein kinase (MAPK) play a role in the regulation of IL-1β expression at the level of transcription. In some macrophage cell lines, p38 MAPK partially controls IL-1β transcription through the CCAAT/enhancer binding protein (C/EBPβ) and/or CEBPδ transcription factors (Baldassare, J.J. et al 1999). Also, NFIL-6 regulatory elements play an important role in the activation of IL-1β transcription.
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(Godambe, S.A. 1994). Several other factors including, PU.1 (Kominato, Y. 1995) and NF-κB (Hiscott, J. 1993), have also been seen to be involved in IL-1β transcription. LPS stimulated casein kinase II (CK II) phosphorylates ets transcription factor, PU.1, which in turn activates transcription of the IL-1β gene (Lodi, T.A. et al 1997). Role of pMAPK through C/EBPβ and C/EBPδ transcription factors and role of CK II through interaction with the PU.1 ets factor, show that at least two divergent signal pathways operate on the IL-1β promoter. Activation of monocytes by LPS results in induction of a number of different second messenger pathways, including the stress-activated transduction pathway, which includes the MAPKs. Three major enzymes make up the terminal kinases of the MAPK transduction pathway and include the extracellular signal-related kinase, p38, and c-jun N-terminal kinase (JNK). CK II is also activated through JNK MAPK, and, in turn, CK II phosphorylates PU.1, which is a necessary component of IL-1β transcription. These two divergent kinase pathways, p38 and JNK, influence the IL-1β promoter at the level of C/EBPβ/δ and PU.1 transcription factors, both of which are necessary for IL-1β transcription (Baldassare, J.J. et al 1999).

2.2.3.2 Tumor necrosis factor-α (TNF-α)

The family of tumor necrosis factors comprises three members: TNF-α, TNF-β (also known as lymphotoxin α, LT α) and LT-β (Eigler. A et al 1997). Macrophages are major source of TNF-α following immune and inflammatory stimulation of the host (Beutler, B. et al 1989). Monocytes/Macrophages produce TNF-α on stimulation with many different exogenous substrates such as lipopolysaccharide (LPS) and β-glucans, or by endogenous mediators such as IL-1 (Eigler. A. et al 1997). TNF-α is produced in large quantities (1-2 % of the total secretory products of activated cells) by both macrophage cell line (RAW 264.7) and thioglycollate elicited peritoneal macrophages (Beutler, B. et al 1989; Beutler, B. et al 1985). High concentrations of plasma TNF-α is produced in a variety of infectious and inflammatory diseases such as sepsis syndrome, bacterial meningitis and cerebral malaria (Tracey, K.J. et al 1994).

TNF-α has a subunit size of 17 kDa. In humans, the molecule is non-glycosylated: in other species like mouse, glycosylation occurs on a single N-liked site in the mature protein, but the sugar moiety is not essential for biological activity. Each monomer
consists of beta-pleated structure, and three monomers combine noncovalently to form the active hormone (Smith, R.A. et al 1987).

TNF-α is only synthesized de novo following activation and is efficiently exported from the cell. Since, its production and release can have deleterious effects upon the organism, its synthesis is tightly governed at multiple levels (Beutler, B. et al 1986; Gifford, G.E. et al 1986). In response to LPS, TNF-α gene transcription is accelerated approximately three fold, although TNF-α mRNA levels rises in the cell by a factor of 50 to 100 fold, and TNF-α protein secretion (which is undetectable in quiescent cultures) rises by a factor of about 10,000. TNF-α gene expression is controlled at post-transcriptional level by AU-rich sequences that reside within the 3'-untranslated region of TNF-α mRNA and imparts instability to the mRNA (Caput, D. et al 1986). Another transcription factor involved in the transcription of TNF-α gene is NF-κB (Collart, M.A. et al 1990; Shakhov, A.N. et al 1990). In uninduced cells, NF-κB is sequestered in the cytoplasm, and its activation involves a posttranslational process in which NF-κB dissociates from an inhibitory protein IκB and move to nucleus. The activation of NF-κB can be induced by a variety of agents such as phorbol esters (Baeuerle, P.A., et al 1988), cytokines (Shirakawa, F. et al 1989) and LPS (Baeuerle, P.A., et al 1988). Activated NF-κB can bind with different affinities to four κB-like elements in the TNF-α promoter. Four elements in the TNF-α promoter includes site at -510 which is very strongly bound, site at -850 which is also well bound and the sites at -655 and -210 that are only weakly recognized by NF-κB. Multiple copies of κB motif binds to these sites and results in activation of the TNF-α gene in macrophages (Collart, M.A. et al 1990).

2.2.4 Phagocytosis

Phagocytosis is a phylogenetically conserved process by which cells internalize large foreign particulate material, is used by unicellular organisms as a source of nutrients. In metazoans, phagocytosis is involved in the clearance of apoptotic cells during development and tissue remodeling. In mammals, phagocytosis is important in innate and adaptive immunity, and contributes to the ability to fight infectious diseases. Phagocytosis takes place in various cell types, ranging from epithelial cells to fibroblasts and circulating cells of the immune system, such as macrophages, neutrophils and
dendritic cells, which are known as professional phagocytes (Rabinovitch, M. 1995). Phagocytosis by macrophages is critical for the uptake and degradation of infectious agents and senescent cells, and it participates in development, tissue remodeling, the immune response, and inflammation (Aderem, A. et al 1999).

The binding of particles to cell surface receptor is the first step of phagocytosis. For recognition of particle, two groups of receptors are involved. First group comprises of receptors that bind to integral surface components of particles. These receptors on phagocytes are known as "pattern-recognition receptors" (PRRs) and the targets for these receptors "pathogen-associated molecular patterns" (PAMPs) (Janeway, C.A.J. 1992). Pathogen-associated motifs include mannans in the yeast cell wall, formylated peptides in bacteria, and lipopolysaccharides and lipoteichoic acids on the surface of Gram negative and Gram positive bacteria. Receptors on phagocytes that recognize these patterns include the mannose receptor (MR), integrins (CD11b/CD18) and scavenger receptors that recognize surface components on bacteria including LPS (Sastry, K. et al 1993). Other receptors include CD36, Scavenger receptor B1, which are involved in phagocytosis of apoptotic cells (Savill, J. 1998). Changes in the composition of cell surface sugars and a relocation of phosphatidylserine to the extracellular leaflet of the plasma membrane provide the ligands recognized in apoptotic cells by these receptors (Pradhan, D. et al 1997).

In contrast, receptors of the second group recognize molecules of host proteins, namely opsonins, by which particles are coated. The major opsonins found in serum are immunoglobulin G (IgG) antibodies and complement fragments C3b and iC3b. For IgG, coating particles, interact with their surface by means of F(ab')2 domains, leaving unoccupied Fc domain, which is recognized by Fcγ receptors (FcγRs). Complement receptor C3 (CR3) belongs to an integrin superfamily that comprises dimeric adhesion proteins.

There are a couple of models extensively analyzed to understand phagocytosis.

In Fc receptor mediated phagocytosis the first event triggered by the binding of particles is clustering of the receptors. Clustering of FcγRs triggers the activation of protein tyrosine kinases (PTK) and tyrosine phosphorylation of the receptor themselves on associated immunoreceptor tyrosine-based activating motif (ITAM), containing subunits, by member of the Src family (Fitzer-Attas, C.J. et al 2000).
phosphorylated receptor/subunit ITAMs then serve as docking sites for Syk leading to activation of the Syk kinase, which in turn triggers different pathways leading to transcriptional activation, cytoskeletal rearrangement, and the release of inflammatory mediators (Greenberg, S. 1995).

The mechanism of actin polymerization and formation of phagosomes is not precisely known, however it involves participation of a number of proteins including PI-3 kinase, the rho family of GTPases, protein kinase C (PKC), and motor proteins. PI3-kinase catalyzes phosphorylation of phosphatidylinositol (PI), PI (4) P and PI (4,5) P2, and is activated by many tyrosine kinase receptors that trigger the polymerization of actin (Toker, A. et al 1997).

Members of the rho family of GTPases regulate the actin cytoskeleton in response to a variety of extracellular signals. In macrophages various members of the rho family are involved in phagocytosis. Cdc42 is a member of rho GTPase family. GTP bound Cdc42 interacts and recruits WASp (Wiskott Aldrich Syndrome protein) to the plasma membrane in the vicinity of activated membrane-associated PTKs (protein tyrosine kinases) (Guinmard, R. et al 1998). PTKs Btk and Lyn phosphorylates membrane recruited WASp. Thus, WASp presents a connection between protein tyrosine kinase signaling pathways and Cdc42 function in cytoskeleton and cell growth regulation in hematopoietic cells (Guinmard, R. et al 1998).

Inhibition of Rac1 and Cdc42, by expression of their dominant negative forms in the RAW mouse macrophage cell line, inhibits phagocytosis by preventing the accumulation of F-actin in the phagocytic cup but has no effect on particle binding to FcRs (Cox, D. et al 1997).

Protein kinase C activated upon particle uptake is shown to accumulate around phagosomes (Allen, L.AH. et al 1995). Also sensitivity of FcR mediated phagocytosis to PKC inhibitors has been shown in monocytes and macrophages (Allen, L.AH. et al. 1996).

Motor proteins like myosin II accumulate on the phagocytic cups of macrophages and neutrophils ingesting yeast, and may act as a mechanical motor during particle internalization (Stendahl, O.I. et al 1980). Myosin I, myosin V, and myosin IX also co-localize with F-actin on forming phagosomes, and might facilitate ingestion (Swanson, J.A. et al 1999).
After internalization and phagosome formation, actin is shed, and the phagosome matures by a series of fusion and scission events with components of the endocytic pathway, culminating in the formation of the mature phagolysosome.

Other model involves mannose receptor mediated phagocytosis. The mannose receptor (MR) on macrophages recognizes mannose and fucose on the surface of pathogens and mediates phagocytosis of the organisms (Stahl, P.D. et al 1998). The high affinity of this receptor for branched mannose and fucose oligosaccharides, prototypic PAMPs, makes the mannose receptor a phagocytic receptor with broad pathogen specificity.

The mannose receptor is a single chain receptor with a short cytoplasmic tail and an extracellular domain including 8 lectin-like carbohydrate-binding domains. The lectin-like carbohydrate-binding domains share homology with other C-type lectins including the mannose binding protein, collectins, and the phospholipase A2 receptor (Taylor, M.E. et al 1990). The cytoplasmic tail is crucial to both the endocytic and phagocytic functions of the receptor, however, little is known about the signals that leads to phagocytosis (Stahl, P.D. et al 1998).

During mannose receptor-mediated phagocytosis of zymosan, the actin cytoskeleton is mobilized around the nascent phagosomes, and proteins such as F-actin, talin, PKCa, and Myosin I are recruited (Allen, L.AH. et al 1996).

2.2.5 Defensive protein against microbial invasion: Natural resistance associated macrophage protein (NRAMP)

In mice, resistance or susceptibility to infection with a number of antigenically and taxonomically unrelated intracellular parasites is determined by alleles of the chromosome 1 locus Bcg, also known as Ity or Lsh. Infections under the control of Bcg include several mycobacterial species such as *Mycobacterium bovis*, *Mycobacterium avium*, *Mycobacterium leprumurium*, *Salmonella typhimurium* and *Leishmania donavani* (Appelberg, R. et al 1990; Gros, P. et al 1981). The candidate gene for Bcg has been isolated using positional cloning and is Nramp1 (Natural Resistance Associated Macrophage Protein) (Vidal, S.M. et al 1993). Nramp is expressed exclusively in
macrophages and polymorphonuclear leukocytes and encodes a polypeptide with features typical of integral membrane proteins (Vidal, S.M. et al 1993; Cellier, M. et al 1994), including 10-12 possible transmembrane domains, a glycosylated extracytoplasmic loop, and an intracytoplasmic consensus transport signature (Bairoch, A. 1993). Sequence analysis of Nramp1 has shown that susceptibility to infection in Bcg^5 inbred strains is associated with a single non-conservative Gly169 to Asp substitution in the predicted TM4 of the protein (Malo, D. et al 1994). Nramp1 is not present in the plasma membrane of macrophages but is rather found in the late endosome fraction. Also, upon phagocytosis Nramp1 gets recruited to the membrane of the phagosome during the course of its maturation from early plasma derived phagosome to phagolysosome, and therefore becomes intimately associated with the membranous compartment containing the ingested parasites (Greunheid, S. et al 1997).

Another member of the Nramp family is Nramp2, that encodes a highly similar protein (77% similarity) (Greunheid, S. et al 1995). As opposed to its phagocyte specific Nramp1 counterpart, Nramp2 is expressed fairly ubiquitously in most tissues tested. Nramp comprises a very ancient family of proteins with highly conserved members in invertebrates (Drosophila melanogaster), plants (Oryza sativa), fungi (Saccharomyces cerevisiae), and bacteria (Mycobacterium leprae) (Cellier, M. et al 1995). This Nramp family has structural organization typical of families of ion transporters and channels (Cellier, M. et al 1995). Also Nramp family possesses an invariant sequence motif with similarity with ion permeation path of mammalian voltage gated channels (Wood, M.W. et al 1995). Together these observations suggest that Nramp1 may be a macrophage specific ion transporter.

A recent model proposes Nramp1 as a Mn^{2+} and/or Zn^{2+} transporter. Following phagocytosis of a parasite into the phagosome, the macrophage produces reactive oxygen and/or nitrogen intermediates that are toxic for the internalized bacteria (Segal, A.W. et al 1993). Phagosomes contain several plasma membrane proteins of which one is the Nramp protein. The survival of the pathogen during the burst of the macrophage respiratory activity is thought to be partly mediated by microbial superoxide dismutase (SOD) and other enzymes (Chan, J. et al 1992) that contain Mn^{2+}, Cu^{2+}/Zn^{2+} or Fe^{2+} in their active centre. Nramp, transports Mn^{2+} and/or Zn^{2+} from the extracellular milieu into the cytoplasm of a macrophage and after the generation of the phagosomes, removes
metal ions from the organelle. Thus the depletion of the metal ion from the phagosome microenvironment by the Nramp gene product may be a rate limiting step in the production of the metalloenzyme by the engulfed bacteria, this will restrict the mycobacterial ability to produce active enzymes such as SOD and prevent the propagation of the ingested microorganisms. Conversely, an increased concentration of metal ions in the phagosome caused by a defective Nramp tansporter (Bcg\textsuperscript{8}) may promote the growth of the mycobacteria and renders the organism sensitive to the pathogen. Thus Nramp acts as a divalent amphoteric cation transporter and modulates the divalent cation composition of the early phagosome, rendering it hostile to the parasite.

2.3 T cell priming functions of phagocytes

Activation of T cells requires two distinct signals (Lafferty, K.J. et al 1993). First signal which confers specificity of the response, is provided by antigen presenting cells (APC) in the form of presentation of major histocompatibility complex (MHC) antigen associated peptides and second in the form of distinct costimulatory signals provided by one or more distinct cell surface molecules expressed by APC interacting with coreceptors on the T cells (Steinmann, R.M. et al 1991). These costimulatory signals are neither antigen specific, nor MHC restricted yet are critical for the induction of maximal T cell proliferation, cytokine production, and development of effector function. There are reports suggesting that T cell receptor in the absence of these costimulatory signals may direct CD4\textsuperscript{+}T cells, particularly of the Th1-type into a state of functional non-responsiveness or towards programmed cell death (Liu, Y. et al 1990). There are large number of APC surface molecules that have been suggested to be involved in transmitting activation signals to counter receptors on CD4 T cells, including B7 (both B7-1[CD80] and B7-2[CD86]) interacting with CD28/CTLA-4 (Harding, F.A. et al 1992; Damle, N.K. et al 1992), ICAM-1 with LFA-1 (Van Seventer, G.A. et al 1990), CD48 with CD2 (Kato, K. et al 1992).
2.3.1 Costimulation

2.3.1.1 B7 superfamily

B7-1 and B7-2, are members of the immunoglobulin supergene family, are encoded by separate genes, and both molecules are capable of providing costimulatory function (Linsley, P.S. et al 1991; Hathcock, K.S. et al 1994). The B7-1/B7-2-CD28/CTLA-4 pathway is the best characterized T-cell co-stimulatory pathway and is crucial in T cell activation (Oosterwegel, M.A. et al 1999). B7-1/B7-2-CD28/CTLA-4 pathway includes two B7 family members, B7-1 (CD80) and B7-2 (CD86), that have dual specificity for two CD28 family members, the stimulatory receptor CD28 and the inhibitory receptor CTLA-4 (CD152). CTLA-4 is the high affinity receptor for B7-1 and B7-2. CD28 is constitutively expressed on the surface of T cells, whereas CTLA-4 expression is rapidly upregulated following T cell activation. B7-1 is normally expressed at low levels on "professional" APCs, such as dendritic cells, macrophages, and thymic epithelial cells. However, B7-1 is upregulated on these APCs as well as on B lymphocytes following activation by soluble factors (e.g. endotoxin and cytokine) or ligation of cell surface molecules (e.g. class II and CD40) (Bluestone, J.A. 1995). Unlike, B7-1, B7-2 is constitutively expressed on dendritic cells and macrophages and is rapidly upregulated on B cells following activation by cross-linking of the immunoglobulin receptor or the addition of a variety of cytokines (Hathcock, K.S. et al 1994).

2.3.2 Interleukin-12: a proinflammatory cytokine with immunoregulatory functions

Early in the infection Interleukin-12 (IL-12) is produced by phagocytic cells, in particular macrophages (D'Andrea, A. et al 1992), as a result of non-specific interaction with bacteria, bacterial products such as LPS and intracellular parasites. It induces production of IFNy from NK and T cells, which contributes to the phagocytic cell activation and inflammation (Trinchieri, G. et al 1995).

IL-12 is a 70 kDa (p70) heterodimeric cytokine, formed by two covalently linked glycosylated chains of approximately 40 kDa heavy chain (known as p40 or IL-12β) and 35 kDa light chain (known as p35 or IL-12α) (Kobayashi, M. et al. 1989). Simultaneous expression of the two genes encoding IL-12 p35 and p40 chains is required for the

IL-12 acts as growth factor for activated NK and T cells, enhances the cytotoxic activity of NK cells, and favors cytotoxic T lymphocyte generation (Kobayashi, M. et al. 1989, Wolf, S.F. et al 1991, Gubler, U. et al 1991). IL-12 directly or through effects on type-1 cytokines such as IFN-γ, enhances activation and production of Th1 associated classes of immunoglobulin. IL-12 also acts on hematopoietic progenitors in synergy with other colony-stimulating factors, and induces proliferation and colony formation (Trinchieri, G. 2003). In vivo IL-12 acts primarily at three stages during the innate/adaptive immune response to infection. Firstly, early in the infection, IL-12 is produced and induces production of IFN-γ from NK and T cells, which contributes to phagocytic cell and activation and inflammation (Chan, S.H. et al 1991). Secondly, IL-12 induced IFN-γ favor Th1 cell differentiation, by priming CD4+ T cells for high IFN-γ production (Kobayashi, M. et al. 1989, Chan, S.H. et al 1991). And lastly, IL-12 contributes to optimal IFN-γ production and proliferation of differentiated Th1 cells in response to antigen (Hsieh, C. et al 1993, Seder, R.A. et al 1993).

The early preference expressed in the immune response depends on the balance between IL-12, which favors Th1 responses, and IL-4, which favors Th2 responses. Thus, IL-12 represents a functional bridge between the early nonspecific innate resistance and the subsequent antigen-specific adaptive immunity (Trinchieri, G. 1995, Trinchieri, G. 2003).

2.3.3. Expression of MHC I and MHC II

Macrophages, B cells and dendritic cells can all act as antigen presenting cells (APCs). MHC molecule expression on macrophage cell surfaces plays a critical role in the initiation of acquired immune responses. Peptides in conjunction with surface molecules encoded by genes of the MHC are seen by mature T lymphocytes. CD8+ T cells use MHC class I molecules as restriction elements and CD4+ T lymphocytes see antigen (Ag) in association with class II molecules (Unanue, E.R. 1992). Resting T cells are not
induced unless they encounter Ag on the surface of appropriate APC. Foreign antigens arising from cellular debris are phagocytosed by host cells and presented in conjunction with MHC class I (Bevan, M.J. 1987). Macrophages act as accessory cells for CD8+ CTL during in vivo priming and cognate interactions between mature T cells and macrophages initiates MHC class I restricted responses. MHC class II molecules on macrophage surfaces are important in acquired immune responses against pathogens. The pathogens are phagocytosed and degraded into peptides, the immunogenic peptides are bound to MHC class II molecules and the MHC peptide complexes are transported to the cell surface.

IFNγ and LPS represent prototype cytokine and bacterial activators of macrophages, respectively. IFNγ, produced by NK and T cells during an infection, converts macrophages from a resting stage to an activated state, priming them for antimicrobial activity, increased killing of intracellular pathogens, and Ag processing and presentation to lymphocytes. These biological effects are mediated by increased proinflammatory cytokines production, NO production and MHC class II expression following IFNγ stimulation. IFNγ induction of MHC class II occurs at the level of gene transcription and requires more than 8 h after stimulation (Sicher, S.C. et al 1995). Also, during gram negative infections of macrophages in vivo, MHC class II expression is upregulated. However, post-transcriptional regulation of MHC class II is responsible for augmentation by LPS (Sicher, S.C. et al 1995). Upregulation of MHC class II levels on macrophages after LPS stimulation is apparent by 2 h of stimulation (Sicher, S.C. et al 1995). Thus, stimulation by both LPS and IFNγ results in an upregulation of MHC class II molecules on macrophages, although, by different mechanisms

2.4 Neutrophils

Neutrophils are most important leukocyte in peripheral blood. The neutrophils are part of the first line of defense against invading microorganisms and are unique among cells by possessing four types of exocytosable storage organelles (azurophil-, specific-, and gelatinase granula and secretory vesicles) (Borregaard, N. et al 1997). Each of these exocytosable organelles is characterized by its specific content of enzymes and antimicrobial proteins which are used by the neutrophil to exert its different biological
functions (Borregaard, N. et al 1997). The three granule types and the secretory vesicles are formed sequentially during granulopoiesis in the bone marrow and their appearance can be used to mark the stage of neutrophil differentiation (Borregaard, N. et al 1997). The maturation of the neutrophil is also characterized by a gradual condensation of the nucleus, which in combination with the cellular granulation results in distinct morphologies of the different neutrophil precursors. These maturation-associated changes of the morphology are accompanied by the sequential appearance of transcripts for granule and membrane proteins and a concomitant synthesis of their cognate proteins (Borregaard, N. et al 1997).

2.4.1 Granulopoiesis
Granulopoiesis is a process by which large number of granulocytes is produced daily from a small pool of pluripotent stem cells. Granulocytes develop from undifferentiated precursors present in bone marrow. The myeloblast is the first morphologically identifiable precursor of the neutrophil lineage, followed by the promyelocyte, myelocyte, metamyelocyte, and band form, which directly precede the mature neutrophil.

Granulopoiesis is a regulated process: its productive capacity can be increased at least 10-fold in response to certain stress conditions such as infection. Several products of activated T-lymphocytes, including GM-CSF, IL-3, and granulocyte colony stimulating factor, participate in the process of neutrophil maturation by direct interactions with their respective receptors, which are present on neutrophil precursor cells.

Granulocyte colony stimulating factor (G-CSF), a polypeptide growth factor, play a major role in regulating both basal and stress granulopoiesis (Demetri, G.D. et al 1991). The biological effects of G-CSF are mediated through its interaction with the granulocyte colony-stimulating factor receptor (G-CSFR), and mutations in G-CSFR is implicated in the etiology of a subset of severe combined congenital neutropenia (Dong, F. et al 1995). Multiple actions of G-CSF contribute to the neutrophilic response. First, G-CSF stimulates the proliferation of granulocytic precursors (Souza, L.M. et al 1986). Second, it reduces the average transit time through the granulocytic compartment (Souza, et al 1986). Finally, G-CSF may prolong neutrophil survival (Colotta, F. et al 1992). The importance of G-CSF to in vivo granulopoiesis is demonstrated in mice
carrying a homozygous null mutation for G-CSF; these mice has approximately 20 % of normal circulating neutrophils and a corresponding decrease in granulocytic precursors in their bone marrow (Lieschke, G.J. et al 1994).

2.4.2 Neutrophil trafficking from bone marrow to blood

Neutrophils, under normal conditions are produced solely in the bone marrow and are released into the blood in a regulated fashion to maintain homeostatic levels of circulating neutrophils. Two observations suggest that neutrophil release from the bone marrow is a highly regulated process. First, in response to inflammatory stimuli, such as infection, the number of neutrophils mobilized from the bone marrow is rapidly increased. Second, under normal conditions, only mature neutrophils and not granulocytic precursors are released in significant numbers into the blood. Though neutrophils play a critical role in innate immune responses, they may also be a major contributor to tissue damage in inflammatory disease. Thus, regulation of circulating neutrophil level is important in controlling both infectious and inflammatory diseases. Moreover, perturbations in the control of leukocyte egress from the bone marrow may contribute to the pathogenesis of leukemias.

The mechanisms that regulate neutrophil release from the bone marrow into the blood are largely undefined. Agents with distinct cellular targets and biological activities such as chemokines and cytokines (granulocyte colony stimulating factor [G-CSF]), microbial products (e.g. N-formyl- methionyl-leucyl-phenylalanine[fMLP]) and various other inflammatory mediators (e.g. C5a) can induce neutrophil release from the bone marrow (Jagels, M.A. et al 1992; Jagels, M.A. et al 1994; Semarad, C.L. et al 2002).

2.4.3 Integration of inflammatory signals by neutrophils

Inflammation is a complex defense mechanism characterized by leukocyte migration from the vasculature into damaged tissues to destroy the injurious agents. Acute inflammation is a limited beneficial response particularly during infectious challenges, whereas chronic inflammation is a persistent phenomenon, which can progress to inflammatory diseases. One hallmark of acute inflammation is that the leukocyte infiltrate is initially mostly neutrophilic but after 24 to 48 h, monocytic cells
predominated (Melnicoff, M.J. et al 1989). By contrast, chronic inflammation is histologically associated with the presence of mononuclear cells, such as macrophages and lymphocytes (Melnicoff, M.J. et al 1989). At sites of inflammation, approximately 95% of the myeloid cells are recruited to, rather than resident at those sites.

In different models of acute inflammation, neutrophils are the first cells to accumulate in tissues (Melnicoff, M.J. et al 1989), possibly because 1) of the high concentration of these cells in the blood compared to monocytes and 2) of the kinetics of expression of leuko-endothelial adhesion molecules.

Adhesion of leukocytes to endothelium is a multi-step process characterized by an initial weak interaction mediated by selectins and their carbohydrate ligands, giving a rolling motion to leukocytes. Rolling enables leukocyte activation by chemotactic agents associated with endothelial-cell membrane, inducing activation of β integrins on the white cell surface. These molecules interact with endothelial members of the immunoglobulin superfamily for firm leuko-endothelial adhesion (Springer, T.A. 1994).

Leukocytes then begin to cross the endothelial layer through homologous interactions of PECAM-1 [platelet endothelial-cell adhesion molecule (CD31)] expressed on both leukocytes and the intercellular membranes of the endothelial cells, and migrate following a chemoattractant gradient initiated in the injured tissue (Newman, P.J. 1997). Neutrophil adhesion is mainly supported by the selectins CD62L on white cells and CD62P on endothelium, the leukocyte β2 integrins CD11a/b (CD18) and the two endothelial members of the immunoglobulin superfamily, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2. Each of these molecules are either constitutively present on leukocytes and endothelium such as CD62L, CD11a (CD18), ICAM-1, ICAM-2 or are very rapidly translocated, activated and upregulated, for example, CD62P, CD11b (CD18) and ICAM-1. Adhesive interactions of β2 integrins are able to initiate intracellular signaling events and participate in the activation of various neutrophil functions (Nathan, C. et al 1989). These effects require integrin aggregation, which is induced by binding to immobilized, but not soluble, ligands (Miyamoto, S. et al 1995). In this context it's seen that the formation of fibrin during clotting may provide an appropriate matrix to induce these β2 integrin-mediated outside-in signaling events, which may regulate and/or activate neutrophil functions.
Additionally, neutrophil recruitment is facilitated by chemotactic agents, such as C5a, leucotriene B4 (LTB4) or the platelet activating factor which is synthesized in minutes. Also, the chemokine IL-8 (CXCL8), which is important for neutrophil recruitment in vivo, exists as preformed pool in endothelial cells (Utgaard, J.O. et al 1998). By contrast, the endothelial selectin CD62E, the β1 integrin VLA-4 on leukocytes and the endothelial cell adhesion molecule-1 (VCAM-1), which are more important for monocyte adhesion, are absent from the surface of quiescent leukocytes or endothelium and require several hours of de novo synthesis. Therefore, the mediators necessary for neutrophil adhesion are present early in the course of inflammation, whereas molecules leading to monocyte adhesion appear several hours later (Kaplanski, G. et al 2003).

A transition from granulocytes to monocyte accumulation at the inflammatory site not only results in a progression of events leading to monocyte recruitment but also results in the disappearance of granulocytes. Activation of granulocytes by phagocytosis of bacteria or more globally by phagocytosis mediated by Fc or complement receptors induces upregulation of programmed cell death genes and apoptosis (Kobayashi, S.D. et al 2002). Some cytokines and growth factors, such as granulocyte colony stimulating factor (G-CSF) prevent granulocytes death but cytokine such as IL-6, induce neutrophil apoptosis (Afford, S.C. et al 1992). Death by apoptosis induces loss of granulocytes biological functions but prevents liberation of granulocytes intracellular toxic contents and further tissue injury (Whyte, M.K.B. et al 1993).

### 2.5 LPS and TLR mediated signaling

The recognition of a pathogen is mediated by a set of germline encoded receptors that are referred to as pattern-recognition receptors (PRRs) and which can directly recognize invariant molecular structures (pathogen associated molecular patterns [PAMPs]) which are shared by a large group of micro-organisms (Medzhitov, R. et al 1997). There are three functional classes of PRRs: signaling receptors, endocytic receptors and secreted proteins. Toll receptors are type I transmembrane proteins that are evolutionarily conserved between insects and humans (Anderson, K.V. 2000). Toll was first identified as an essential molecule for embryonic patterning in *Drosophila* and was subsequently shown to be key in antifungal immunity. Homologous families of Toll receptors, the so-
called TLRs, exist in mammals. Based on the similarity in the cytoplasmic portions (designated the Toll-IL-1R, or TIR domain), TLRs are related to IL-1 receptors (IL-1Rs). However, the extracellular portion of TLRs contains leucine-rich repeats, whereas IL-1Rs contains three immunoglobulin-like domains.

TLR family members are expressed differentially among immune cells and appear to respond to different stimuli. TLR expression is also observed in a variety of other cells, including vascular endothelial cells, adipocytes, cardiac myocytes and intestinal epithelial cells.

Toll-like receptor 4 (TLR4) was identified as the receptor for the gram-negative bacterial product lipopolysaccharide (LPS). This receptor was identified through analysis of a strain of mice, C3H/HeJ, that is hyporesponsive to LPS. The C3H/HeJ mouse strain carries a missense point mutation within the Tlr4 gene region encoding the cytoplasmic tail and this mutation changes a highly conserved proline to histidine (Poltorak, A. et al 1998). TLR4−/− mice are also hyporesponsive to LPS as C3H/HeJ mice, confirming that TLR4 is required for LPS signaling (Hoshino, K. et al. 1999).

TLR mediated signaling involves MyD88 (myeloid differentiation factor) and IRAKs (IL-1 receptor associated kinases), as key proximal signaling components regulating activation of the pro-inflammatory transcription factor, nuclear factor kappa B (NFkB), in response to LPS (O’Neill, L.A. 2002). Both TLR2 (receptor for bacterial products such as peptidoglycan) and TLR4 recruit an adapter protein homologous to Myd88, which has been termed Mal (Myd88 adapter-like protein) or TIRAP (Toll/IL-1 receptor adapter protein), and shown to interact with TLR4 and MyD88 and mediate NFkB signaling (Yamamoto, M. et al 2002).

Recent study has shown role of Btk in TLR-mediated signaling (Jefferies, C.A. et al 2003). Btk has been shown to interact with the TIR domain of TLR4, 6, 8 and 9. Btk interacts with Myd88, Mal and IRAK-1 but not TRAF-6, suggesting that Btk is recruited to a multiprotein complex containing these proteins. Also, involvement of Btk in LPS signaling via TLR4 was found and it was shown that inactive mutants of Btk inhibited LPS-signaling to NFkB activation.