A Peptide Array for Bovine-Specific Kinome Analysis: Comparative Analysis of Bovine Monocytes Activated by TLR4 and TLR9 agonists

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ABSTRACT

As phosphorylation represents the pivotal mechanism for regulation of biological processes, kinases belong to one of the most biologically significant enzyme classes. The development of analytical techniques for characterization of kinase activity, in particular at a global scale, is a central priority for proteomic and cell biology researchers. In order to facilitate global analysis of cellular phosphorylation, a new paradigm of microarray technology which focuses on analysis of total cellular kinase activity, kinome, has emerged in the past few years. As the specificity of many kinases is dictated primarily by recognition of residues immediately surrounding the site of phosphorylation a logical methodology is to employ peptides representing these immediate sequences as experimental substrates. Microarray chips carrying hundreds of such substrate targets have been developed for human kinome analysis, however, lack of similar tools for species outside research mainstream has limited kinome analysis in these species.

Based on sequence alignment of orthologous phosphoproteins from mammalian species, conservation of amino acid identity is reported to be 80 %. Accordingly, the potential exists to utilize phosphorylation sequence databases to extrapolate phosphorylation sites in other species based on their genomic sequence information. Peptides representing these proposed phosphorylation sites can then be utilized as substrates to quantify the activity of the corresponding kinase. Based on these principles, a bovine microarray of 300 unique peptide targets was constructed. The bovine phosphorylation targets were selected to represent a spectrum of cellular events but with focus on processes related to innate immunity.

Initial application and validation of the bovine peptide arrays was carried out for kinome analysis of bovine blood monocytes stimulated with either lipopolysaccharide (LPS) or CpG-ODNs; ligands for Toll-like receptors (TLR) 4 and 9, respectively. The arrays confirmed activation of the known TLR signaling pathway as well as identifying receptor-specific phosphorylation events. Phosphorylation events not previously attributed to TLR activation were also identified and validated by independent bioassays. This investigation offers insight into the complexity of TLR signaling and more importantly verifies the potential to use bioinformatics approaches to create tools for species-specific kinome analysis based on genomic information.

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LIST OF ABBREVIATIONS

AP-1	activating protein 1
APC	antigen presenting cell
ATP	adenosine triphosphate
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
Casp8	caspase 8
cDC	conventional dendritic cell
cDNA	complementary DNA sequence
COX-2	cyclo-oxygenase-2
CpG	cytosine-phosphate-guanosine
DD	death domain
dsRNA	double-stranded RNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular regulated MAP kinase
EST	expressed sequence tag
FADD	Fas-associated death domain
FAK	Focal Adhesion Kinase
FBS	fetal bovine serum
GPI	glycosyl phosphatidylinositol
H. pylori	*Helicobacter pylori*
HIV	human immunodeficiency virus
HRP	Horse radish peroxidase
HSV	herpes simplex virus
IFN	Type I Interferon
IKK	IκB kinase

IL	interleukin
IL-1R	interleukin-1 receptor IRAK interleukin-1 receptor-associated kinase
iNOS	nitric oxide synthetase
IRF	interferon regulatory factor
IκB	inhibitor of kappaB
JNK	c-Jun N-terminal kinase
L. pneumophila	*Legionella pneumophila*
LBP	lipopolysaccharide binding protein
LP	lipoprotein/lipopeptide
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LTR	long terminal repeat
MAP3K	mitogen-activated protein kinase kinase kinase
MAPK	mitogen-activated protein kinase
MHCI/II	major histocompatibility complex class I/II
MMTV	mouse mammary tumor virus
MyD88	myeloid differentiation primary response gene 88
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa B
NK	natural killer cell
NVOC	nitroveratryloxycarbonyl- γ -amino butyric acid
ODN	oligodeoxynucleotide PAMP pathogen-associated molecular pattern
ORNs	synthetic oligoribonucleotides
PAMPs	pathogen-associated molecular patterns
PBSA	phosphate-buffered saline
PBMC	peripheral blood mononuclear cell
PD	phosphodiester
pDC	plasmacytoid dendritic cell
PKA	protein kinase A
PMSF	Phenylmethylsulphonyl fluoride
poly(I:C)	polyriboinosinic:polyribocytidylic acid

PRR	pattern recognition receptor
PTO	phosphorothioate
RNase	RNA endonuclease
RSV	respiratory syncytial virus
SAM	self assembled monolayer
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
SPR	surface plasmon resonance
ssRNA	single-stranded RNA
TBST	Tris-buffered saline with Tween-20
TICAM-1	Toll-IL-1 receptor domain containing adaptor molecule-1
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TLR-/-	TLR knockout
TNF	tumor necrosis factor
TRAF	TNF-receptor-associated factor
Y. pestis	*Yersinia pestis*

1.0 LITERATURE REVIEW

1.1 Kinases and Phosphorylation

The reversible modification of proteins through the enzymatic addition of phosphoryl groups to the hydroxyls of specific amino acids such as tyrosine, serine and threonine is speculated to occur on up to half of the human proteome (Zhang *et al*., 2002). While the phosphohydroxyl linkages are chemically stable, they are readily reversed through the action of opposing phosphatase enzymes. The net phosphorylation status of the phosphoproteome, therefore, represents the collective influences of the protein kinases and phosphatases.

An estimated 2% of the genes within the human genome code for either protein kinases or phosphatases (Hunter, 1995; Manning *et al*., 2002; Alonso *et al*., 2004). This corresponds to over 500 distinct kinases, only a fraction of which have been characterized with respect to their substrate targets and physiological functions. The frequency of protein phosphorylation is matched by its functional importance. Phosphorylation serves to regulate a variety of critical aspects of protein behavior including activity, stability, cellular localization and interacting partners. Regulation of protein function through phosphorylation is observed in virtually every cellular process including metabolism, cell division, apoptosis and signal transduction. As important players in signal transduction pathways, protein kinases play roles in transducing, amplifying or integrating upstream signals.

There are two main groups of kinases: protein serine/threonine and tyrosine kinases. Such classification is based on the substrate specificity of kinases. Eukaryotic protein kinases belong to a single superfamily of protein kinases (ePKS) and within their catalytic domains, share extensive sequence and structural homologies. The kinase catalytic domain consists of 11 subdomains (Hanks and Hunter, 1995) and is folded into a smaller N-terminal and a larger C-terminal lobe. While the N-terminus is the ATP binding site and contains a glycine rich loop (P-loop), the C-terminus is considered the activation lobe and consists of a conserved activation loop (T-loop) which is regulated by phosphorylation. A substrate binds to the cleft between the N and C-terminal lobes (Kobe *et al*., 2005). Human kinases, in addition to the catalytic domain, carry other functional domains. To date, 83 different domains have been reported in the human kinome (Krupa and Srinivasan, 2002; Manning *et al*., 2002). These domains are involved in

other aspects of kinase functions such as molecular interactions, localization and regulation. For example, Src homology 2 (SH2) and SH3 domains are two commonly encountered kinase domains (Manning *et al*., 2002).

1.1.1 Kinase Specificity

Cellular signaling accuracy and integrity depends highly on kinase specificity. Two critical elements determine kinase specificity: 1) Identity of phosphorylation target and residues around it and 2) recruitment of substrate.

Binding of protein kinases to their substrates have been investigated through the analysis of the three-dimensional structures of protein kinases bound to peptide substrates. It is been reported that both classes of kinases bind their substrates in an extended conformation with the same orientation of the substrate peptide chain relative to the protein kinase (Hubbard *et al*., 1997; Brinkworth *et al*., 2003). Early investigations into the kinase specificity were focused on the importance of phosphorylation sites and residues around it (Tessmer *et al*., 1977; Kemp *et al*., 1990) leading to the subsequent tabulation, compilation of databases and rationalization of phosphorylation motifs (Kreegipuu *et al*., 1998).

The second factor that plays an important role in kinase specificity is recruitment of substrate by the kinase which plays a critical role in determining substrate preference. Substrate recruitment is any process that can bring a kinase and a substrate in close proximity, thus increasing the effective concentration of the substrate and the chance of forming the enzyme–substrate complex. Some of the mechanisms of substrate recruitment inclue binding of the substrate to the regulatory domain of a kinase, or to a site distinct from the active site on the catalytic domain of the kinase (Lee *et al*., 2004); binding of the kinase and substrate to the same scaffolding protein (Faux and Scott, 1997); or co-localization of the enzyme and the substrate to a small subregion of the cell.

1.2 Methods of Studying Phosphorylation

While a variety of experimental approaches have been developed for analysis of cellular phosphorylation, they can be functionally divided into two categories, kinome and phosphoproteome analysis. The distinction is based upon whether the focus is on the protein kinases that mediate cellular phosphorylation events, the kinome, or the protein targets of these

enzymes, the phosphoproteome. While these terms are often used interchangeably, they represent distinct experimental approaches, albeit to the same biological phenomena.

The value of either kinome or phosphoproteome analysis is determined by the ability to accurately describe the underlying biology of cellular phosphorylation. Phosphoproteome and kinome investigations are generally comparative in nature with interest in characterizing differential patterns of phosphorylation in response to a particular stimulus or cellular event. Within this context there are a number of questions that are typically of interest: 1) Which proteins are phosphorylated and at what sites? 2) Under what conditions are particular proteins differentially phosphorylated? i.e under what conditions are the associated kinases activated? 3) Which kinases mediate these phosphorylation events? and 4) What are the functional consequences of phosphorylation both to individual proteins being modified as well as to the overall cellular response? There is no one analytical technique that can single-handedly address all these questions, but through the application of the complimentary approaches of phosphoproteome and kinome analysis a more comprehensive description of phosphorylation events is obtained.

While the analytical capabilities for the description of the phosphorylation status of a single protein, or the quantification of the enzymatic activities of a single kinase, are becoming increasingly routine, the major challenge to both phosphoproteome and kinome analysis is for the development of capabilities for global characterization. This reflects a growing trend in biological investigations towards more systems approach to more fully appreciate the context and interplay of biological responses.

1.2.1 Phosphoproteome Analysis

The objective of phosphoproteome analysis is to define the members of this sub-population of proteins and to identify their specific points of modification. While this shares many of the challenges associated with traditional proteomic analysis, it is experimentally complicated by the scarcity of many of the members of the phosphoproteome, the dynamic nature of this entity and the tendency of the remainder of the proteome to overwhelm, or actively suppress, detection of the phosphoproteome.

One of the most significant challenges to phosphoproteome analysis is the low abundance of phosphoproteins relative to the proteome. This consists of two elements. Firstly,

while a significant fraction of the proteins undergoes phosphorylation, many of these proteins, in particular those which are involved in signal transduction, are expressed at very low levels. Therefore the proteins that are of greatest interest are among the most poorly represented. This is exasperated by the fact that many of these low abundance proteins are phosphorylated in sub-stoichiometric levels such that only a small fraction are modified at any given time. It is estimated that only 1-2% of the total amount of an individual member of the phosphoproteome is present in the phosphorylated form (Schlessinger, 1993; Mann *et al*., 2002; Kalume *et al*., 2003; Raggiaschi *et al*., 2005).

As a consequence of their low abundance, one of the central challenges and prerequisites for phosphoproteome analysis is the ability to discriminate these modified proteins from the remainder of the proteome. This can be achieved either through enrichment of phosphorylated proteins on the basis of the post-translational modification, prior to experimental analysis, or by separation of all the members of the proteome with subsequent phosphorylation-specific detection methods. In either strategy, the techniques employed need to be reproducible, specific for phosphorylation, but generally applicable to different types of phosphorylation. A number of experimental approaches have been employed to this end, most notably two-dimensional electrophoresis, antibody microarray and mass spectrometry.

1.2.2 Kinome Analysis

While the well-defined and highly conserved chemistry of phosphorylation reactions make it straightforward to characterize the enzymatic activity of an isolated kinase and its substrate, the challenge is in the application to global models. There are two primary obstacles to the development of a technology that permits global analysis of kinase activity. These are the creation of appropriate kinase substrates and the expansion of scale to permit simultaneously evaluation of hundreds of kinases to thousands of phosphorylation sites. Assays for the evaluation of the activity of a single kinase enzyme are relatively straight-forward provided an appropriate substrate molecule is available. The challenge of expansion of scale parallels those encountered in gene expression analysis in the transition from Northern blot analysis of selected genes to analysis of global patterns of gene expression. Not surprisingly, as array technology permits global analysis of gene expression, arrays have similar potential for investigations of protein-based biological events.

1.2.2.1 Peptides as Kinase Substrates

For many protein kinases, recognition of phosphoacceptor substrates has been shown to be dependent on amino acid residues surrounding the site of phosphorylation rather than higher order tertiary structure effects (Kemp *et al*., 1975). Synthetic peptides modeled on the site of phosphorylation can be excellent kinase substrates with values of Vmax and Km approaching that of the natural substrate (Zetterqvist *et al*., 1976; Kemp *et al*., 1977).

Utilization of such peptides has various advantages over complete protein sequences: they are easily synthesized and manipulated, are inexpensive to produce and highly stable. Preparation of arrays having hundreds to thousands of immobilized peptides offers an unprecedented opportunity for investigating the activities of hundreds of kinase enzymes and serve as valuable tools for profiling cellular activities.

In some cases peptide substrates may not serve as ideal ligands for the corresponding protein kinase. There is the danger for the assignment of false positives as a result of phosphorylation of a particular peptide by a kinase other than the one that mediates cellular phosphorylation. For this reason it is important to verify kinomic results through alternative methodologies that permit direct characterization of phosphorylation status such as phospho-specific Western blots. That the peptides are recognized by the correct protein kinase, but with lower efficiency than when the sequence is in the context of an intact protein, also presents the danger for false negatives where the activity of a kinase may be underestimated. For this reason it is difficult to make quantitative comparisons of the levels of activities of different kinases based upon the extents of phosphorylation of their corresponding peptides substrates. It is more appropriate to make comparison of the relative levels of activity of a particular kinase under different experimental conditions.

1.2.2.2 Peptide Arrays for Kinome Analysis

Array technology is being applied to monitor protein-based biomolecular interactions, including protein-substrate interactions (Blackburn and Hart, 2005), receptor-ligand interactions, antibody-antigen recognition (Kukar *et al*., 2002), determination enzymatic activities (Zhu *et al*., 2003), and specificities (Kim *et al*., 2005). Not surprisingly, it appears as though similar application of array technology will permit the advancement of kinome analysis.

One of the earliest applications of array technology for kinome analysis was screening the majority of kinases of yeast against a limited number of selected substrates. In this investigation full-length proteins were printed onto the arrays and evaluated for their ability to act as substrates for the yeast kinases (Zhu *et al*., 2000). While these arrays were simple relative to the total proteome, this was a pivotal study for demonstrating the potential for array technology to permit rapid screening of substrates for determination of kinase specificity.

Synthetic peptide libraries have served as valuable tools for investigation of kinase specificity determinants (Till *et al*., 1994; Songyang *et al*., 1995). A critical outcome of these investigations was the demonstration of the ability for peptides, specifically in the context of arrays, to function as comparable ligands as the natural protein targets. Through the marriage of peptides as protein kinase substrates, and the application of array technology, it becomes feasible to construct an array that will permit large scale evaluation of the kinome. The potential for such an approach was first demonstrated by Houseman and Mrksich with an array constructed with peptides substrates to a non-receptor tyrosine kinase c-Src (Houseman and Mrksich, 2002). It was verified that this tool could be used to quantitatively evaluate kinase activity. Hence, in principle, an array exhibiting specific consensus sequences for protein kinases across the entire kinome would allow a comprehensive detection of signal transduction events in whole cell lysates. The application of peptide arrays for studying signal transduction events that are associated with specific stimuli or cellular events have been reported. One of the earliest reports involved generation of the first comprehensive description of the temporal kinetics of phosphorylation events induced by LPS. This methodology identified a novel kinase whose involvement in Toll-like signaling was not previously suspected and the results were confirmed through more traditional forms of analysis (Diks *et al*., 2004). Peptide arrays also proved a powerful tool for investigation of effects of glucocorticoid on insulin signaling pathways. An array with 1176 different kinase consensus substrates was employed to demonstrate inhibitions of the insulin receptor and several downstream signaling intermediates in response to glucocorticoid treatment. Importantly these effects were transcriptional-independent and would have been undetectable by traditional genomic analysis (Lowenberg *et al*., 2005b). In a related investigating the application of the same peptide array was able to demonstrate that the immunosuppressive effects of glucocorticoids are mediated through the tyrosine kinases Lck and Fyn (Lowenberg *et al*., 2005a).

1.2.2.3 Detection of Peptide Array Signals

A number of strategies are available for the detection and quantification of the incorporated phosphoryl groups to immobilized peptides. These include radiolabeling, fluorescent antibodies and chelators as well as surface plasmon resonance (**Figure 1.1**).

Radioisotopes: When [γ-^{32}/^{33}P]-ATP is added to the kinase reaction mix, transfer of a radiolabeled phosphoryl group is catalyzed to a threonine, tyrosine or serine residue of the consensus sequences on the array (MacBeath and Schreiber, 2000; Falsey *et al*., 2001; Diks *et al*., 2004). Phosphorylation can be detected and quantified by exposing the array to a Phosphor Imager screen (Falsey *et al*., 2001; Diks *et al*., 2004).

Care must be taken in employing [γ-^{32}P]-ATP with peptides spotted on cellulose supports as the high peptide densities can create electrostatic traps for ATP, in particular with cationic peptides, creating false positives. Glass slides are more amenable to radioactive detection methods as their peptide density are approximately six orders of magnitude lower. Even though the usage of isotopes provides a very sensitive way to measure phosphorylation, due to the risk of handling and usage of radioactive material, researchers are turning to alternative techniques.

Surface Plasmon Resonance Imaging: Surface plasmon resonance (SPR) imaging is a well established technique for studying molecular interactions (Rich and Myszka, 2000). However, it is still in its infancy for use with peptide array technology. An SPR biosensor characterizes interaction of molecules on a solid surface and a solution. The solid surface of a peptide array is required to be coated with gold or other noble metals (Wegner *et al*., 2002). As a proof-of-concept Wegner et al. monitored interaction of peptides on a microarray with antibodies in solution (Wegner *et al*., 2002). Inamori's group established a new method: capturing phosphorylated residues of peptides with biotinylated zinc (II) complex and detecting them with streptavidin by SPR technique (Inamori *et al*., 2005). This detection method is independent of the amino acid residues so that detection all phosphorylated peptides on an array is possible using a single-probe complex. This system is quantitative for phosphorylation efficiency.

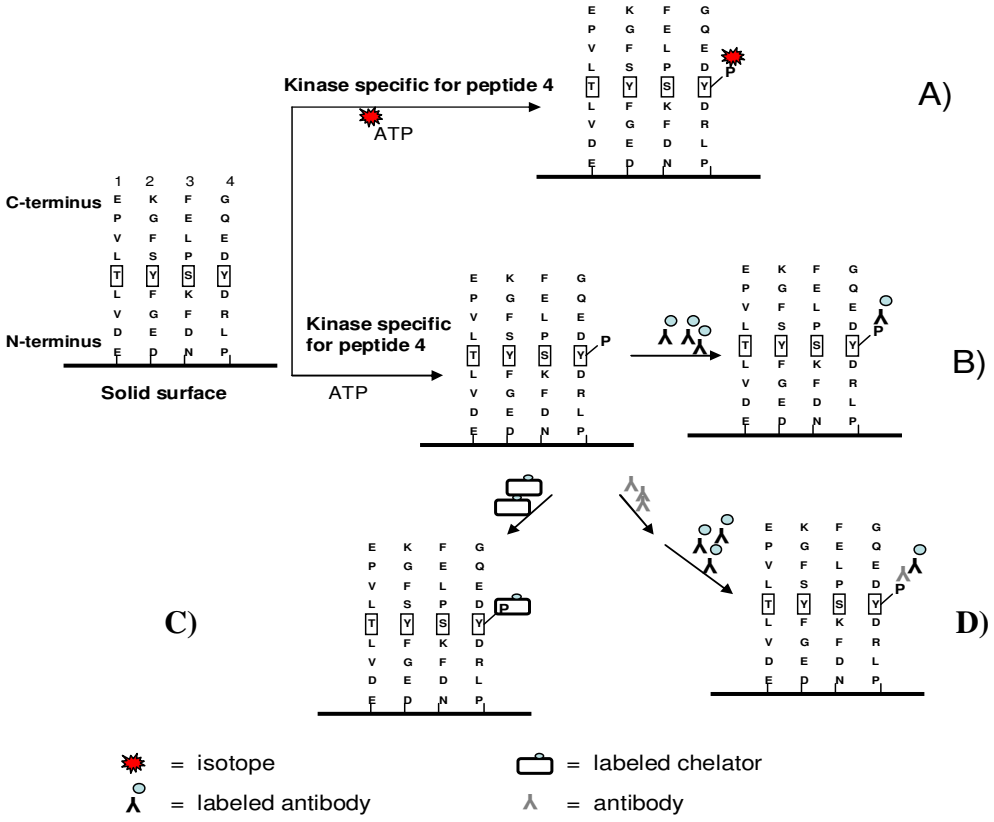

Figure 1.1. Summary of methods for detection of phosphorylated peptides. A) ATP labeled with an isotope such as $^{32/33}$P is added during the kinase reaction and phosphorylation is detected with a Phospho-Imager. B) Fluorescently labeled Phosphorylation-specific antibodies are added after the kinase reaction is complete, and phosphorylated peptides are detected by a fluorescence scanner. C) A labeled phosphoamino acid chelator is added after the kinase reaction is complete. Surface plasmon resonance, MALDI-TOF and MS is used for detection D) Phosphorylation-specific antibodies are added first to bind to phosphorylated residues and then fluorescently labeled antibodies are added to bind phosphorylation-specific antibodies. Adapted from Jalal 2007.

Fluorescence: This technique relies on fluorescently-labeled antibodies or chelators that bind to phosphorylated residues. Lesaicherre *et al.* were the first to develop this detection method (Lesaicherre *et al*., 2002). After the kinase reaction is completed, fluorescently-tagged antibodies are added onto the slide to bind the phosphorylated residues of the peptides. Binding of antibodies to the peptides is very sensitive; as little as femtomol antibodies of phosphorylated epitopes can be recognized by the antibodies (Lesaicherre *et al*., 2002). A fluorescence scanner is used to scan an image of the array. Unlike the radioisotope based technique, this method is safe and phosphorylation can be detected immediately after antibody binding.

The concerns associated with antibodies for detection of phosphorylation events parallel those presented with the application of phosphospecific antibodies in 2D-gels. A recent investigation examined the ability for phosphospecific antibodies to recognize their epitopes in the context of a peptide array. While monoclonal antibodies to phosphotyrosine residues demonstrated a high degree of specificity, antibodies for phosphoserine residues gave high rates of false positives and phosphothreonine antibodies showed concerning levels of cross-reactivity with the phosphoserine residues (Panse *et al*., 2004).

1.2.2.3.1 Quantification of Kinome Activity

While the extent of phosphorylation of a particular peptide is readily quantified, comparative analysis across different peptides is problematic as the ability for individual kinases to recognize and act on the appropriate sequences in the context of a peptide array will be unique for each kinase. Therefore while the absolute activities of the kinases cannot be determined, it is possible to report relative levels of activities of individual kinases under different physiological conditions.

1.2.2.4 Peptide Array Design

1.2.2.4.1 Knowledge-based Peptide Design

Online databases such as Phosphosite (www.phosphosite.org) and Phosphobase (phospho.elm.eu.org) are compilations of experimentally reported and manually curated peptide substrates for human, mouse and rat kinases. *In silico* analysis of such online resources can

allow identification of substrates of interest. *In silico* analysis of the Phosphobase enabled identification of consensus amino acid phosphorylation sequences for most kinases present in the mammalian genome (Kreegipuu *et al*., 1999).

For example, Diks *et al.* produced a peptide array, in which peptides were derived from *in silico* analysis of Phosphobase data. Their analyses revealed that peptides of nine amino acids in length could provide sensitive and specific binding to the kinases of interest. The nanomers were chemically synthesized and covalently immobilized onto glass slides. This array proved of considerable value in characterizing signal transduction events associated with activation of the Toll-like system (Diks *et al*., 2004).

1.2.2.4.2 Bioinformatics for Sequence Determination

Phosphobase and Phosphosite contain phosphorylation based data for well studied species such as human, rat and mouse. While such data are beneficial to researcher concerned with these species, not much data is available for other species. Our research group is interested in bovine kinomic analysis which requires a bovine-specific array.

To obtain bovine-specific consensus sequences we relied on sequence comparison and homology to collect a list of consensus peptides for bovine kinases of interest. By first identifying human peptides from Phoshobase and Phosphosite and employing the Blastp program from National Center for Biotechnology Information(NCBI) to compare collected human peptides against the NCBI bovine protein database. Blastp was set to retrieve short exact matches.

1.3 The Innate Immune System

A pathogen's detection and disposal, without damage to the host tissue, involves various mechanisms by the host immune system. Given the high molecular diversity of pathogens, and the frequent degree of mutations, the mammalian immune system has evolved mechanisms to meet such challenges. The mammalian immune system has been traditionally divided into two components: the innate and adaptive immunity (Hoffman *et al*., 1999). Adaptive immunity is responsible for specific responses and long-term memory of encountered immunological challenges while innate immunity responses have broader specificity and are generated within minutes to limit early invasion and spread of pathogens (Iwasaki and Medzhitov, 2004).

The adaptive immune response depends on two types of specialized immune cells, B and T lymphocytes. Each of these lymphocytes displays a structurally unique antigen receptor as a result of random gene rearrangements. The large repertoire of T and B receptors is highly diverse and it is extremely probable that one or more of these receptors will recognize an encountered antigen. Recognition of an antigen by the lymphocyte receptor triggers activation of the lymphocyte. To produce an efficient immune response, clonal expansion of the relevant lymphocytes is necessary. This typically takes 4-7 days, causing a delay in the immune response (Iwasaki and Medzhitov, 2004). Such a delayed response against rapidly replicating pathogens can be detrimental to the host. Hence the innate immune system is in place as the first line of defense against encountered pathogens.

Innate immunity is considered the more evolutionarily ancient arm of the immune defense system. The same molecular modules of innate immunity are conserved across plants and animals indicating that the system existed before the split into these two kingdoms (Hoffman *et al*., 1999; Janeway and Medzhitov, 2002). From insects to large animals, the innate immune system serves as an indispensible component of the immune defense system. While the vertebrates' immune system consists of both innate and adaptive components, invertebrates lack adaptive immunity and rely entirely on the innate immunity (Medzhitov and Janeway, 1997).

An array of chemical, mechanical and cellular elements make up the innate immune system. First, the mechanical elements such as the epidermis and mucosa inhibit early penetration of pathogenic microorganisms. Binding of the pathogen to the host epithelium can trigger alarm signals resulting in the release of chemical elements such as chemokines and recruitment of other cellular elements, such as natural killer cells (NK), phagocytic cells, mast cells etc. These elements function in parallel to mount appropriate immune responses to rapidly eliminate infectious microorganisms. Major functions of the vertebrate innate immune system include: activation of the complement cascade and recruitment of immune cells to the site of infection, up-regulation of cytokines and chemokines expression resulting in inflammation, activation of immune cells to identify the pathogen and activate the adaptive immune system (Murray *et al*., 2002).

Specific and appropriate engagement of the innate immune system is ensured by targeting highly conserved microbial molecules, pathogen-associated molecular patterns

(PAMPs), which are produced by microbes but not by the host organism, such as bacterial lipopolysaccharide. Recognition and response to PAMPs is facilitated primarily through the pathogen recognition receptors (PRRs) (Gordon, 2002). PAMPs are invariant between microbes within the same class which allows recognition of a large group of the microbes by a single PRR. All PAMPs are essential for survival of the microorganisms making it difficult to produce mutated PAMPs which can escape recognition by the innate immunity (Ulevitch, 2004).

1.3.1 Pattern Recognition Receptors (PRRs)

Central to the innate immune recognition mechanism is a set of receptors appropriately termed PRRs. These germline encoded protein structures have evolved over time and have defined specificities for their ligands. A limited number of PRRs exist, although with a broad level of specificity, they can recognize many ligands which share a common molecular pattern such as DNA, RNA, and bacterial lipopolysaccharide.

As the innate immune system recognizes infectious microorganisms in the early stages of an infection, these PRRs are expressed on cells that first encounter pathogens such as surface epithelium, antigen presenting cells (APCs), macrophages and dendritic cells (DCs) (Gordon, 2002). In addition, a variety of other PRRs are expressed in intracellular compartments, or secreted into the bloodstream and tissue fluids (Janway and Medzhitov, 2002). PRRs are classified based on their cellular localization, target ligand group or function. For example, Toll-like receptors (TLRs) are the class of PRRs that are categorized based on their target ligands.

1.4 The Toll-like Receptors

The Toll-like nomenclature reflects similarity to Toll, a *Drosophila melanogaster* receptor essential in the establishment of dorsoventral polarity during embryogenesis, which was the first member of the Toll family to be identified (Hashimoto *et al*., 1988). Analysis of *toll* gene sequence revealed that it encoded a transmembrane protein with a Leucine-rich extracellular domain and a cytoplasmic domain that was similar to the cytoplasmic domain of the mammalian interleukin-1 receptor (IL-1R) (Gay and Keith, 1991). Moreover, it was discovered that *D. melanogaster* Toll was required for protection against fungal infection in the

adult fly (Lemaitre *et al.,* 1996), as well as being involved in the Nuclear Factor kappaB (NF-κB) signaling pathway similar to the mammalian IL-1R (Belvin and Anderson, 1996).

A great deal of research has been directed towards discovering homologues of *D. melanogaster* Toll, referred to as Toll-like receptors (TLRs) in mammals (Medzhitov *et al*., 1997; Rock *et al*., 1998). Human Toll-like receptor 4 (TLR4) was the first TLR recognized to activate the NF-κB signaling pathway similar to the *D. melanogaster* Toll. TLR4 was also reported to induce expression of cytokines and adaptive immune molecules through the NF-κB pathway (Medzhitov *et al*., 1997). Further studies in mice established that TLR4 acted as a receptor and signal transducer for bacterial lipopolysaccharide (LPS) to initiate innate immune responses (Poltorak *et al*., 1998; Hoshino *et a*., 1999; Qureshi *et al*., 1999).

Mammalian TLRs belong to type I transmembrane proteins and are involved in recognition of a range of PAMPs (Janeway Jr. and Medzhitov, 2002). A wide variety of biomolecules have been identified which serve as TLR ligands, including microbial DNA for TLR9 (Krieg, 2002a) and LPS for TLR4 (Hoshino *et al*., 1999). Most of these ligands are conserved microbial products (PAMPs) that indicate the presence of an infectious agent. They share some common characteristics, although each mammalian TLR has a unique function. First, all TLRs recognize one or more structurally unrelated ligands. Second, some TLRs are known to require additional proteins in order to bind their target ligands. Finally, TLRs act as PRRs by recognizing PAMPs as well as prompting activation of the adaptive immune responses (Bowie and O'Neill, 2000; Takeshita *et al*., 2004).

1.4.1 Members of the Toll-like Receptor Family

To date, thirteen TLR family members have been discovered in mammals, including 11 human, 12 mouse and 10 bovine homologues. Based on expression patterns and activation of cellular functions, TLR members show diversity among mammalian species. TLR1 through 9 are conserved in humans, mice and bovine. TLR10 is expressed in humans and bovine, and a non-functional TLR10 has been detected in mice. TLR11 (also knows as TLR12) and TLR13 are present in mice with a pseudo-gene for TLR11 found in humans (Leulier and Lemaitre, 2008).

TLR1, TLR2, TLR6: These three TLR members belong to a subfamily based on their highly homologous amino acid sequences. Specifically at 66%, TLR1 and TLR6 have the highest amino acid sequence similarity among TLR family members and being located on the same chromosome, the two are thought to have duplicated from the same ancestral gene (Du *et al*., 2000; Takeda *et al*., 2003). Expression of TLR2 is seen on innate immune cells, as well as T and B cells (Lien *et al*., 1999; Borsutzky *et al*., 2005; Xu *et al*., 2005; Imanishi *et al*., 2007). Up-regulation of TLR2 is observed on mononuclear cells during influenza virus infection (Lee *et al*., 2006), chronic obstructive pulmonary disease (Pons *et al*., 2006) and sepsis (Harter *et al*., 2004).

TLR2 recognizes a repertoire of lipoproteins/lipopeptides (LP) that are present in the cell wall of various micro-organisms. For TLR2 to recognize such a broad range of LP molecules, it has developed the ability to form dimers with TLR1 or TLR6 which prompts its activation. Whether TLR2 binds with TLR1 or TLR6 is dependent on the length and arrangement of fatty acids of the LP (Ozinsky *et al*., 2000; Takeuchi *et al*., 2001; Takeuchi *et al*., 2002; Morr *et al*., 2002). Reports also indicate that TLR2 activation by some LPs is independent of TLR1 or TLR6 (Buwitt-Beckmann *et al*., 2005, 2006).

TLR4: TLR4 was the first member of the TLR family to be identified. LPS from Gram negative bacteria is the best studied ligand for TLR4. However, evidence also suggests that several other PAMPs such as fusion protein from respiratory syncytial virus (RSV) and the envelope protein from mouse mammary tumor virus (MMTV) act as TLR4 ligands (Kurt-Jones *et al*., 2000, Rassa *et al*., 2002). In addition, host cellular molecules such as heat-shock proteins, hyaluronic acid and β-defensin 2 have also been reported to interact with TLR4 (Ohashi *et al*., 2000; Biragyn *et al*., 2002; Termeer *et al*., 2002).

The immunostimulatory responses induced by LPS include up-regulation of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1 (Akira, 2004). If hyperinflammatory responses are produced, tissue injury and damage to the host body can follow. Sepsis is the consequence of hyperactivity of TLR4 and is the leading cause of death in the intensive care units, causing approximately 200,000 deaths in the United States annually (Angus *et al*., 2001).

TLR5: Smith and colleagues were the first to identify bacterial flagellin as the agonist for TLR5 (Hayashi *et al*., 2001). Flagellin protein is abundantly expressed in flagella of Gram-negative bacteria which is necessary for locomotion and survival of these micro-organisms (Macnab *et al*., 1992). In recent years, Smith's group - through mutagenesis experiments- reported a 13 amino acid region of bacterial flagellin as the recognition site for TLR5. They discovered that the 13 amino acid cluster participated in intermolecular interactions within flagellar protofilaments and that were crucial for bacterial locomotion (Smith *et al*., 2003a). In contrast, the complementary interaction site on TLR5 was not identified until recently. Last year a new study by Smith's research group identified that interaction between TLR5 and its agonist depended on the tertiary structure of the TLR5 (Andersen-Nissen *et al*., 2007). Furthermore, they refuted previously published work by two research groups who had claimed the interaction to be based on the primary sequence of TLR5 (Jacchieri *et al*., 2003; Mizel *et al*., 2003). Two conserved regions that are expressed on the surface regions of TLR5 extracellular domain over a 228 amino acid span are reported as the interaction site between flagellin and TLR5. Binding of flagellin to a beta-sheet structure that is comprised of theses sites, activates TLR5 (Andersen-Nissen *et al*., 2007).

TLR3, TLR7, TLR8, TLR9: These TLRs are grouped together as they are expressed in intracellular compartments as well as identify nucleic acid ligands. These TLRs specifically identify foreign nucleic acids due to two reasons. First, intracellular localization is suggested to provide cytoplasmic shielding from self nucleic acid and allow recognition of foreign nucleic acids. Endogenous nucleic acids do not enter the endosomal compartment, while pathogens commonly transit through this compartment (Akira, 2006). Second, vertebrate nucleic acids carry several modified nucleosides present in rRNA and tRNA or methylated nucleosides in DNA which inhibits binding by these TLRs (Krieg *et al*., 1995; Kariko *et al*., 2005). Nevertheless, some researchers have reported self DNA and RNA causing autoimmune responses (Krieg, 2002b; Leadbetter *et al*., 2002; Viglianti *et al*., 2003; Baccala *et al*., 2007).

TLR3: By recognizing viral double-stranded RNA (dsRNA), TLR3 is reported to initiate immune responses and confer protection against some viral infections (Alexopoulou *et al*., 2001; Matsumoto *et al*., 2002). Moreover, it is established that particular sterically and

spatially organized synthetic dsRNAs can act as TLR3 agonists as observed with polyriboinosinic:polyribocytidylic acid (poly(I:C)) (a synthetic analogue of dsRNA). Activated TLR3 signals via the adaptor protein Toll-IL-1 receptor (TIR) domain containing adaptor molecule-1 (TICAM-1) which in turn activates transcription factors such as NF-kB and AP-1 and leads to induced expression of Type I Interferon (IFN), inflammatory cytokine/chemokine production and myloid dendritic cell maturation (Oshiumi *et al*., 2003; Yamamoto *et al*., 2003b).

TLR3 is predominantly expressed in the endosomal compartment of immune cells such as myeloid DCs and in the endosomal compartments as well as cell surface of fibroblast and epithelial cells (Cario *et al*., 2000; Muzio *et al*., 2000; Visintin *et al*., 2001). Myloid DCs act as antigen presenting cells and activation of TLR3 in myloid DCs is suggested to generate adaptive immune responses, protection and memory against some viral infections (Heath, 2004). The mechanism of dsRNA recognition by TLR3 is still not understood.

TLR7/8: Single stranded RNA (ssRNA) from pathogenic viruses functions as ligand for TLR7/8 in humans as well as TLR7 in mouse (Heil *et al*., 2004; Forsbach *et al*., 2007; Hornung *et al*., 2005; Melchjorsen *et al*., 2005; Diebold *et al*., 2006; Wang *et al*., 2006). Uracil or uracil and guanosine rich ssRNA found in untranslated genomic end regions of RNA viruses are suggested to act as physiological pathogenic ligands for TLR7/8(Heil *et al*., 2004; Forsbach *et al*., 2007; Hornung *et al*., 2005; Diebold *et al*., 2006). As uracil rich sequences are also present in eukaryotic RNA (Vollmer *et al*., 2005), it is unclear how TLR7/8 discriminates between self and foreign RNA. Although, some factors playing a role in such specificity of TLRs have been suggested such as the intracellular localization of TLR7/8 (Nishiya *et al*., 2004, 2005; Sioud *et al*., 2005) and the ability of cells to rapidly degrade unprotected self RNA in interstitial fluids by cellular RNAase allows discrimination of self from foreign RNA (Barton *et al*., 2006).

Synthetic oligoribonucleotides (ORNs) containing guanosine and uracil complexed to liposomal or similar formulations have been shown to mimic pathogenic RNA effects and stimulate potent innate immune responses *in vivo* and *in vivo* (Diebold *et al*., 2004; Heil *et al*., 2004; Scheel *et al*., 2004; Forsbach *et al*., 2007; Vollmer *et al*., 2005). Studies in mouse models demonstrate that such immune modulatory ORNs are efficient vaccine adjuvants and induce strong innate and adaptive immune responses (Scheel *et al*., 2004; Bourquin *et al*., 2007;

Hamm *et al*., 2007). Moreover, in cancer research some small molecular compounds, imidazoquinolines in particular, induce the antitumor activity of TLR7/8 (Gorden *et al*., 2005, 2006; Jurk *et al*., 2006; Ma *et al*., 2007). While a few compounds are currently applied for topical treatment of skin cancer, others are still in clinical trials (Schon and Schon, 2008)

TLR9: In the year 2000, TLR9 was cloned and identified as a receptor for bacterial and viral DNA rich in unmethylated cytosine-phosphate-guanine dinucleotides (CpG-DNA). TLR9 has evolved to discriminate between the host and pathogenic DNA as CpG-DNA either appears at low frequency or is highly methylated in the vertebrate's DNA (Hemmi *et al*., 2000). The phosphodiester backbone of the native DNA is the target of cellular nucleases and as such native CpG-DNA is rapidly degraded. To create a more stable CpG-DNA, synthetic CpG-DNA (CpG-ODN) with a phosphorothioate (PTO) backbone is utilized in TLR9 investigations. TLR9 seems to be required for all immune stimulatory responses to synthetic PTO ODN which is supported by a complete loss of activity in mice genetically deficient for TLR9 (Hemmi *et al*., 2000, 2003; Vollmer *et al*., 2004a).

Expression patterns of TLR9 on immune cells differ among mammalian species. A limited number of cell types, B cells and plasmacytoid DCs (pDCs), and monocytes in humans express TLR9. Similar expression of TLR9 is also observed on bovine cells with the exception of monocytes (Griebel *et al*., 2005). In contrast, B cells, monocytes, and probably all DC subsets express TLR9 in mice (Iwasaki and Medzhitov, 2004). Consequently, TLR9 immune responses vary among different organisms making it unfeasible to extrapolate research conclusions across organisms.

Both innate and adaptive responses are reported via TLR9 activation. For example, while conventional dendritic cells (cDCs) and macrophages produce proinflammatory cytokines such as IL-6, 1L-12 and TNF-α, pDCs secrete Type I IFNs which in turn activates cytotoxic T cells and helper T cells. Mature DCs activated through TLR9 induce T cells to mount adaptive immune responses (Hemmi *et al*., 2000, 2003; Liu, 2005; Havenar-Daughton *et al*., 2006).

Although much data is available for TLR1 - TLR9 and TLR11 regarding their sequence, structure and biological significance, in comparison TLR10 - TLR13 have not been characterized to a significant level. For these TLRs their ligands, expression patterns and signaling pathways are not understood (West *et al*., 2006).

1.5 Structure of the Toll-like Receptors

The TLRs are type I integral membrane glycoproteins with a high degree of structural conservation in particular in the N-terminal extracellular leucine-rich domain (LRR), a C-terminal intracellular Toll/Interlukin-1 receptor (TIR) domain, and a transmembrane domain. A TLR ligand interacts with the extracellular domain of TLR and intracellular signals are triggered via the transmembrane and TIR domains (Takeda *et al*., 2003).

1.5.1 Extracellular Domain

The ectodomains of TLRs are situated either outside the cell on the cell surface or in intracellular compartments. LRR motifs are a conserved feature of all TLR ectodomains. TLR ectodomains are comprised of 19-25 repeats of LRR motifs and each LRRs are comprised of 24 amino acid residues. LRRs are categorized according to their primary sequences and conservation of amino acid residues. The consensus sequence for TLR-LRR is $xL^{2}xxL^{5}xL^{7}xxN^{10}x\varphi^{12}xx\varphi^{15}xxxxF^{20}xxL^{23}x$ where x is any amino acid, L and F are occasionally replaced with other hydrophobic residues, φ is any hydrophobic amino acid and N^{10} is consistently replaced with C, S or T (Bell *et al*., 2003).

Despite efforts by a number of research groups to resolve the crystal structures of TLRs, TLR3 is the only solved structure to date. The ectodomain of TLR3 structure reveals that individual LRR form a loop where the first 10 amino acids form a beta-strand and the remaining 14 a.a are an alpha-helix. A tertiary solenoid-structure results from the combination of the 23 LRRs. The conserved hydrophobic amino acids of each LRR form a tight hydrophobic core of the solenoid-structure. While the beta-strands occupy larger space than alpha-helices and give shape to a concave outer surface, the helices come together to form the convex center (Bell *et al*., 2005, Choe *et al*., 2005).

Evidence based on the crystal structure of TLR3 suggests a direct interaction between the TLR3 ectodomain and its ligand (dsRNA) (Bell *et al*., 2005, Choe *et al*., 2005). The basis of an interaction between a TLR ectodomain and its ligand is suggested to be the LRR motifs. Such an interaction is also reported for human and mouse TLR1, TLR2, TLR4 and TLR5 (Mizel *et al*., 2003; Andersen-Nissen *et al*., 2007; Jin *et al*., 2007), as well as murine TLR9 (Cornelie *et al*., 2004; Rutz *et al*., 2004). Furthermore, other LRR containing proteins such as

NACHTLRR in vertebrates and NBS-LRR in plants have displayed similar properties (Kufer *et al*., 2005, Martinon *et al*., 2005).

Two other conserved features among TLR ectodomains are the C- and N-caps. These non-LRR components are located at the C and N termini and flank the LRR regions similar to CD42b N-terminus cap. CD42b N-cap forms a β hairpin where disulfide bonds connect the β strands. By covering the hydrophobic core of the first LRR motif, CD42b N-cap stabilizes the solenoid-structure (Huizinga *et al*., 2002). The C-cap structure unlike the N-cap has conserved sequence among TLRs as well as *Drosophila* Toll. Mutations introduced in the C-cap of Drosophila significantly reduced its activity and it is suggested that the C-cap plays an important role in maintaining Toll conformation (Schneider *et al*., 1991; Bell *et al*., 2003).

1.5.2 TIR Domain

Intracellularly the TLRs share considerable homology with the interleukin-1 receptors on the basis of a signaling domain known as the Toll interleukin-1 receptor (TIR) (Bowie and O'Neill, 2000). The primary sequence of TIR domains are composed of 135 to 160 amino acids. Conservation of three sequence regions referred to as Box 1, Box 2 and Box 3 has been identified in all TLRs. Box1 is unique to each TLR; Box 2 forms a loop referred to as "BB loop" which is essential in intracellular signaling activity; and Box 3 contains residues which are also essential for signaling (Xu *et al*., 2000). Mutations introduced in Box 2 of the *tlr4* gene altered its ability to signal (Poltorak *et al*., 1998; Qureshi *et al*., 1999). Another important feature identified in the TIR domain is the "DD loop". A recent investigation reported an interaction between the "DD loop" of TLR2 and the "BB loop" of TLR1 (Gautam et al., 2006). Such interaction is suggested to be the basis for TIR-TIR interaction between TLR-TIR domains and adaptor molecules containing TIR domains such as Myeloid differentiation primary response protein 88 (MyD88) (West *et al*., 2006).

Through the resolution of TLR1 and TLR2 molecular structures, it is evident that the TIR domain is comprised of a central five-stranded, parallel β-sheet which is surrounded by a total of 5 α-helices on both sides. The core of the TIR domain starts at an (F/Y)DA amino acid motif and ends eight residues C-terminal to a conserved phenylalanine or tryptophan residue, and most of the conserved residues of the TIR domain lie within this hydrophobic core (Xu *et al*., 2000).

The underlying cellular signaling events are initiated by the TIR domain. As the TIR domain is conserved across different TLRs in different species, it is proposed that similar signaling pathways are conserved across various species (Gay and Keith, 1991).

1.6 Cellular Localization of Toll-like Receptor

Due to the conservation of structural components such as an LRR containing ectodomain, a transmembrane region and a TIR domain among all TLRs, it was initially hypothesized that all TLR family members were expressed on the cell surface. On the contrary, it has been established that while TLR1, -2, -4, -5 and -6 are expressed on the cell surface, TLR3, -7, and -9 are localized in the intracellular endosomal compartments. While TLR8 is often expressed on the cell surface, it is more frequently expressed intracellularly (Matsumoto *et al*., 2003; Funami *et al*., 2004; Nishiya and Defranco, 2004).

1.6.1 Intracellular Members

1.6.1.1 TLR9 and Oligodeoxynucleotide Ligand

It has been known for more than two decades that bacterial DNA acts as a key immunostimulatory PAMP (Tokunaga *et al*., 1984). TLR9, now known as the receptor for bacterial DNA, was not discovered until 2000 (Hemmi *et al*., 2000). Through sequence comparison of previously discovered TLRs (TLRs 1-6) to expressed sequence tags (ESTs), Hemmi and his colleagues, discovered a high level of sequence similarity between an EST and selected TLRs. After retrieving a full-length complementary DNA sequence (cDNA), they were able to identify the conserved regions including TIR and extracellular domains on the newly discovered *Tlr9* gene. Furthermore, they established that bacterial DNA carried unmethylated CpG motifs which were recognized by TLR9. This was evident from TLR9-deficient ($TLR9^{-/-}$) mice lacking immune responses to CpG DNA (Hemmi *et al*., 2000). Recent studies show that TLR9 recognizes viral-derived CpG DNA as well (Lund *et al*., 2003; Krug *et al*., 2004b). More specifically, activation of TLR9 on pDC cells by mouse cytomegalovirus (MCMV) (Krug *et al*., 2004a) herpes simplex virus (HSV) type 1 and type 2 (Lund *et al*., 2003; Krug *et al*., 2004b) and adenovirus (Zhu *et al*., 2007) have been reported to result in production of IFNα and other cytokines.

Short single-stranded oligodeoxynucleotides containing CpG motifs (ODNs) can mimic the immunostimulatory actions of bacterial DNA. While bacterial DNA has a phosphodiester (PD) backbone which can be degraded quickly by cellular nucleases, ODNs have phosphorothioate modified (PTO) backbones with one or more non-bridging backbone oxygen atoms replaced with sulfur. Optimally, ODNs are 8-30 bases in length with the most potent ODNs usually containing two or more CpG motifs (Krieg, 2002a).

ODNs are classified into three subtypes on the basis of their unique immunostimulatory effects, backbone modification targets, and the number and positioning of CpG motifs (Krieg, 2002a). Class-A ODNs, also known as D-type ODNs, are characterized by a phosphodiester backbone CpG motif and phosphorothioate-modified poly G stretches at the 5′ and 3′ ends (Krieg, 2006). These oligonucleotides elicit production of IFNα by pDCs which subsequently activates natural killer (NK) cells and induce IFNγ secretion (Hemmi *et al.*, 2003). Class-B ODNs, also referred to as K-type, encompasses ODNs that are phosphorothioate-modified throughout their sequences, contain more than one CpG motif, and typically have linear conformations. Their immunostimulatory effects include DC maturation, B cell proliferation and activation resulting in high expression levels of Major Histocompatibility Class II complex (MHCII) (Klinman, 2004). Finally, the sequence for Class-C ODNs is structurally typified by complete phosphorothioate-modified backbone, multiple CpG motifs, and a 5' TCG dimer. Class-C ODNs possess Class-A and Class-B immunostimulatory abilities such as activation of B cells and stimulation of IFNα production (Marshall *et al.*, 2003).

Some characteristics of ODNs, such as positioning of CpG motifs, formation of secondary structures such as hairpin structures, and residues flanking the CpG motifs are reported to dictate some of the immunostimulatory activity of the ODNs (Verthelyi *et al.*, 2001). Hairpin structures on the 5' end of the ODNs greatly affect their immune stimulatory responses but to a lesser extent if located on the 3' end. Given that the three classes of ODNs possess variable sequence properties, each class of ODNs can adopt a unique secondary structure. Although all ODNs can interact with TLR9, Class-specific responses may result from differential mechanisms of cellular localization, sequence/structure difference or unique costimulatory molecules. Species-specific responses to the ODNs are partially attributed to the residues flanking the CpG motif. For example, mouse TLR9 responds best to an ODN containing GACGTT motif because of the purine residues on the 5' side and pyrimidine

residues on the 3' side of the CpG. ODNs containing GTCGTT or TTCGTT are most effective with human TLR9 (Yu *et al*., 2003). Bovine TLR9 is reported to respond effectively to Class-B ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT) where CpG motifs are flanked by one or more thymine residues (Nichani *et al*., 2006).

1.6.2 Extracellular Members

1.6.2.1 TLR4 and Lipopolysaccharide Ligand

The first characterized mammalian Toll member was human TLR4. Currently expression of TLR4 is reported in a variety of cell types such as macrophages and DCs in humans, mouse, bovine, rabbit and other species. LPS is the structural component of the outer membrane of Gram-negative bacteria (Tobias *et al*., 1987). It is composed of a core oligosaccharide linked to an O-antigen, and lipid moieties referred to as lipid A (Raetz and Whitfield, 2002). Lipid A, or endotoxin, unlike other regions of LPS is recognized by TLR4 and elicits innate immune responses.

A high degree of diversity exists among lipid A structures of different Gram-negative bacteria. Common variations include: difference in the number and length of fatty acid side chains, as well as presence of terminal phosphate groups and modifications associate with them. Effective TLR4 recognition and immune responses to different lipid A structures is species specific. Reports indicate that maximal TLR4 stimulation of human cells occurs by hexa-acylated *E. coli* lipid A with 12-14 carbons in length while modification introduced to the side chain reduced the magnitude of the signal (Schromm *et al*., 1998, 2000; Somerville *et al*., 1999). Some reports also suggest that variations of lipid A from *Helicobacter pylori* (*H. pylori)*, *Legionella pneumophila (L. pneumophila)* are poor stimulators of human TLR4 (Girald *et al*., 2003; Smith *et al*., 2003b). In comparison, mouse TLR4 can recognize a broad range of lipid A structures and mount protection against various human pathogenic bacteria such as *E. coli*, *H. pylori* and *L. pneumophila* (Kawasaki *et al*., 2000).

Formation of a TLR4 complex with accessory molecules including LPS binding protein (LBP), CD14, MD-2 and TLR4 facilitates recognition of LPS by TLR4 (Gioannini and Weiss, 2007; Miyake, 2007). Initially LBP, a soluble shuttle protein, directly binds LPS and converts oligomeric micelles of LPS to a monomer for delivery to CD14, which is a glycosyl phosphatidylinositol (GPI)-anchored, high-affinity membrane protein that can also circulate in a

soluble form. Association of LPS to CD14 allows transfer to TLR4/MD-2 receptor complex and positions LPS for recognition by the TLR4 receptor (Wright *et al*., 1989, 1990; Shimazu *et al*., 1999).

1.7 TLR Signal Transduction

The primary consequence of TLRs is expression and activation of immune molecules such as cytokines and chemokines, class II Major Histocompatibility complex and activation of transcription factors such as nuclear factor-kB (NF-κB) which shape the critical immunological response to counter pathogens. With the exception of TLR3, all TLRs signal through the universal adaptor molecule MyD88. Some TLRs recruit other adaptor molecules and trigger activation of receptor-specific pathways. This differential induction pattern depends heavily on cytoplasmic adaptor molecules that can associate with the intracellular region of the TLRs (Akira and Takeda, 2004).

1.7.1 General TLR Signaling Pathway

The majority of what is known about the TLR signaling pathways started with the discovery of the TLR adaptor molecule, MyD88. In 1994 Dan Hultmark suggested that MyD88 was a transducer molecule, and together with IL-1R receptor, had ancestral function in activation of the immune system (Hultmark, 1994). Shortly after, Wesche and colleagues provided support for this hypothesis by showing that MyD88 interacted with both IL-1R-associated kinase (IRAK) and the cytoplasmic domain of IL-1R mediating binding of IRAK to the receptor (Wesche *et al*., 1997). Given that there is a very high degree of similarity between IL-1R and TLRs, IL-1R research helped in understanding the details of TLR signaling pathways. In 1998, Medzhitov provided evidence that human TLR4 activated NF-κB by signaling through MyD88, IRAK-1 and TNF-receptor-associated factor 6 (TRAF6) (Medzhitov *et al*., 1998). Current research shows that MyD88 is the central adaptor molecule in the NF-κB signaling pathway that is commonly shared by all TLRs with the exception of TLR3 (Alexopoulou *et al*., 2001).

TLR signals are mediated through a central protein complex composed of MyD88, IRAK-1 and IRAK-4, and adaptor protein TRAF6 (**Figure 1.2**). Ligand binding to TLRs leads to formation of the protein complex as MyD88 recruits IRAK-4 and TRAF6 proteins.

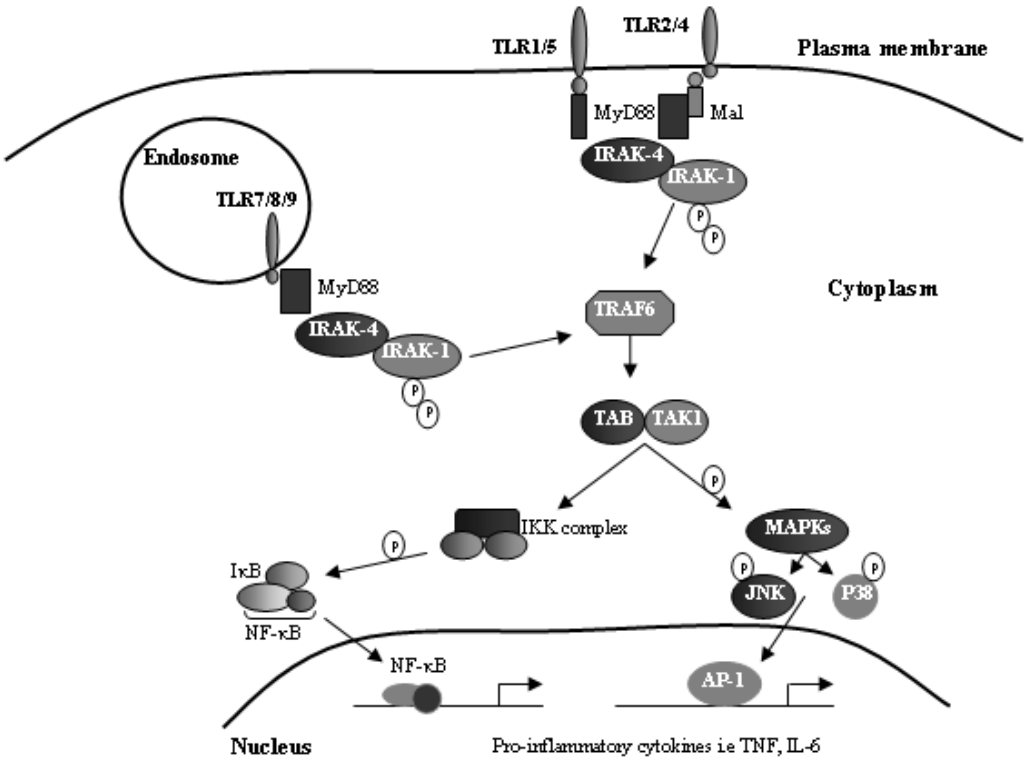

Figure 1.2. A common signal transduction pathway shared by TLR family members. This pathway is initiated when a TLR ligand binds to its respective TLR receptor and induces TLR dimerization. The ligand-bound dimer then recruits MyD88 which in turn recruits IRAK-4. Another member of IRAK family, IRAK-1, is phosphorylated and activated by IRAK-4 and subsequently interacts with TRAF6. The TAB/TAK complex is recruited by TRAF6 and when activated, the complex triggers activation of the MAPK cascade. TAK also activates the IKKs, which phosphorylate IκB. Phosphorylated IκB dissociates from NF-κB and is degraded. NF-κB translocates to the nucleus to induce expression of NF-κB depended genes such as proinflammatory cytokines. In addition, two MAPK proteins, JNK1 and p38, activate transcription factor AP-1 which also translocates to the nucleus and activates expression of proinflammatory cytokines.

IRAK-1 is then phosphorylated by IRAK-4 and dissociates from MyD88, which results in TRAF6 activation. Subsequently, TRAF6 binds an ubiquitin ligase complex and activates mitogen-activated protein kinase kinase kinase (MAP3K or TAK1) in an ubiquitin-dependent manner. TAK1 acts as an activation node for NF-κB and the mitogen-activated protein kinase (MAPK) pathway which in turn activates extracellular signal-regulated kinase (ERK), and effector proteins c-Jun N-terminal kinase (JNK) and p38 MAPK kinase (**Figure 1.2**) (Kawai and Akira, 2007).

The adaptor protein Myd88 is composed of a TIR domain that interacts with the TLR-TIR domains and a death domain (DD) that recruits IRAK proteins. Similarly, IRAK family members carry a DD domain that mainly interacts with Myd88 (Akira and Takeda, 2004; West *et al*., 2006). There are currently four known members of the IRAK family: IRAK-1, IRAK-2, IRAK-4 and IRAK-M. Whereas IRAK-1 and IRAK-4 are catalytically active, IRAK-2 and IRAK-M are inactive as a result of an absence of key residues in their respective kinase domains (Cao *et al*., 1996; Muzio *et al*., 1997; Li *et al*., 2002).

Upon activation of TLR signaling, IRAK-1 and IRAK-4 are recruited to the receptor complex. At the receptor, IRAK-1 associates with MyD88, and TRAF6, and phosphorylation by IRAK-4 triggers IRAK-1 hyperphosphorylation and dissociation of IRAK-1 from the complex while still maintaining its engagement with TRAF6. IRAKs are subject to regulation at a number of levels including interaction with inhibitory molecules and inhibition of expression (Kawai and Akira, 2007). A number of these inhibitory molecules are well defined. For example, IRAK-M is an induced negative regulator that blocks IRAK-4 activation and subsequent IRAK-1 phosphorylation (Wesche *et al*., 1999).

TRAF6 belongs to the TRAF family of proteins which is comprised of six members. The unique characteristic of these proteins is a novel TRAF domain at the C terminus which is essential for association with upstream receptors and other signaling molecules (Chung *et al*., 2002). Each TRAF family member participates in distinct singaling pathways. TRAF6 is an essential signaling protein in TLR signaling pathways. Reports indicate participation of other TRAF members in TLR signaling pathways, however, in a regulatory manner (Wang *et al*., 2006).

NF-κB transcription factor belongs to the Rel-homology domain-containing protein family and is comprised of homodimers and heterodimers of five related proteins: RelA (p65), NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelB and c-Rel (Kawai and Akira, 2007). Under normal cellular conditions, the inactive form of NF-κB is retained in the cytoplasm by an inhibitory molecule called inhibitor of κB (IκB). Upon TLR signaling through the MyD88, TAK1 leads to the activation of inhibitor of the κB kinase (IKK) complex which is composed of two catalytic (IKKα and IKKβ) and a regulatory (IKKγ or NEMO) subunits. IKK in turn phosphorylates IκB causing its degradation by the proteasome pathway, and allowing transcription factor NF-κB to translocate into the nuclei and regulate transcription of genes containing the κB regulatory elements (Kawai and Akira, 2007)..

In order to prolong survival inside the host cells, pathogens have developed mechanisms to affect the NF-κB pathway. While a few pathogens, such as *Shigella* and *Chlamydia,* activate the NF-κB pathway to assist them in survival and replication by avoiding apoptosis of infected cells, most pathogens commonly inhibit the NF-κB pathway. For example, some bacteria insert toxins into the host cells to halt the NF-κB pathway by disrupting the MAP3K activation and subsequent activation and release of NF-κB by its inhibitor IκB. *E. coli*, *Salmonella* and the Measles virus have evolved ways to interfere with the degradation of IκB as well. A more interesting mechanism by Vaccinia virus involves expression of a MyD88 homolog by the virus to interfere with the binding of host MyD88 adaptor to TLR receptors and causing inhibition of NF-κB pathway (Wilson *et al*., 2002).

1.7.2 Receptor-specific Pathways

In addition to MyD88 adaptor, there are three other functional adaptors: Mal/TIRAP, Trif and Tram which interact with specific TLR receptors and initiate a number of different signaling networks in a MyD88-dependent or independent manner (**Figure 1.3**). Such TLR specific pathways present a mechanism by which the TLR system may tailor distinct responses for different types of pathogens.

Some TLRs that signal through MyD88 activate a group of interferon regulatory factors (IRFs) leading to up-regulation of downstream immune response genes. IRF7, for example, was shown to participate in TLR7 and TLR9 signaling in a cell specific manner (Hochrein et al., 2004; Honda et al., 2004; Kawai et al., 2004; Honda et al., 2005; Uematsu et al., 2005).

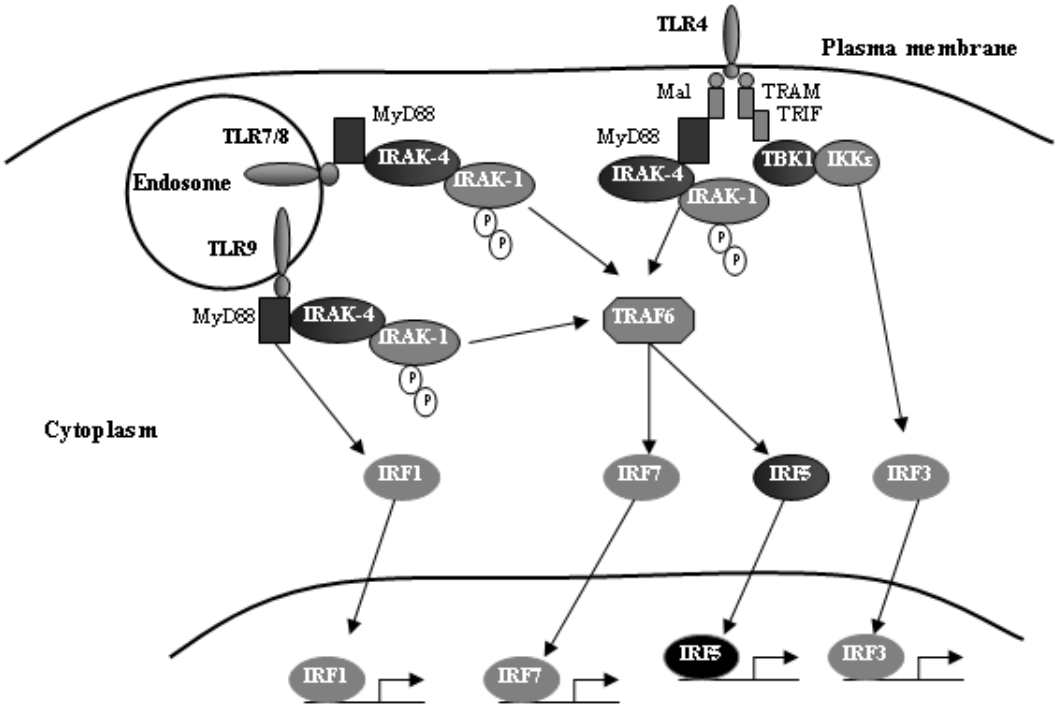

Figure 1.3. Receptor-specific signaling pathways. Some members of the TLR family participate in activation of IRF transcription factors through unique signaling pathways. Activation of IRF3 by TLR4 is achieved through recruitment of TRAM and TRIF adaptor molecules by TLR4 and interaction of these adaptor proteins with TBK1 and IKK kinases. Activation of IRF3 has not been reported by other TLR family members. TLR7, -8 and -9 as well as -4 activate other IRF members through interaction with MyD88 adaptor protein. After activation, IRF transcription factors translocate to the nucleus and regulate expression of immune genes such as type I IFN.

IRF5 is another transcription factor from the same family that is activated via MyD88 (**Figure 1.3**). IRF5 has also been found in a complex with MyD88 and TRAF6 in TLR4 and TLR9 signaling (Takaoka et al., 2005).

Mal/TIRAP was discovered based on sequence similarity to MyD88. Mal and MyD88 knock out mice responded in a similar manner to TLR4 signaling (Horng et al., 2002; Yamamoto et al., 2002a). Although both of these knocked out mice responded to TLR4 agonists, their responses were delayed. During TLR4 signaling, Mal acts as a bridging adapter, enabling MyD88 to be a component of the TLR4 signaling complex, and MyD88 is the signaling adapter, leading to the downstream events. Mal is able to interact with Bruton's tyrosine kinase (Btk) which phosphorylates Mal and facilitates its downstream signaling (Gray et al., 2006). Btk has been shown to participate in TLR2 and TLR4 signaling (Jefferies et al., 2003; Liljeroos et al., 2007) through phosphorylation of NF-κB on its p65 subunit (Doyle et al., 2005). However, the tyrosine phosphorylation of Mal is also required for its degradation providing a means to terminate its signaling (Mansell et al., 2006).

One of the adaptors that were independent of MyD88 was discovered recently and was named TRIF (Yamamoto et al., 2002b; Hoebe et al., 2003; Oshiumi et al., 2003a). Role of TRIF in TLR4 signaling was established through TRIF-deficient mice. TLR4 requires TRAM to engage TRIF (Fitzgerald, et al., 2003b; Oshiumi et al., 2003b; Yamamoto et al., 2003b; McGettrick et al., 2006; Rowe et al., 2006). Thus, TRAM is also a bridging adapter like Mal and is different than all the other adapters in the fact that it is strictly utilized by TLR4. TRIF signaling is amplified downstream by three molecules: an IKK-like kinase named the TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) (Fitzgerald et al., 2003a), the IKK homolog IKKε and IRF3. TRIF interacts with the three molecules resulting in phosphorylation and activation of IRF3 which subsequently acts as a transcription factor (Sato et al., 2003). Similar to IRF3, IRF7 becomes activated via its phosphorylation by TBK1 and IKKε (Kawai et al., 2004; Honda et al., 2005; Uematsu et al., 2005). TLR4 also leads indirectly to NF-κB activation. This is happening via TRIF, which induces TNFα production through activation of IRF3. The TNFα is then secreted from the stimulated cells and binds to its receptor (TNFR) (Covert et al., 2005).

1.8 Levels of TLR Signaling Control

1.8.1 Regulated TLR Expression on Different Cell Populations

1.8.1.1 B Cells

B cells are lymphocytes that arise from the bone marrow and act as APCs. A naïve B-cell circulating in the blood or lymphatic sites recognizes, ingests and presents an antigen to an antigen-specific T-cell. The interaction between a B-cell and T-cell triggers proliferation and clonal-expansion of the B-cell leading to a humoral immune response. Recently, TLR-dependant activation of B cells was reported to be required for eliciting humoral immune responses. Such an activation event leads to maturation of these cells and enables presentation of processed antigen to T cells in an immunogenic form. Mice carrying TLR deficient B cells lacked production of antibodies against protein antigens given with adjuvants (Pasare *et al*., 2005). Consistent with this observation, previous work had also suggested that murine B cells could be stimulated *in vitro* by TLR4 and TLR9 ligands to proliferate and secrete antibodies (Whitlock and Watson, 1979; Krieg *et al*., 1995).

Through gene expression profiling of TLRs and measurement of co-stimulatory molecules corresponding to activation of TLR molecules, a new investigation has shown distinction between the TLR repertoires on murine and human B cells. Given that B cells are comprised of subsets, delving into details of TLR expression on each subset of human and mice is beyond the scope of this thesis. Suffice it to say that controversy exists regarding expression of TLRs on specific subsets of B cells (Oliver *et al*., 1997; Mansson *et al*., 2002; Bernasconi *et al*., 2003; Huggins *et al*., 2007) and details of expression of TLRs on all subsets is still not clear. Barr and colleagues suggest that in general murine B cells express TLR1-9. In contrast, other reports have shown that human B cells express TLR1-TLR10 with the exception of TLR3, TLR5 and TLR8 (Dasari *et al*., 2005; Barr *et al*., 2007).

B cells produce cytokines similar to other APC populations such as DCs. The pattern of cytokine expression by B cells is dependent on the activation of a particular subset of B cells. Barr and colleagues investigated the pattern of cytokine expression by DCs and B cells. Their report indicates distinct response patterns from different APC populations stimulated via the same TLR. B cells make IL-10 and IL-6 following TLR4 and 9 ligation, in contrast to DCs

which produce IL-12, IL-6 and IL-10. In addition, IL-6 is elicited in B cells via TLR2, 4 and 9, but through TLR3, 4 and 9 in DCs (Barr *et al.*, 2007). This suggests that the composition of the membrane-proximal TLR signalling elements (adaptors) differs between these two important APC populations.

1.8.1.2 Monocytes

Monocytes originate from progenitor cells of the bone marrow and are mononuclear phagocytes circulating in blood. Once a monocyte exits blood, it differentiates into a macrophage or DC (Chomarat *et al.*, 2003), and the cell is no longer referred to as a monocyte, but rather as a monocyte-derived cell. Overall, monocytes are defined as cells that (a) are mononuclear and circulate in blood (b) have phagocytosis capabilities and (c) maintain some repertoires of macrophages and monocyte-derived DCs (Chomarat *et al.*, 2003).

Two main subsets of monocytes have been identified in mice and humans. Assignment of monocytes to a subset is on the basis of the expression of surface markers such as chemokines receptors and adhesion molecules. A subset of monocytes in mice is referred to as inflammatory monocytes, express chemokines receptor CCR2, cell surface protein Ly6c ($Gr1^+$), and adhesion molecule L-selectin. Their names rather reflect their recruitment to sites of inflamed tissues and lymph nodes (Geissmann *et al.*, 2003). By differentiating to inflammatory DCs, these monocytes replenish repertoires of these cells in skin, digestive tract and lung (Varol *et al.*, 2004; Ginhoux *et al.*, 2006). The second subset of murine monocytes referred to as resident monocytes are recruited to both inflamed and resting tissues. They lack markers unique to inflammatory monocytes and instead express chemokine receptor CX_3CR1 and LFA-1 integrin (Geissmann *et al.*, 2003). Human monocytes are divided into $CD14^+$ $CD16^-$ and $CD14^{low}$ $CD16^+$ subsets. While $CD14^+$ $CD16^-$ monocytes resemble murine inflammatory monocytes, $CD14^{low}$ $CD16^+$ monocytes are similar to murine resident monocytes (Geismann *et al.*, 2003). In comparison to human and mouse monocytes, bovine monocytes are not characterized extensively; however, reports confirm expression of $CD14^+$ and L-selectin on bovine blood monocytes (Wang *et al.*, 1997; Eun *et al.*, 2004).

Monocytes have the ability to quickly recognize pathogens, mediate phagocytosis and respond with the production of reactive oxygen and nitrogen species, antimicrobial peptides, and inflammatory mediators (Medzhitov *et al.*, 2000). Such important immune functions are

initiated by TLR modulation of signaling pathways. Human monocytes are reported to express TLR 1, 2, 4, 5, 8 and 9 (Hornung *et al*., 2002; O'Mahony *et al*., 2008)

1.8.1.3 Dendritic Cells

Dendritic cells (DCs) are APCs and originate from the bone marrow. Their ability to recognize an extensive range of antigens and their presence in virtually all tissues interfacing the host and environment make them the most important APCs. DCs are broadly categorized into two subsets: myeloid (mDCs) and plasmacytoid (pDCs). In human, these subsets of DCs express a variety of TLRs. Myloid DCs reportedly express the complete repertoire of TLRs while plasmacytoid DCs express limited number of TLRs such as TLR7 and TLR9 (Muzio *et al*., 2000; Seya *et al*., 2005). The *in vivo* requirement of DCs to initiate immune responses to specific TLR agonists is not fully understood.

1.8.2 Phosphorylation-Mediated Signal Transduction in TLR Activation

Proteins comprising TLR signaling adaptor molecules, kinases and effectors are regulated through various post-translation modifications including phosphorylation, ubiquitination, acetylation, sumoylation and nitrosylation (Perkin, 2006). The degree and type of modification can vary, depending on the nature of the TLR-inducing stimulus. Moreover, such modifications frequently have distinct, sometimes antagonistic, functional consequences and the same modification can have different effects depending on the context.

Phosphorylation serves as an important regulatory mechanism for the TLR signaling pathways. Critical aspects of a protein function such as activity, stability, cellular localization and interacting partners are regulated through this post-translation modification. Phosphorylation of adaptor molecules, MyD88, Trif, TRAM as well as transcription factors such as NF-kB, IRFs, c-Jun, p38, and activity of IKK and MAPK family of kinases comprise essential signaling molecules of TLR pathways (Miggin and O'Neill, 2006; Perkin, 2006).

2.0 HYPOTHESIS AND OBJECTIVES

Comparison of short amino acid sequences representing human phosphorylation targets to a bovine protein database will identify bovine orthologues. These bovine peptides can be used to create a bovine-specific array for kinome analysis of bovine cells.

Objectives:

1. Identify bovine phosphorylation targets and create a bovine peptide array.
2. Test new peptide arrays by performing kinome analysis on bovine monocytes treated with TLR agonists.
3. Verify kinome analysis with bioassays.

3.0 MATERIALS AND METHODS

3.1 Reagents and Chemicals

All chemical reagents required to carry out experiments throughout this thesis are listed in **Table 3.1**.

3.2 Bovine Peptide Array Development

3.2.1 Peptide Selection Process

Peptides for the array were selected to represent a spectrum of biological events but with an emphasis on pathways and processes associated with innate immunity. A total of 194 proteins representing signal transduction kinases, transducers and effectors were selected. Many of these proteins are functionally complex and undergo phosphorylation at multiple sites to control discrete aspects of protein behavior. Therefore, for many of these proteins multiple peptides, representing distinct phosphorylation events, were selected to allow more detailed insight into cellular responses under different treatment conditions.

3.2.2 Designing Bovine Peptides

Phosphosite (www.phosphosite.org) and Phosphobase (phospho.elm.eu.org) are publically available online databases that hold information on manually-curated and literature-based serine, threonine and tyrosine phosphorylation sites. This information is primarily focused on phosphorylation events characterized for either human and mouse with limited representation of other species such as rat. Search results for a specific protein return short peptide sequences corresponding to characterized phosphorylation sites. Where available, information on the corresponding kinase, as well as links describing the biological function of the specific modification, is also included. These two databases were searched for proteins of interest and corresponding human peptides were saved.

Table 3.1. List of selected chemicals, reagents and their suppliers.

Chemicals	Supplier
Acrylamide	Sigma
Aim V media	Gibco, Invitrogen Corp.
Aprotenin	Sigma
Adenosine triphosphate(ATP)	Calbiochem
Bovine serum Albumin(BSA)	Biorad Laboratories
Bromophenol Blue	Sigma
CD14 microbeads	Miltenyi Biotec Inc.
CpG 2007	Merial
Dithiothreitol (DTT)	Biorad Laboratories
Ethylene glycol tetraacetic acid(EGTA)	Sigma
Ethylenediamine tetraacetic acid (EDTA)	Fluka BioChemika
Fetal Bovine Serum (FBS)	Gibco
Glycerol	Amersham Bioscience
Horse radish peroxidase(HRP)	Sigma
Leupeptin	Sigma
Lipopolysaccharide(LPS)	Sigma
3-Aminophthalhydrazide(Luminol)	PerkinElmer BioSignal, Inc.
Magnesium Chloride	Sigma
Percoll	Amersham Biosciences
Phenylmethylsulphonyl fluoride(PMSF)	Sigma
Polyoxyethylene Lauryl Ether(Brij-35)	ICN Biomedicals Inc.
Sodium dodecyl sulfate(SDS)	Calbiochem
Sodium floride	Sigma
sodium orthovanadate	Sigma
Sodium pyrophosphate	Sigma
Triton 100	Sigma
Fura-2, AM	AnaSpec Inc.

Antibodies	**Supplier**
Fluorescent antibody	LI-COR Biosciences
Phospho-Etk(Y40)	Cell Signaling Technology
anti-bovine CD14	VMRD
FITC-conjugated anti-mouse	BD Bioscience
Radioactive Chemicals	**Supplier**
gamma-^{32}P Adenosine Triphosphate(ATP) salt	Amersham Bioscience
Supplier	**Address**
Fluka BioChemika	Sigma-Aldrich Canada Ltd., Oakville, ON, Canada
Gibco	Invitrogen Ltd., Gaithersburg, MD, USA
Calbiochem	EMD Chemicals Inc., Gibbstown, NJ, USA
ICN Biomedicals	ICN Biomedical Canada Ltd., Saint Laurent, PQ, Canada
Sigma	Sigma-Aldrich Canada Ltd., Oakville, ON, Canada
Miltenyi Biotec Inc.	Miltenyi Biotec Inc., Auburn, CA, USA
LI-COR Biosciences	LI-COR Biosciences, Lincoln, Nebraska, USA
Cell Signaling Technology	New England Biolabs Ltd., Pickering, Ontario, Canada
Amersham Bioscience	GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA
AnaSpec Inc.	AnaSpec Inc., San Jose, CA, USA
Merial	Merial Canada Inc., Montreal, Quebec, Canada
Biorad Laboratories	Biorad Laboratories, Hercules, CA, USA

PerkinElmer BioSignal, Inc.	PerkinElmer BioSignal, Inc., Montreal, Quebec, Canada
BD Bioscience	
VMRD	VMRC, Inc., Pullman, WA, USA

To obtain bovine-specific consensus sequences, we identified a series of human phosphorylation events of interest and then employed the Blastp (version 2.2.13) program (ncbi.nlm.nih.gov/BLAST) from NCBI to compare the collected human peptides against the NCBI-NR database. Blastp was set to search bovine protein records and retrieve short exact matches for the list of human peptides. A comparison of the protein descriptions for the query and hit sequences was performed to ensure that they referred to orthologous protein.

3.2.3 Synthesis and Spotting of Peptides

All peptides were synthesized and printed according to the following protocol by JPT Peptide Technologies (www.jpt.com). Amino-oxy-acetylated peptides were synthesized on cellulose membranes in a parallel manner using SPOT synthesis technology (Frank, 1992; Wenschuh *et al*., 2000). Following side chain deprotection the solid phase bound peptides were transferred into 96 well microtiter filtration plates (Millipore, Bedford, USA) and treated with 200 µL of aqueous triethylamine (0.5 % by vol) to cleave the peptides from the cellulose. Peptide-containing triethylamine solution was filtered off and solvent was removed by evaporation under reduced pressure. Resulting peptide derivatives (50 nmol) were re-dissolved in 25 µL of printing solution (70% DMSO, 25% 0.2 M sodium acetate pH 4.5, 5 % glycerol; by vol.) and transferred into 384-well microtiterplates. Two droplets of 0.5 nL peptide solution were deposited per spot on aldehyde functionalized glass slides using the non-contact printer Nanoplotter of GESIM (Großerkmannsdorf, Germany) equipped with 8 piezoelectric NanoTips (GESIM, Großerkmannsdorf, Germany). Printed peptide microarrays were kept at room temperature for 5 hours, washed with deionised water, quenched for 1 hour with 50% aqueous hydroxylamine, washed extensively with water followed by ethanol, and dried under a stream of nitrogen. Resulting peptide microarrays were stored at 4 °C and maintained reactivity for more than 18 months. To determine intra-experimental variability in substrate phosphorylation, each block of 300 peptides was printed in triplicate on each slide. The final physical dimensions of the array were 19.5 X 19.5 mm with each peptide spot having a diameter of ~350 µm and peptide spots being 750 µm apart.

A total of 298 bovine/human peptides and 2 negative peptide controls, of 15 amino acids in length, were printed on each block of the array according to the map presented in **Appendix A- Figure A1**. The identities of the proteins and the sites of phosphorylation are

presented in **Appendix A- Figure A2**. The sequences of all peptides are presented in **Appendix A- Table A1**. Seven positive control (Histon 1-4, Myelin basic protein- MBP bovine, and alpha/beta Casein) proteins were printed around each block on the array by JPT Peptide Technologies. Each positive control is a full length protein and is known to be phosphorylated in cells in presence of ATP. Signals from these controls are not included in our peptide array analysis as these proteins are mainly printed for visualization and gridding of the blocks.

3.3 MACS Isolation of Bovine Blood Monocytes

Blood was collected by venupuncture from mature cattle using 0.3 % EDTA as an anticoagulant. Blood was transferred to 50 mL polypropylene tubes and centrifuged at 1400 x g for 20 minutes at 20 °C. Mononuclear leukocytes were isolated from the buffy coat and mixed with calcium and magnesium free phosphate-buffered saline (PBSA) (1 x solution; PH 7.3) to a final volume of 35 mL. The cell suspension was then layered onto 15 mL of 54 % isotonic Percoll (GE Healthcare) and centrifuged at 2000 x g for 20 minutes at 20 °C. Peripheral blood mononuclear cells (PBMCs) from the Percoll-PBSA interface were collected and washed twice with cold PBSA (1 x solution; PH 7.3) supplemented with 0.1% EDTA and once with MACS buffer. Monocytes were purified from isolated PBMCs by MACS® purification using CD14 microbeads (Miltenyi Biotec). Monocytes were plated in Aim V medium (Gibco^{TM}, Invitrogen) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Gibco^{TM}). The purity of monocytes was assessed by flow cytometry (BD FACSCalibur) following cell labeling with an anti-bovine CD14 antibody (VMRD). Cells were cultured for 19 hours prior to stimulation.

3.4 Flow Cytometry Analysis

A volume of 50 µL (2 x 10^7/mL) of isolated $CD14^+$ monocytes was placed with 50 µL of mouse anti-bovine CD14 monoclonal antibody (mAb) (VMRD, clone number:MM61A). Monocytes were incubated on ice for 15 minutes and pelleted by centrifugation at 349 x g for 2 minutes at 4°C. Next, three washes were performed with an initial 100 µL and subsequent 200 µL of flow cytometry solution [PBSA (1 x solution; PH 7.3), 0.03 % sodium azide and 0.2% gelatin]. Finally a goat anti-mouse Ig polyclonal antibody (BD Bioscience) was added to the well and three washes similar to the first set of washes were performed. Monocytes were then

fixed with 200µL of 2% formaldehyde and their purity was assessed with a flow cytometry instrument (BD FACSCalibur) using Cellquest software.

3.5 Treatment of Monocytes

Isolated monocytes were resuspended ($10x10^6$ per mL) in Aim V medium (Gibco™, Invitrogen Corp., San Diego, CA) enriched with 10 % fetal bovine serum (FBS) and were incubated at 37 °C overnight before any treatment.

3.5.1 CpG-treatment

CpG ODNs are often species-specific in their ability to induce innate immune responses and ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT) has been shown to be effective for cattle (Nichani *et al*., 2006). Rested monocytes (10×10^6/mL) were stimulated with 5 µg/mL CpG 2007 (Merial) or cultured in media for 4 hours at 37 °C. After removing the media from the tubes, cells were stored at -80 °C before use with the peptide arrays. A total of 2.5×10^7 monocytes were required per peptide array.

3.5.2 LPS-treatment

Rested bovine monocytes (1.0×10^7/mL) were stimulated with 100 ng/mL *E. coli* LPS (Sigma-Aldrich). This quantity of LPS has been shown to induce cellular responses in monocytes isolated from humans (Mookherjee *et al*., 2006).Cells were pelleted and stored at -80 °C before use with the peptide arrays. A total of 2.5×10^7 monocytes were required per peptide array.

3.6 Peptide Array Analysis

3.6.1 Kinome Analysis of Bovine Monocytes

Cell lysates were prepared and incubated with the arrays as reported previously (Diks *et al*., 2004) with the exception that lysate from 25×10^6 monocytes was incubated with each chip. Cell pellets were lysed with 100 µl lysis buffer (20 mM Tris-HCL pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/mL leupeptin, 1 g/mL aprotinin, 1 mM PMSF), incubated on ice for 10 minutes and

then spun in a microcentrifuge for 10 minutes at 4 ºC. A 70 µl aliquot of this supernatant was mixed with 10 µl of the activation mix (50% Glycerol, 50 uM ATP, 60 mM $MgCl_2$, 0.05% v/v Brij-35, 0.25 mg/mL BSA, 2 mCi/mL γ-^{32}P-ATP) and incubated on the chip for 2 hours at 37 ºC. Finally, slides were washed once with Tris-buffered saline (PBS) (1 x solution; PH 7.3) containing 1 % Triton X-100, twice with 2 M NaCl containing 1% Triton X-100 and in demineralized H_2O.

Following air drying the arrays were exposed to a phosphoimager screen for one week. Images were obtained by scanning the screen with a Typhoon scanner (GE Healthcare) and then loaded on Arrayvision software (Image Research). Intensity values for the spots and background were obtained and normalization and statistical analyses were performed with Genespring software (Agilent Technologies).

3.6.2 Measuring Signal Intensity for Array Peptides

Radioactive signals were captured from the array by exposure to a phosphoimager screen over a period of one week. A phosphoimager was utilized due to ease of handling and availability of a scanner that would allow detection of weak signals. A Typhoon scanner (GE Healthcare) was set to obtain array images from the phosphoimager screen using the highest sensitivity settings with a pixel size of 25 microns. The captured image of the phosphoimager plate was then processed using ImageQuant TL v2005 software (Amersham, GE Healthcare) to obtain individual peptide array images. ArrayVision software (GH healthcare) was used with a protocol describing the spacing between spots and blocks of the array to obtain spot and background intensity of the spots.

3.6.3 Signal Intensity Calculation

Arrayvision software was utilized to process peptide arrays and collect signal intensity data for peptide spots. First, a protocol file was created to contain the coordinates of each spot and the measurements of spacing between spots/blocks as well as the dimension of spots/blocks. More specifically, a horizontal and vertical spacing of 0.125 mm between spots, a distancing of 0.125 mm between two blocks, and dimension of 0.4 mm for each spot was specified. Three replicate blocks on the array were numbered as 1, 2, and 3 with 1 being the block adjacent to the slide label. In order for the program to correlate the correct spot identifier

to its intensity proper orientation of the slide on the image screen was necessary. When loading a peptide array it was rotated so that the label side was positioned to the left and the corner with a row as well as a column of positive controls was oriented to the bottom right.

When drawing grids on a slide, each spot was represented as a circle and a total of 362 circles were present in each block. In addition to 298 bovine and 2 negative control peptides, there were 7 positive controls printed on four sides of each block (**Appendix A- Figure A1**). Blank spots were used to complete a row or column containing positive controls in order to create a proper square shape for a block. Background intensity for a spot was calculated as the average of pixels from 4 regions in the immediate vicinity of each spot.

Prior to analyzing the array data with Genespring software, data points for positive controls and blank spots were removed from the data file. Positive control spots and blanks were merely used for gridding purposes. In addition, very high intensity values for positive control spots would skew lower signal data from bovine peptides and preclude proper normalization and statistical analysis.

3.6.4 Statistical Analysis on Signal Intensities

For peptide array data, global normalization to the 50th percentile was performed on the raw data by Genespring software. One sample t-test was performed on the normalized data from the 18 replicates to evaluate reproducibility of the signal by comparing peptide signals to a baseline value of 1. There were two biological samples for each treatment and 9 technical replicates. Together, there were a total of 18 replicates for each spot under each treatment.

A student t-test was performed on PKA and the superoxide assay results. By comparing results for replicates of media control to CpG or LPS samples, confidence in the differential production of superoxide or PKA activity was measured as a result of LPS or CpG stimulation.

3.7 Western Blot Analysis

3.7.1 Detection of Phosphorylated Etk

Human blood was collected using 0.3% EDTA as an anticoagulant. PBMCs were isolated and $CD14^+$ monocytes were MACS purified following the same protocols used to isolate bovine monocytes. Monocytes were cultured overnight, resuspended at 2 x 10^6/mL and

stimulated with 100 ng/mL LPS (Sigma-Alrich), 5 µg/mL CpG 2007 (Merial) or cultured in media for 4 hours at 37 °C. Stimulated cells were then pelleted and lysed with 100 µl SDS sample buffer [62.5 mM Tris-HCl (pH 6.8 at 25 ºC), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue, 1 mM PMSF], denatured (5 mins at 95 ºC) and 30 µl of it was loaded on a 10% SDS gel and transferred to a nitrocellulose membrane. Phospho-Etk antibody (catalogue number 3211) (Cell Signaling Technology) and a fluorescent-conjugated secondary antibody (LI-COR biosciences) were used in accordance to the Supplier's protocol. An image of the blot was captured using an Odyssey scanner (LI-COR Biosciences).

3.8 Measuring Protein Kinase A (PKA) Activity

Omnia ^{TM} Lysate Assay kit (Catalog number KNZ0021; Biosource) was utilized to measure PKA activity. Ten million bovine CD14+ monocytes were stimulated with either LPS, CpG or media control for 4 hours and were lysed with 50 µl Omnia Cell Extraction Buffer. The protein concentration was determined using Quick Start^{TM} Protein Assay (Bio-Rad) and 50 µg of protein was used to carry out kinase reactions following the manufacturer's protocol.

3.9 Quantification of Oxidative Burst

Superoxide production was measured by chemiluminescence. The protocol that was followed was as described (Thrasher *et al.*, 1992) with the exception that 4 x 10^6 bovine monocytes were used without phorbol myristate acetate activation and before reading the luminescence, the 96 well plate was wrapped in foil and incubated at 37°C, 5% CO_2 for 20 minutes. Luminescence was measured at 30 second intervals using a Victor^{3}V 1420 Multilabel Counter (PerkinElmer).

4.0 RESULTS

4.1 Development of Bovine Peptide Array

4.1.1 Available Phosphorylation Motifs for Human Proteins

Phosphorylation of serine, threonine and tyrosine phosphorylation occurs at an approximate ratio of 1,000:100:1(Hunter, 1998). In addition to the large number of proteins that undergo phosphorylation, many of these proteins are modified at multiple sites, giving rise to an estimated 100,000 unique phosphorylation events within the human proteome (Zhang *et al*.,2002). Currently there are two main publically available phosphorylation based databases: Phosphosite and Phosphobase. There are 32,250 human phosphorylation sites on Phosphosite as of March, 2008. The majority of the approximately 29,000 phosphorylation sites are curated based on published work. The rest of the phosphorylation sites are based on unpublished MS/MS data. In comparison, Phosphobase holds approximately 16,000 phosphorylation sites as of July, 2007. Phosphobase records are based on experimentally verified phosphorylation sites as well.

4.1.2 Comparison of Human phosphorylation motifs to Bovine Proteins

To determine the degree of sequence conservation surrounding sites of phosphorylation in humans and bovine, a series of 880 peptides representing sites of phosphorylation within human proteins were searched against the NCBI bovine protein database by Blastp program to generate orthologous bovine peptides. Blastp results revealed that half of the hit sequences had 100% identity to their query sequences (**Table 4.1**). Moreover, a comparison of the protein descriptions for the query and hit sequences confirmed that they referred to the same protein.

From these 880 phosphorylation sites, we selected peptides for the array based on their biological significance and the degree of conservation of the sequence surrounding the phosphoacceptor site based on the premise that higher degrees of sequence conservation may also imply functional conservation. Of the bovine peptides included on the array 65 % displayed 100 % sequence conservation and the remainder showed various degrees of sequence similarity to their human homologous peptides. About 5 % of the array peptides were comprised of human peptides with no bovine hits detectable by the Blastp program (**Table 4.1**).

Table 4.1. Analysis of Bovine Kinase Target Peptides Selected from Queries of Human Peptides.

Sequence Differences[1]	All Bovine Peptides[2]	Peptides on Array[3]	Comparison of Bovine and Human Peptide[4]
0	50%	65%	Same descriptions
1	13%	15%	Same descriptions
2	7%	8%	Same descriptions
3	4%	5%	Same descriptions
4	1.5%	1%	Same descriptions
5	0.4%	1%	Same descriptions
6	0.6%	0%	Same descriptions
0 -15	22%	5%	Different descriptions or no hits

1) A brief report of Blastp (version 2.2.13) results for human peptides against the NCBI-NR protein records for bovine organism with increasing number of peptide differences used to differentiate among human peptides compiled from Phosphosite and Phosphobase.
2) An initial list of 880 human peptides was searched against the NCBI-NR protein database to find bovine orthologous peptides. The list is categorized based on conservation of sequence similarity between the query peptide and the bovine hit.
3) Final list of 298 bovine peptides on the array is divided into 8 sections based of the number of amino acids that were different between a human query and bovine hit peptide.
4) For each group of peptides, this column indicates whether query human peptides and their bovine homologs referred to the same proteins based on the description of their proteins.

4.2 Phosphorylation Activity Detection in LPS and CpG Treated Monocytes

4.2.1 FACS analysis of isolated monocytes

Monocytes were isolated by MACS purification technique using CD14 microbeads that would bind to monocytes expressing $CD14^+$ receptors. As $CD14^+$ monocytes were isolated from PBMC populations, it was necessary to ensure that purified monocytes did not include other cell types from the PBMC population. Purity of isolated monocytes was tested by FACS analysis (**Figure 4.1**). For this set of isolated monocytes, 98.2 % of the cells expressed $CD14^+$ marker which indicates that these cells are pure monocytes.

4.2.2 Bovine Peptide Array Results

Cellular extracts of isolated bovine monocytes treated for four hours with media, 100 ng/mL LPS or 5 µg/mL CpG ODN 2007 were subjected to kinome analysis by the arrays. Pseudo-images representing the averaged normalized signal for each peptide on the array were generated for each treatment condition (**Figure 4.2**).

Individual peptide signals displayed high technical and biological (n = 2) reproducibility when one-sample t-test on replicate data for CpG and LPS samples produced high confidence levels ($p<0.05$) for 219 and 220 of the peptides, respectively (**Appendix A- Table A3**). Of these peptides, 92 and 98 were differentially phosphorylated following LPS and CpG treatment as compared to media treated cells (**Figure 4.3**).

Over half of the peptides, many relating to known TLR-associated signaling molecules, were conserved in both identity and phosphorylation change as predicted from previous studies in human cell lines. These data support the conclusion that TLR4 and TLR9 signaling is highly conserved in bovine monocytes with many similarities in kinase activation following LPS and CpG stimulation (**Figure 4.4**). This is not unanticipated as previous reports (Miggin and O'Neill, 2006) have implicated similar adaptor molecules are involved in signaling by TLR family members and these data confirm the capacity of the current peptide arrays to reproducible reflect kinome changes. The remaining, differentially phosphorylated, peptides were either specific to a particular treatment or had different trends of phosphorylation for either LPS or CpG. These peptides presumably represent differential responses achieved by activation of the two different TLRs (**Figure 4.4**) (**Table 4.2**).

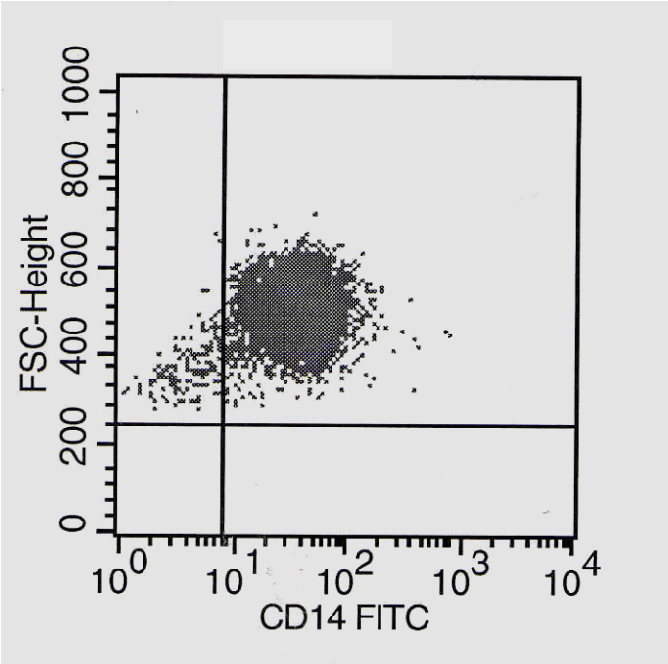

Figure 4.1. FACS analysis of purified CD14+ bovine blood monocytes. Isolated monocytes were stained with CD14 FITC and analyzed by flow cytometry. For this sample purity of monocytes was at 98.2%.

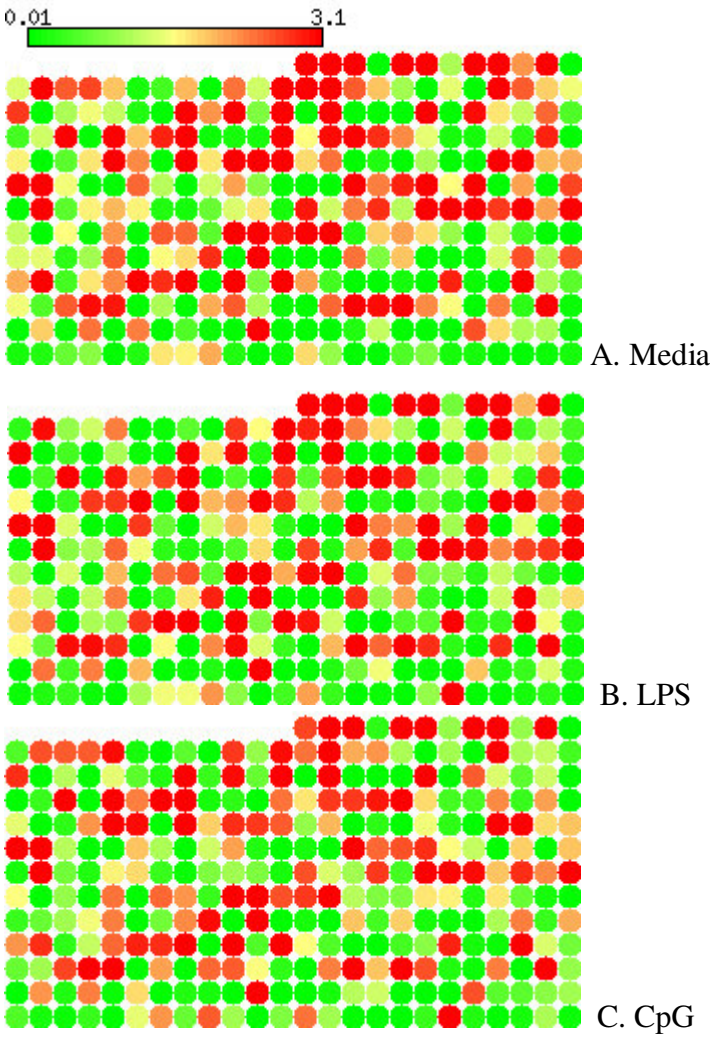

Figure 4.2. Phosphorylation of substrate targets on peptide arrays by cellular lysates from bovine monocytes stimulated with medium (A), LPS (B), or CpG ODN (C). Purified $CD14^+$ bovine monocytes (25×10^6) were cultured overnight, stimulated with 100 ng/mL LPS, 5 µg/mL CpG ODN 2007, or cultured in media for 4 hours at 37 °C. Monocyte lysates were prepared and incubated with peptide arrays in the presence of γ-^{32}P-ATP. After original slide images were acquired by a scanner, normalized and corrected for background noise then signal strength for each spot was calculated. The average signal for each peptide was calculated from the 18 replicates and this value was used to generate pseudo-images of peptide arrays for each condition assayed. For peptide identity and protein information, please refer to supplemental figure A1 and table A1.

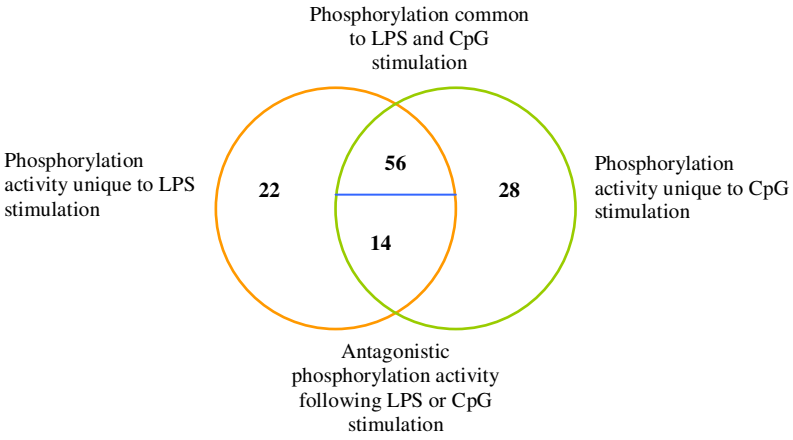

Figure 4.3. Altered phosphorylation events following LPS and CpG ODN stimulation. The Venn diagram summarizes the number and pattern of significant and differentially phosphorylated peptides observed with LPS- and CpG ODN-stimulated cell lysates when compared to responses from media treated cells. Altered phosphorylation events by LPS and CpG ODN were identified with fold changes less than or equal to 0.7 and greater than or equal to 1.4 when normalized signals were compared to signals from media control.

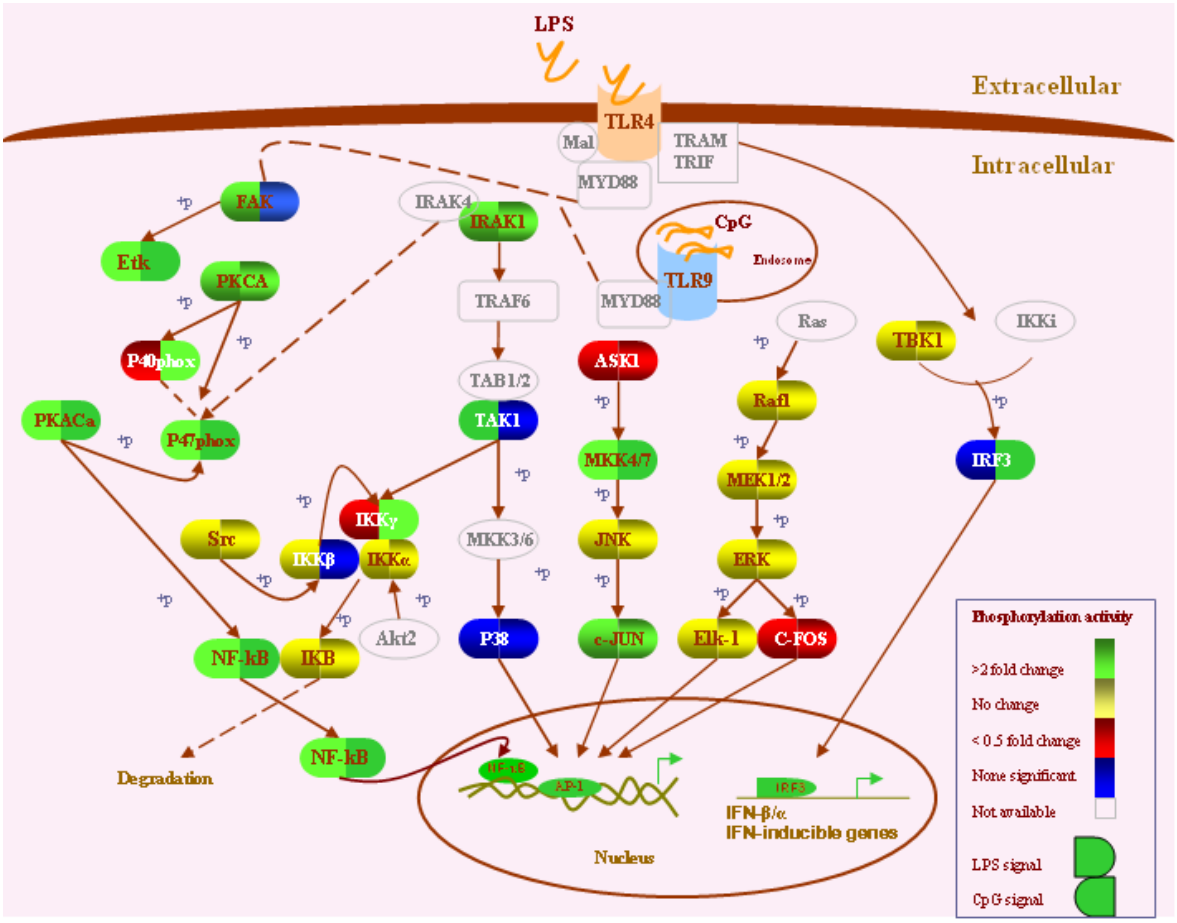

Figure 4.4. A signaling pathway based on known phosphorylation events of TLR4 and TLR9 in a variety of human cell lines and reflecting phosphorylation signals in bovine blood monocytes stimulated with LPS or CpG. A coloring scheme is used to illustrate phosphorylation events which were detected with the peptide array when analyzing lysates from bovine monocytes stimulated with LPS (TLR4) or CpG ODN (TLR9). A (+p) on a path indicates addition of a phosphate group to a phospho-site of the substrate protein the arrow points to. Solid pointed lines indicate known phosphorylation activities while dashed pointed lines refer to previously suggested phosphorylation interaction. Non-pointed lines show non-phosphorylation based protein interactions. Both TLR4 and TLR9 signal through common adaptor proteins although the degree of phosphorylation at specific sites on the signaling transducers may differ. Details of the phosphorylation of peptides for individual signal transducers in these pathways are available in Table 2.

Table 4.2. Phosphorylation of substrate peptides by kinases in LPS and CpG ODN stimulated monocytes.

Phospho -protein[1]	Kinase Target[2]	Fold Change[3]		p-value[4]		Kinase Mediating Phosphorylation[5]	Effect of Phosphorylation[6]
		LPS	CpG	LPS	CpG		
IRAK-1	T209	3.2	3.7	0.01	0.01	IRAK-1	Required for self phosphorylation and activation
IRAK-1	S376	1.1	7.3	0.01	0.22	IRAK-4	Activation
IRAK-1	S568	0.2	0.2	0.01	0.01		
IRAK-1	T387	1.0	1.1	0.01	0.01		
IRAK-1	T100	1.3	1.2	0.01	0.01	Akt1	regulates transcription
FAK	Y397	2.9	2.5	0.14	0.01	FAK	Regulates apoptosis, and protein degradation
Etk	Y40	7.2	1.6	0.01	0.01	FAK	Enzymatic activation, altered intracellular location, regulates molecular association and cell motility
PKCA	S657	1.1	1.0	0.01	0.01		Altered intracellular location
p40phox	T154	1.7	0.4	0.01	0.01	PKCA	
p47phox	S370	4.6	3.1	0.01	0.01	PKCA, PKACa	Enzymatic activation, and altered intracellular location
PKACa	S338	3.9	2.5	0.02	0.02		
PKACa	T195/7	7.7	1.9	0.02	0.01		
Src	S74	1.0	1.0	0.01	0.01		
IKKα	T23	1.0	1.0	0.01	0.01	Akt2	Regulate apoptosis, and enzymatic activation
IKKβ	Y188	0.4	1.1	0.56	0.01	Src	Regulates transcription, and activation
IKKγ	S31	0.1	0.0	0.01	0.01	IKKβ	Regulates proper NFkB activity
IKKγ	S43	2.6	0.5	0.01	0.01	IKKβ	
IkBα	S32/6	0.8	0.5	0.06	0.62	CK2-A1(S32/6), IKK-alpha(S32/6), Nik(S32/6)	activation(S32), protein degradation and more(S32/6)
IkB-β	T19	0.8	0.8	0.01	0.01		Protein degradation
IkB-ε	S18	0.5	0.6	0.01	0.01		Protein degradation
NFkB-p100	S870/72	3.5	13.2	0.06	0.71	Nik(S870), IKK-α (S872)	Protein processing(S870), regulates association with cellular proteins(S870)
NFkB-p105	S337	18.4	20.5	0.02	0.01	PKACa	Activation, and regulates transcription
NFkB-	S276	11.4	11.0	0.01	0.01	MSK1, PKACa	Activation, regulates

p65							transcription, and more
NFkB-p65	S311	4.3	2.3	0.01	0.01	PKCZ	Activation?
TAK1	S192	15.0	22.9	0.15	0.09	TAK1	Activation
P38-γ	T185	0.4	1.1	0.16	0.32		
p38-α	T179/Y181	1.3	1.4	0.88	0.76	MKK3/6(T179/Y181)	Activation, Enzymatic activation, and more (T179/Y181)
ASK1	S966	0.2	0.4	0.01	0.01	PKD	Inhibition
MEK1	S217	1.3	1.4	0.01	0.01	Raf1 and Cot	Activation
MEK2	Y216	0.4	0.7	0.11	0.29		Inhibition
MEK5	S311	0.9	1.1	0.01	0.01		
MEKK1	T1383	0.8	0.7	0.01	0.01	MEKK1	Activation
MKK4	S257/0	4.1	2.9	0.01	0.01	ASK1(S261), MLK3(S261)	enzymatic activation(S261)
JNK2	T404/7	0.7	1.2	0.01	0.01	CK2-A1(T404/7), MKK7(T404/7)	Activation(T404/7)
Jun	S63	1.9	1.6	0.01	0.01	JNK1,JNK2	Protein activation, stabilization and more
Jun	S73	0.9	1.1	0.04	0.01	JNK1	Protein activation, stabilization and more
ERK1	T202/4	1.0	1.1	0.01	0.01	Cot(T202/4), MEK1/2(T202/4), Lck(T204), ERK1(T204)	Activation (T202/4), enzymatic activation (T202/4)
Elk-1	S389	1.2	1.2	0.01	0.01	ERK1	Activation, and regulates transcription
Raf1	S259	1.0	1.0	0.01	0.01	PKACa	Inhibition
Raf1	S499	1.0	2.3	0.01	0.01	PKACa	Activation
Fos	T232	0.2	0.2	0.05	0.01	ERK5, ERK2	Altered intracellular location, and activation
ERK2	Y204	1.2	1.0	0.01	0.01	MEK1	
TBK1	S172	1.0	1.1	0.01	0.01		Enzymatic activation
IRF-3	S385/6	0.6	3.0	0.01	0.13	TBK1(S385/6), IKK-ε(S385)	Activation(S385), and regulates association with cellular proteins and more (S385/6)
IRF-3	S396/8	7.7	27.1	0.04	0.27		activation, regulates transcription, and more(S369), altered intracellular location, regulates association with cellular proteins(S368)
FADD	S194	10.6	5.9	0.08	0.01		
Casp8	S347	3.4	2.8	0.01	0.01		

1) Common name for substrate protein with a peptide for a phosphorylation site on the array.
2) Position and name of target amino acid on the substrate protein.
3) Fold changes for LPS and CpG samples were calculated by comparing the background corrected and normalized signal values of these samples to the media control.

4) P-values reported by Genespring software for normalized phosphorylation signals. A p-value represents confidence level on reproducibility of a signal and is evaluated by one-sample t-test which compares normalized signals (n = 18) for each peptide to a baseline value 1.
5) For phosphorylation sites with known upstream kinases, Phosphosite database provides names of these kinases. Available information from Phosphosite is listed here.
6) Curated data on Phosphosite database maintain information on effects of phosphorylation on cellular activity of a substrate protein. When available, such information was presented here.

4.2.2.1 Activation of the Known Toll-like Receptor Pathways

Signaling events associated with TLR activation have been identified using a variety of techniques, including immunoprecipitation, site mutagenesis and mass spectrometry. These analyses have been performed with a variety of human and mouse cells and provide a framework to determine if TLR signaling pathways are conserved in bovine monocytes (Miggin *et al*., 2006). Patterns of phosphorylation observed for proteins of this pathway which are present on the array are presented in (**Figure 4.4**) along with phosphorylation details in (**Table 4.2**). Selected phosphorylation events, and their potential biological significance are further discussed.

IRAK-1: This is an important upstream kinase in TLR signaling pathways (Miggin *et al*., 2006). IRAK-1 undergoes a series of phosphorylation events during activation resulting in its release from the receptor complex to interact with TLR adaptor molecules such as TRAF6 (Miggin *et al*., 2006). It has been established that phosphorylation of T209 of IRAK-1 is a prerequisite for TLR-mediated activation in human embryonal kidney cells (Kollewe *et al*., 2004). Consistent with this observation, a peptide corresponding to this site of bovine IRAK-1 undergoes a 3.2 and 3.7-fold increase in phosphorylation following LPS and CpG stimulation, respectively (**Table 4.2**). The increased phosphorylation of this site under both treatment conditions indicates that IRAK-1 is a conserved signal transduction kinase for both TLR4 and TLR9 in bovine monocytes.

Our array also contained peptides representing putative IRAK-1 phosphorylation sites which have yet to be defined with respect to biological roles. For example, the degree of phosphorylation of peptides representing T100 and T387 was independent of TLR4 or TLR9 activation. In contrast, phosphorylation of the peptide corresponding to S568 of IRAK-1 displayed a 5-fold decrease in phosphorylation following either LPS or CpG treatment. Finally, levels of phosphorylation of S376 increased 7.3-fold in response to CpG treatment but remained unchanged following LPS stimulation relative to the media control (**Table 4.2**).

Collectively these results confirm the prerequisite modification of IRAK-1 at T209 for TLR-induced signal transduction but also suggest that regulation of IRAK-1 through phosphorylation is more complex and multi-faceted involving aspects of regulation through

phosphorylation which are independent of TLR activation, conserved for various TLRs and specific to particular TLRs.

NF-κBs: The NF-κB family of transcription factors is one of the key effectors for TLR signaling by both TLR4 and TLR9. In addition to regulation through programmed degradation of inhibitory proteins, NF-κB is controlled through phosphorylation. Phosphorylation events relating to the regulation of key NF-κB proteins, such as p65, p100 and p105 are represented on our peptide array. As anticipated (Perkins, 2006) increased levels of phosphorylation of critical regulatory sites within NF-kB and related kinases were observed following both the LPS and CpG treatment conditions.

NF-kB p105/p50: The ability for the NF-κB p105 sub-unit to bind DNA and function as a transcription factor depends upon phosphorylation of S337, as reported through studies in a variety of mammalian cell systems (Hou *et al*., 2003). A peptide representing this phosphorylation event undergoes one of the most dramatic changes in phosphorylation as a result of both LPS and CpG treatment with 20 and 18-fold increases in phosphorylation, respectively (**Table 4.2**).

NF-kB p100/p52: Activation of p100 is dependent upon phosphorylation of a series of C-terminal sites including S870 (Xiao *et al*., 2004). While both LPS and CpG stimulation resulted in increased phosphorylation of this site the effect was more pronounced with CpG (13.2-fold) versus LPS (3.5 fold) stimulation (**Table 4.2**).

NF-kB p65: Similarly, investigations of the transactivation domain of the p65 NF-κB subunit indicate that S276 is the major phosphorylation site for this protein and modification of this residue is essential for p65-dependent cellular responses in murine embryonic fibroblasts (Okazaki *et al*., 2003). Consistent with the established role of the p65 subunit in mediating cellular responses for various Toll-like receptors we observed a 11-fold increase in phosphorylation of this residue upon stimulation with LPS and CpG (**Table 4.2**). Phosphorylation of S311 has also been implicated in activation of p65 NF-κB in murine

embryonic fibroblasts (Duran *et al*., 2003) and the bovine peptide corresponding to displayed significantly increased phosphorylation following LPS and CpG stimulation (**Table 4.2**).

TAK1/MAP3K7: TAK1 is a member of the mitogen-activated kinase kinase kinase (MAP3K) family which serves as an important link between the Toll-like and MAPK pathways. TAK1 is activated by an early intermediate of the TLR pathway, TRAF6, which forms active signaling complexes with TAK1 following its activation by IRAK-1. The bovine peptide array included a peptide representing S192, a known phosphorylation site for TAK1 activation in human embryonic kidney cells (Kishimoto *et al*., 2000). This kinase target underwent 22.9 and 15.0-fold increases in phosphorylation with LPS and CpG treatments, respectively. Conversely, a second peptide corresponding to T184 of TAK1, which has not been implicated in TLR-mediated signaling, did not undergo differential phosphorylation as a consequence of either LPS or CpG treatment (**Table 4.2**). TLR activation of the MAPK signaling pathway was supported by increased phosphorylation of peptides corresponding to activating events of v-raf-1 murine leukemia viral oncogene homolog 1(Raf1), jun oncogene (Jun), and mitogen-activated protein kinase kinase 4(MKK4) (**Table 4.2**). While Raf1 is an upstream protein kinase of the MAPK pathway, MKK4 lays mid point and Jun is a downstream transcription factor.

IRF3: In response to microbial nucleic acids, IRF3 is activated through phosphorylation of S396 in human embryonic kidney cells (Servant *et al*., 2003). Phosphorylation of this site has also been reported with LPS (McCoy *et al*., 2008). IRF3 therefore presents an interesting opportunity to test the hypothesis that differential phosphorylation plays a role in determining cellular responses to TLR signaling. Following LPS stimulation there was a 7-fold increase in S396 phosphorylation but no significant change in phosphorylation was observed following CpG treatment (**Table 4.2**). This observation provides independent confirmation that the peptide arrays can reveal discreet events involved in the regulation of complex biological responses. Furthermore, this observation provides substantial evidence for the conservation of TLR signaling events across disparate species and cell types.

FADD/Casp8: In addition to its apoptotic function FADD also plays a role in TLR-induced proliferative responses (Imtiyaz *et al*., 2006). FADD is activated through a direct interaction with the IRAK-1 MyD88 signaling complex following TLR stimulation (Zhande *et al*., 2007) which then induces cellular responses through activation of Casp8 (Beisner *et al*., 2005). FADD-mediated activation of Casp8 has been observed following LPS, and to lesser extent, CpG treatment (Alappat *et al*., 2005). We also observed increased phosphorylation of peptides corresponding to activating events for FADD and Casp8; S194 of FADD by 10.6 and 5.9-fold and S347 of Casp8 by 3.4 and 2.8-fold following LPS and CpG treatments, respectively (**Table 4.2**). The activation of these proteins, and the higher degree of activation observed with LPS treatment, provides further evidence that TLR signaling is highly conserved across species and within a variety of cell types.

FAK and Etk: Based on reported crosstalk with MyD88, activation of FAK through the TLR system has been suggested (Zeisel *et al*., 2005). Interestingly, a peptide corresponding to Y397 of FAK, a phosphorylation event associated with activation of this kinase in human embryonic kidney cells (Glover *et al*., 2004) was increased by 2.9 and 2.5-folds following LPS and CpG stimulation of bovine monocytes.

FAK is known to activate members of the Bruton's tyrosine kinase (Btk) family which has defined roles in integrin signaling, promoting cell migration (Chen *et al*., 2001), and has been implicated in TLR signaling (Doyle *et al*., 2007). Etk is a Btk family member, although a specific role for Etk in TLR signaling has not been reported. Activation of Btk by FAK (Chen *et al*., 2001) does, however, provide a possible link with TLR signaling. The bovine peptide arrays included a peptide corresponding to Y40, the key regulatory phosphorylation site in Etk. Phosphorylation of this peptide was increased over 7-fold following LPS stimulation and 1.6-fold following CpG (**Table 4.2**).

Consistent with the bovine kinome results from our peptide array, stimulation of the human monocytes with LPS, but not CpG resulted in phosphorylation of Y40 of Etk (**Figure 4.5**). To our knowledge this is the first report of evidence linking Etk activation with LPS stimulation. This association was first revealed by data obtained from the peptide array and highlights the utility of this approach for analyzing cell signaling events.

4.2.2.2 Activation of Receptor-specific Pathways

In addition to the general TLR4 signaling pathway activated through MyD88 and TRAF6 adaptor molecules, TLR4 specific pathways include adaptor molecules TRIF/TRAM and subsequent activation of effector proteins such as IRF3 and NF-kB (Yamamoto et al., 2002b; Hoebe et al., 2003; Oshiumi et al.,2003a). A peptide representing phosphorylation site S396, that is implicated in activation of IRF3, is present on the peptide array. Following LPS stimulation there was a 7-fold increase in S396 phosphorylation but no significant change in phosphorylation was observed following CpG treatment (**Table 4.2**). Clearly, TLR4 signaling through TRIF/TRAM has allowed phosphorylation activity of this site following LPS stimulation.

Activation of IRF3 is reported to induce TNFα production which is then secreted from the stimulated cells and binds to its receptor (TNFR) (Covert et al., 2005). Signaling induced by TNFR also culminates in NF-κB activation. In this way endotoxin can cause a prolonged transcriptional activation that is important for the defense of the host. TRIF in contrast to the other adapter proteins is shown to be important for the induction of apoptosis in response TLR4 (Han et al., 2004; Ruckdeschel et al., 2004; De Trez et al., 2005; Kaiser and Offermann, 2005). Along with TRIF this pathway requires involvement of the proteins RIP1, FADD, and caspase-8. We observed increased phosphorylation of peptides corresponding to activating events for FADD and Casp8; S194 of FADD by 10.6 and 5.9-fold and S347 of Casp8 by 3.4 and 2.8-fold following LPS and CpG treatments, respectively (**Table 4.2**). Although, these results indicate activation of FADD and Casp8, it is evident that LPS stimulation results in a higher level of phosphorylation for these peptides. It is possible that these proteins are activated through a conserved pathway for TLR4 and TLR9 as well as an LPS specific pathway through TRIF/TRAM adaptor molecules.

Mal is another cytoplasmic receptor that binds the TIR domain of TLR4. Previous reports indicate phosphorylation of this adaptor by a Btk kinase which is required for degradation of Mal (Mansell et al., 2006), providing a means of termination of its signaling. Etk is a member of the Btk family of kinases. The bovine peptide arrays included a peptide corresponding to Y40, the key regulatory phosphorylation site in Etk. Phosphorylation of this peptide was increased over 7-fold following LPS stimulation and 1.6-fold following CpG (**Table 4.2**).

Figure 4.5. Etk protein phosphorylated due to LPS stimulation of CD14$^+$ human monocytes. Phosphorylation of Etk in human monocytes cultured with media, or stimulated with LPS (100 ng/mL), or CpG ODN 2007 (5 µg/mL) for 4 hours was detected by a phospho-Etk antibody reacted with a Western blot.

Consistent with the bovine kinome results from our peptide array, stimulation of the human monocytes with LPS, but not CpG resulted in phosphorylation of Y40 of Etk (**Figure 4.5**).

4.3 Physiological Responses of LPS and CpG Treated Monocytes

4.3.1 Western Blot with Phospho-Ekt Antibody

The unknown role of Etk in TLR-mediated responses, the differential phosphorylation of the Y40 peptide under the LPS and CpG stimulation conditions and the availability of a commercial phosphospecific antibody for Y40 of Etk made it a strong candidate for more detailed investigation.

A human Etk-specific monoclonal antibody is available for the Y40 site. In spite of the high degree of conservation of the human and bovine Etks, in particular around the phosphoacceptor site, this antibody did not cross react with the bovine protein (data not shown), highlighting one of the technical limitations of antibody-based kinome analysis. Based on the anticipated shared biological role of Etk in humans and bovine cells, we examined the phosphorylation status of Etk from human $CD14^+$ monocytes in response to LPS and CpG treatments. Human monocytes are previously reported to express TLR4 and TLR9 and respond to LPS and CpG ligands respectively (Bosisio *et al*., 2002; Hornung *et al*., 2002; Doyle *et al*., 2007).

Consistent with the bovine kinome results from our peptide array, stimulation of the human monocytes with LPS, but not CpG, resulted in phosphorylation of Y40 of Etk (**Figure 4.5**). To our knowledge this is the first report of evidence linking Etk activation with LPS stimulation. This association was first revealed by data obtained from the peptide array and highlights the utility of this approach for analyzing cell signaling events.

4.3.2 Measuring Protein Kinase A (PKA) Activity

A previous kinomic investigation with peptide arrays indicated increased phosphorylation of numerous targets of PKA in response to LPS stimulation of human peripheral blood mononuclear cells (Diks *et al*., 2004). PKA is a central cellular kinase which is regulated through both cAMP availability as well as phosphorylation of the catalytic sub-unit of PKA (PKACa) at T197. The bovine peptide arrays revealed increased phosphorylation at the

orthologous region of PKACa. LPS stimulation increased T197 peptide phosphorylation nearly 8-fold higher than the media control and 4-fold more than CpG ODN stimulation (**Table 4.2**). Another PKACa target S338 was phosphorylated due to LPS and CpG stimulation at 3.9 and 2.5 fold increase, respectively (**Table 4.2**).

To determine if increased phosphorylation of PKACa corresponded directly to increased PKA activity we used a substrate assay to monitor PKA activity in cell lysates. Purified bovine monocytes were stimulated with LPS, CpG ODN, or control medium using the same culture conditions and time used for the peptide arrays. Within 4 hours, LPS stimulation induced a significantly ($p = 0.02$) greater increase in PKA activity than media stimulated cells and CpG ODN stimulation did not significantly ($p = 0.2$) increase PKA activity relative to the media control (**Figure 4.6**). Although phosphorylation of two sites, T195/7 and S338, were observed with peptide arrays following CpG and LPS stimulation, the PKA assay suggests that phosphorylation of T195/7 is more reflective of PKACa activation. Furthermore, it can be argued that a 1.9 phosphorylation fold change increase of T195/7 by CpG during the kinome analysis is not a significant increase to reflect cell signaling responses. Therefore, it is critical that kinome studies be complemented by functional assays to determine the biological significance of kinome changes.

4.3.3 Oxidative Burst

As a mechanism of innate immune defense the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase catalyzes the reduction of oxygen to superoxide (O2-) at the expense of NADPH (Johnson *et al*., 1998). NADPH oxidase is not constitutively active but exposure of cells to appropriate stimuli results in rapid activation of enzymatic activity. Activation of the oxidative burst is closely associated with signaling by the Toll-like system with p47 (PHOX) serving as a direct target for IRAK-4 in human neutrophils (Pacquelet *et al*., 2007). Differential levels of oxidative burst in response to TLR ligands have been reported for chicken and turkey monocytes and heterophils. Not all TLRs utilize the same signaling pathway to activate this response (Farnell *et al*., 2003; He *et al*., 2006).

P47(PHOX) activation involves phosphorylation at numerous sites but modification of S370 has been identified as one of the earliest and most significant activation events in human leukocytes (Johnson *et al*., 1998).

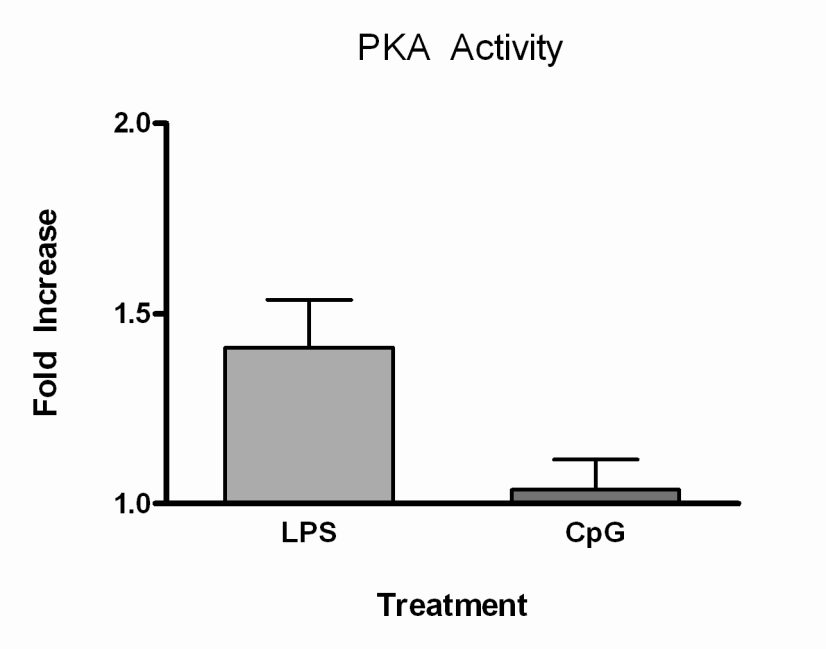

Figure 4.6. PKA activity in bovine monocytes following LPS and CpG ODN stimulation. Purified $CD14^+$ monocytes were stimulated with 100 ng/mL LPS, 5 µg/mL CpG, or culture medium for 4 hours before being lysed. Reagents were added to the lysate and incubated for 20 minutes at 37°C, 5% CO_2 before reading chemiluminescence every 30 seconds during a 30 minute interval. Monocytes were isolated from three animals and triplicate cultures were analyzed for each condition. The slope of the PKA activity was calculated for each replicate and these values were averaged for each biological replicate. Student t-tests carried out on slopes of PKA ($n = 9$) showed significant increase for LPS ($p = 0.02$) but not CpG ($p = 0.2$) stimulated cells when compared to medium cultured cells. Fold changes for LPS and CpG were calculated in relation to results from medium cultured cells.

The bovine peptide array revealed increased phosphorylation of $P47^{PHOX}$ at the S370 position following both LPS (4.6 fold) and CpG ODN (3.1-fold) stimulation of monocytes. These data suggest activation of oxidative burst following both stimuli, but that this activation was more efficient following TLR4 stimulation.

The activity of NADPH oxidase is also influenced by p40(PHOX) which interacts with p47(PHOX) in a phosphorylation-dependent manner to regulate oxidative burst. Phosphorylation of p40(PHOX) at position Thr154 has been reported to inhibit NADPH oxidase in a semirecombinant cell-free system (Lopes *et al*., 2004). Oxidative burst is therefore dependent upon both activation of p47(PHOX) through phosphorylation at S370 as well as removal of p40(PHOX) mediated inhibition through dephosphorylation of this regulatory protein at T154. Phosphorylation of the bovine peptide orthologous to T154 of p40(PHOX) was decreased 2.5-fold with lysate from CpG stimulated monocytes but increased 1.7-fold following LPS stimulation. This differential phosphorylation of T154 in p40 (PHOX) led us to hypothesize that CpG ODN, but not LPS stimulation, results in increased oxidative burst activity in bovine monocytes.

Purified $CD14^+$ monocytes were stimulated using the same stimulation conditions as employed for the peptide arrays. CpG ODN stimulation as compared to media cultured cells induced a significant level ($p = 0.04$) of oxidative burst while LPS stimulation caused lower oxidative burst than media control cells but not by a significant amount ($p = 0.5$) (**Figure 4.7**). The oxidative burst assay was consistent with phosphorylation changes for the regulatory p40(PHOX) and support the conclusion that p47phox phosphorylation does not directly correlate with cellular responses. This kinome analysis provides direct evidence for one mechanism by which TLR signaling may result in differential activation of the oxidative burst in monocytes.

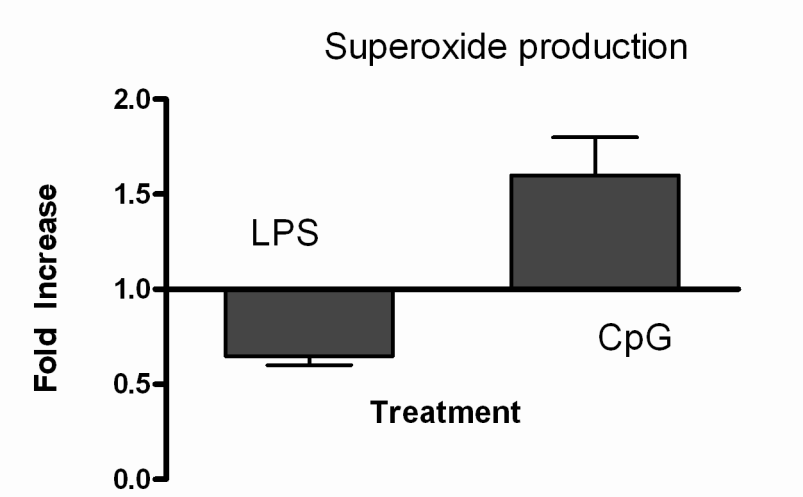

Figure 4.7. Superoxide production in LPS and CpG ODN stimulated monocytes. Bovine monocytes were stimulated with 100 ng/mL LPS, 5 µg/mL CpG or cultured in medium for 4 hours before the addition of luminol and HRP. The monocytes were then incubated for 20 minutes at 37°C, 5% CO_2 to allow the reaction to begin. Following incubation a luminescence reading was taken every 30 seconds for 30 minutes. Fold change calculation was based on data from the mean and one standard deviation of values from 3 biological samples which were analyzed in duplicate. Fold changes for LPS and CpG were calculated in relation to results from media cultured cells. Student t-tests (n = 6) confirmed a significant increase in superoxide by CpG ($p = 0.04$) but not LPS stimulation ($p = 0.5$) when compared to cells compared to medium.

5.0 DISCUSSION

5.1 Kinome and Phosphoproteome

In spite of the undetermined function of many protein kinases, the medical significance of this class of enzymes is readily apparent. For example, in cancer, approximately half of the genes directly linked to the disease encode protein kinases, the remainder encode products that either regulate kinases or are acted upon by them (Blume-Jensen and Hunter, 2001). Similar underlying defects in phosphorylation physiology have been linked to diabetes (Chrissobolis and Sobey, 2006), Alzheimers (Avila, 2006), inflammation (Vermeulen *et al*., 2006) as well as numerous other disorders. The medical significance of the protein kinases has prompted extensive research efforts with an estimated quarter of drug discovery efforts of pharmaceutical and biotechnology groups being focused on protein kinase inhibitors (McInnes, 2006): As a result, 60 such kinase inhibitors are currently in advanced clinical trials. With the appreciation that many cellular responses occur independent of changes in transcription or translation there is an increasing interest in defining cellular responses at the level of post-translation modification of proteins. Analysis of cellular phosphorylation events can be conceptually divided into two categories, kinome and phosphoproteome analysis. Kinome analysis focuses on the protein kinases while phosphoproteome analysis focuses only on the protein targets of kinases.

5.2 Design of a Bovine Peptide Array

5.2.1 Non-Specific Peptides

This category defines peptides on the bovine array that are either human peptide sequences without bovine orthologous peptides or peptide sequences that are conserved across members of a protein family. Therefore, prior to assigning biological functions to these peptide targets on the basis of bovine experimental results, validation of phosphorylation activity is required.

Although, each peptide on the array uniquely identifies a single bovine protein, a set of peptides were created to represent conserved phosphorylation motifs across members of the same protein family. For example, peptide sequence TSFMMTPYVVTRYYR with a target

tyrosine is 100 % conserved between JNK1 and JNK3 with one amino acid difference in JNK2. Similarly three peptides representing conserved phosphorylation motifs for members of MKK(3,6), Smad(1, 2, 3), and Akt(1, 3) as well as peptides conserved across isomers of PKC(A, B, C) and IKK (A, B) are printed on the array (**Table 5.1**).

A peptide target (QNRSGAMSPMSWNSD) for human FADD (S194) was included in the pool of peptides on the array (**Table 5.1**). Blastp query did not return a bovine orthologous peptide for this sequence; however, due to the significance of FADD in some of the signaling pathways, it was decided to print the human peptide sequence on the array. A protein record for bovine FADD protein (accession number Q645M6) was available. Closer investigation of Q645M6 revealed conservation of S194 on the bovine FADD. We acquired a peptide sequence of 15 amino acids with S194 in position 9 (QSGSANPGSFTAWDS) which was printed on the array. Although, S194 is conserved between human and bovine FADD proteins, the degree of similarity surrounding S194 is very low.

There are 13 additional human peptide sequences (**Table 5.1**) on the array. Similar to human FADD (S194), for a number of phosphorylation targets involving proteins such as IκB-β, TNF-R1 and Nik no equivalent bovine peptides were identified, although bovine orthologous proteins are available. Either these bovine sequences are not correctly annotated or the phosphorylation targets in the orthologous proteins have not been sufficiently conserved to match the human sequence when using the current search criteria utilized.

5.2.2 Non-Reacting Peptides

TRAF6 is an important adaptor protein in early stages of TLR signaling pathways. Searches of Phosphosite and Phosphobase databases did not produce any previously reported phosphorylation motifs for TRAF6. However, some research groups have generated potential phosphorylation motifs based on computer analysis of TRAF6 sequence. After consulting a generated list of phosphorylation motifs by JPT Peptide Technologies, seven potential target peptides for S12, S48, S97, T83, T317, T486 and T429 were selected. Data analysis of bovine experiments revealed that S12, S97 and T486 did not produce significant phosphorylation signals due to exposure to cell lysates from CpG, LPS stimulated, or media cultured bovine monocytes. It is possible that these predicted targets are not involved in phosphorylation supported by the fact that no previous phosphorylation reports are available. Alternatively, it is

possible that these sites are not phosphorylated in the context of the cell type and stimulants used during this investigation. Although S48, T83, and T429 were phosphorylated by active kinases in medium cultured lysates, in contrast to LPS and CpG stimulated monocytes.

Consistent lack of phosphorylation signals for 21 additional peptides was an indication these peptides may not be phosphorylation sites (**Table 5. 2**). These peptides either require additional substrate structural components/domains to interact with the appropriate kinases or they are not phosphorylated in the context of the cell type and stimulants used during this investigation.

5.3 Biological Relevance of Kinome Responses of LPS and CpG Treated Monocytes

5.3.1 Activated Signaling Pathways

With our bovine peptide arrays we demonstrated the ability to characterize complex signaling events through two different TLRs. We confirmed activation of known and shared signaling pathways as well as discovering novel signaling events both shared by, or specific to, each Toll-like receptor, including oxidative burst, protein kinase A (PKA) activity and activation of kinases not previously associated with Toll-like signaling.

The bovine peptide arrays confirmed the utility of this approach for characterizing complex signaling events. We used the arrays to analyze cell signaling events within bovine monocytes following stimulation by two agonists known to target distinct TLRs. TLR4 and TLR9 are known to share conserved signaling pathways but there are also reports that each TLR may also utilize unique signaling proteins (Miggin and O'Neill, 2006) This would be consistent with emerging evidence that leukocytes display distinct cellular responses to each TLR agonist (Muzio *et al*., 2000). Our kinome analysis confirmed that TLR4 and TLR9 signaling in bovine monocytes displayed a remarkable level of conservation when comparing between TLRs (**Figure 4.3**).

Table 5.1. A list of non-specific peptides.

Phospho-protein	Kinase Target	Human peptide Sequence	Bovine peptide Sequence
JNK1/2/3	Y185	TSFMMTPYVVTRYYR	TSFMMTPYVVTRYYR
PKCA/B/C	T323	GVTTKTFCGTPDY	DGVTTKTFCGTPDYI
MKK3/6	S98	GYLVDSVAKTMDAGC	GYLVDSVAKTMDAGC
IKKα/β	T180	DQGSLCTSFVGTL	LDQGSLCTSFVGTLQ
Smad1/2/3	S405	GSPSVRCSSMS	LTQMGSPSVRCSSMS
Akt1/3	T310	DGATMKTFCGTPEY	KDGATMKTFCGTPEY
Akt1/3	S173	RPHFPQFSYSASS	RPHFPQFSYSASATA
FADD	S194	QNRSGAMSPMSWNSD	
NF-κB (p105)	S907	QAHSLPLSPASTRQQ	
P53	S6/9	MEEPQSDPSVEPPLSQ	
PKCE	T710	TREEPVLTLVDEAIV	
IRAK-1	S568	WQPLAAPSGASAQAA	
P300	S893	SELLRSGSSPNLNMG	
P300	S1834	MLRRRMASMQRTGVV	
TRAIP	S497	IKTEVPGSPAGTEGN	
TRAIP	S690	IDGATQSSPAEPKSE	
IkB-β	S131/15	SGPCSSSSDSDGGDE	
IkB-β	T19/23	DEWCDTGLGSLGPDA	
TNF-R1	S274	LAPNPSFSPTPGFTP	
Akt3	T447	TAQTITITPPEKYDE	
Nik	T559	TGDYIPGTETHMAPE	

Table 5.2. A list of non-reacting peptides

Phospho -protein	Kinase Target	LPS-fold	CpG-fold	Human peptide	Bovine peptide
ATF-4	S245	1.0	0.8	TRGSPNRSLPSPGVL	SRGSPNKSLLSPGAL
NFAT2	S245	0.9	1.1	PSTSPRASVTEESWL	PSTSPRTSVTEESWL
BATF	T48	1.0	2.0	QKSRQRQTQKADTLH	QKSRQRQTQKADTLH
Jak1	Y1022/3	0.3	0.6	AIETDKEYYTVKDDR	AIETDKEYYTVKDDR
ITK	Y512	0.8	0.4	RFVLDDQYTSSTGTK	RFVLDDQYTSSTGTK
Btk	Y222	1.0	1.8	LKKVVALYDYMPMNA	LKKVVALYDYMPMNA
DOCK2	Y221/4	1.0	1.0	MSKDQPDYAMYSRIS	MSKDQPDYGMYSRIS
RelB	S573	1.0	1.5	AFGGGLLSPGPEAT	AGFGGGLLSPGPEAT
BLNK	Y72	1.0	1.0	SDDFDSDYENPDEHS	SDDFDSDYENPDEHS
VEGFR-2	Y1214	1.0	1.0	VCDPKFHYDNTAGIS	VCDPKFHYDNTAGIS
RAC1	S71	1.0	1.0	YDRLRPLSYPQTDVF	YDRLRPLSYPQTDVF
FGFR1	Y463	1.0	1.0	MLAGVSEYELPEDPR	MLAGVSEYELPEDPR
FGFR3	Y760	1.4	0.9	TVTSTDEYLDLSAPF	TVTSTDEYLDLSVPF
Src	S74	1.0	1.0	NSSDTVTSPQRAGPL	NSSDTVTSPQRAGPL
PKG1	T58	1.0	1.0	THIGPRTTRAQGISA	THIGPRTTRAQGISA
Fes	Y713	1.0	1.5	REEADGVYAASGGSR	REEADGIYAASGGLR
RasGAP	Y460	0.4	0.8	TVDGKEIYNTIRRKT	AVDGKEIYNTIRRKT
Fas	Y291	1.0	1.0	LHGKKEAYDTLIKDL	SHGKKNAYCTLTKSL
ASK1	S83	1.0	1.0	ATRGRGSSVGGGSRR	ATRCRGNSGGGGGRR
TRAF6	S12	1.1	1.2	ENSCGSSQSESDC	HCENSCGSSQSESDC
TRAF6	S97	0.9	0.9	KACIIKSIRDAGH	CKACIIKSIRDAGHK
TRAF6	S48	0.8	0.4	LSSSFMEEIQ	TVTLSSSFMEEIQGY
TRAF6	T486	0.9	0.8	EALRQRTFIKDDT	LEALRQRTFIKDDTL
TRAF6	T317	0.4	0.3	HQIRELTAKMETQ	DHQIRELTAKMETQS
TRAF6	T83	0.1	0.1	LREAVQTPCGHRF	ALREAVQTPCGHRFC
TRAF6	T429	0.6	0.1	PWPFQGTIRLTIL	LPWPFQGTIRLTILD

Furthermore, a comparison with previous analyses in other species and cell types also revealed a remarkable conservation of kinome activity for TLR4 and -9 in bovine monocytes (**Figure 4.4**). This observation supports the conclusion that there was a relatively high level of accuracy when annotating orthologous genes in the bovine genome.

Kinome analysis also revealed a substantial component of cell signaling that was unique to each TLR (**Figure 4.3**). Some of the phosphoproteins implicated in the TLR cell signaling pathways had been identified in previous studies using a variety of other techniques and cell lines (**Figure 4.4**). There were also a number of kinase targets which had not been previously implicated in TLR signaling. We have shown that by using a peptide array many cellular phosphorylation activities that were previously investigated by laborious methodologies in various cell types can be quickly studied in a single cell population and species of interest.

The development and use of peptide arrays for kinome analysis are in early stages of development. Therefore, careful interpretation of data and application of functional assays to validate phosphorylation results is necessary. The rationale behind these arrays is to provide a relative measure of activity for individual kinases under different treatment conditions.
Most appropriately these arrays can be used to provide a broad overview of kinome activity which can then be validated through independent, species-specific techniques.

As demonstrated here, validation of peptide array observations can be done with functional assays or phosphorylation-specific antibodies. A PKA assay, for example, allowed us to identify the specific site of PKACa corresponding to activation of this enzyme. Moreover, a superoxide assay facilitated proper interpretation of a complex interaction between p47(PHOX) and its inhibitor p40(PHOX) which was not apparent from our peptide array results. Finally, a phospho-Etk antibody was utilized to validate a phosphorylation activity of this substrate in human monocytes due to the lack of a bovine specific phospho-antibody.

5.3.2 Biological Rationale for Distinct TLR Responses

TLR9 and TLR4 belong to two different subsets of the TLR family: TLR4 is a cell membrane receptor and TLR9 is located intracellularly in endosomal compartments. Being located on the plasma member, TLR4 can detect its ligand rapidly. On the other hand detection of CpG by TLR9 depends on intracellular localization of DNA first. Such cellular activity can cause a delay in cell responding to DNA as compared to LPS. Previous reports indicate that

TNF production by human monocytes occurs at 4 hours as compared to 18 hours by CpG (Hartmann and Krieg, 1999). We would expect earlier activation of the general TLR signaling pathway and NF-kB as well as earlier production of TNF by LPS as compared to CpG. At 4 hour time point during our experiments, it is evident that activation of this TLR pathway is variable by LPS and CpG stimulation (**Figure 4.3**).

TLR4 location on the cell surface allows its exposure to external stimuli. Thus, TLR4 has evolved to trigger the innate immune responses in a more efficient manner through two sets of adaptor molecules. Our results show that pathways specific to TLR4 are also activated during the LPS stimulation of bovine monocytes. More specifically, we were able to show that IRF3 which is phosphorylated through TRIF/TRAM adaptor complex is activated and subsequently induced activation events of FADD and Casp8 proteins (**Table 4.2**).

Kinome responses and functional assays on bovine monocytes were performed at a 4-hour time point. From kinome responses of bovine monocytes to CpG ODN and LPS, we observed conserved and differential phosphorylation activities in these monocytes due to LPS and CpG ODN. Previous research indicates that long term stimulation of cells with LPS inhibits activity of IRAK-4 which in turn affects downstream signaling pathways by TLR4 (Hatao *et al*., 2004). In addition, comparison of LPS and CpG stimulated human monocytes have shown high sensitivity and earlier responses of monocytes to LPS at 4 hour time and a longer 18 hours time point to CpG (Hartmann and Krieg, 1999). As this investigation was carried out at a single time point, the differences that were observed between TLR4 and TLR9 signaling could have been transient and not reflective of biological differences. A series of experiments at early and late time points can shed more light into the complex TLR signaling networks.

5.4 Advantages and Disadvantages of Peptide Arrays

It is now possible to use information from phosphoproteome investigations to create tools to investigate both the kinases; the kinome, and their targets the phosphoproteome. One advantage of kinome analysis is that focusing on enzymatic activity, rather than product abundance, enhances the sensitivity of detecting changes in kinase activity. This approach also enables development of high throughput tools which can simultaneously assess changes in a large number of preselected phosphorylation events.

The present investigation clearly demonstrated an additional advantage of using peptide arrays for kinome analysis. The conservation of many phosphorylation sites within orthologous proteins enables the rapid creation of peptide arrays for kinome analysis in species for which limited phosphorylation databases exist but genomic information is available. This bioinformatics approach eliminates the need to generate and characterize species-specific and phosphorylation-specific antibodies. The present data (**Table 4.1** and **Table 4.2**) confirm that it is possible to identify conserved and divergent phosphorylation sites in orthologous proteins using protein databases from other species. This enables the rapid design of kinase substrate peptides to incorporate species-specific variances. While many of the resulting sites are absolutely conserved with respect to the protein sequences surrounding the phosphoacceptor sites, there is sufficient species-specific variation to justify the utility of a bioinformatics approach (**Table 4.1**). Such customization of arrays can be taken a step further by development of arrays focused on specific biological responses or cell functions. The bovine array used in the current investigation was biased for phosphoprotein targets known to be involved in innate immune signaling pathways.

The use of peptide arrays for kinome analysis is still in the early stages of development and there are a number of technical and biological challenges which need to be addressed. For example, while the arrays are designed to monitor kinase activity as a marker of signal transduction activity biologically, the net degree of phosphorylation of a particular target reflects the dual and opposing contributions of both kinase and phosphatase activity. The presence of a panel of phosphatase inhibitors within the cellular extracts analyzed here negates the contribution of these enzymes to the net phosphorylation status of a particular target in order to simplify analysis of kinase activity.

In some cases peptide substrates may not serve as ideal ligands for the corresponding protein kinase. This could manifest itself in a number of ways. Firstly, a peptide may be recognized and phosphorylated by kinases other than those which mediate physiological phosphorylation. This would result in false positives or overestimation of the extent of phosphorylation which would occur with the cellular protein. It is also possible that peptides on the array make available substrate targets that are not present within the particular cell type. Alternatively, that individual peptides may be recognized with differing degrees of efficiency

by their respective kinases makes it difficult to use the absolute measure of phosphorylation of a given peptide as an indicator of the level of activity of the corresponding kinase.

A species-extrapolated peptide array will experience the same limitations as a peptide array created for species with well-defined phosphorylation information with one additional challenge. A paralog protein rather than an ortholog may produce matches of up to 100 % sequence similarity for the phosphorylation motif of interest. Inclusion of such peptides on the array will produce false positive signals.

5.5 Future Direction

Peptide array technology has evolved in the past few years and like any new technology, it has not reached its full potential. Currently commercial arrays are available for human research. As progress in peptide array technology continues, arrays of increasing complexity will become available to researchers. These advancements will undoubtedly include an expansion of the breadth of arrays available for different organisms, as well as kinome subsets, such as for specific organelles. One method to develop focused and species-specific array is reported here.

Based on the principle that a high degree of amino acid conservation for phosphorylation targets exists among mammalian species, a peptide array for bovine species was developed encompassing phosphorylation targets for innate immune signaling transducers and effector molecules. Development of this tool was achievable mainly because bovine genome was sequenced in 2005 and much effort has been directed towards prediction of proteins and functional annotations of bovine genes. As a result, many sequences for bovine proteins of interest are available in public databases. However, for many predicted kinase substrates, phosphorylation sites are not calculated and their biological significance is unknown. We successfully inferred phosphorylation information for approximately 140 proteins and 298 phosphorylation targets of bovine species by utilizing publically available databases and bioinformatics tools. Furthermore, we were able to validate biological function of many of the inferred phosphorylation sites quickly by applying the peptide array to a well studied signaling pathway. Currently, efforts are directed towards genome sequencing of many organisms and the methodology developed here can be utilized to annotate phosphorylation sites for other species as soon as their genomes become available. In addition, similar focused

arrays can be developed for subsets of the kinome such as all kinases involved in a particular disease or cell function.

The quality of our array can be improved in the future by removing non-specific peptides from the pool and adding controls for each peptide. In addition, current array holds some peptides which carry more than one phosphorylation targets. Phosphorylation signal for such a peptide may result from activity of more than one kinase. While measuring phosphorylation signals for such a peptide, we can not predict whether all the targets or one site is phosphorylated. In such cases, we can have representative peptides where one phosphorylation site is present and the rest are replaced with amino acids that do not participate in phosphorylation. A variation of this method can be utilized to investigate significance of having a combination of the phosphorylation sites present simultaneously. In addition, for peptides holding a single phosphorylation target, controls can be created by replacing the target amino acid with a non-phosphorylation amino acid.

A tremendous amount of phosphorylation data was generated during this investigation. There are approximately 100 peptides that are differentially phosphorylated by CpG or LPS when compared to media control. By applying functional assays we have validated few of these responses and interpreted interactions between signaling molecules in superoxide production, PKA activity and Etk phosphorylation. However, much work is needed to validate the remaining peptide array results and interpret phosphorylation based signaling pathways such as Smad and Stat pathways whose members are present on the array. In addition, we have identified new phosphorylation targets for some of the proteins that were not reported previously. New targets of TLR9 and TLR4 signaling pathways can be identified by understanding the significance of these previously unknown phosphorylation targets.

As our bovine peptide array holds transcription factors, such as NF-kB, a parallel investigation of transcription activity in stimulated monocytes will enhance the validity of our kinome analysis. As phosphorylation may correspond to activation of a transcription factor, by performing real-time PCR to test expression of target genes, we can validate our kinome results. Alternatively, a large scale gene expression analysis can be carried out by utilizing oligo or cDNA microarrays.

As kinome analysis of cells was carried out at a single time point, the differences that were observed between TLR4 and TLR9 signaling could have been transient and not reflective

of biological differences. To further investigate, a series of experiments at early and late time points need to be carried out. These peptide arrays are excellent tools to evaluate transient and time dependency nature of these kinome responses.

Controversy exists regarding expression of TLR9 on human, bovine and mouse monocytes. Recent investigations have reported that human monocytes express TLR9 and respond to CpG through NF-kB pathway (Hornung *et al*., 2002; Doyle *et al*., 2007). Here we have demonstrated that bovine monocytes respond to CpG through activation of NF-kB as well. We assume that such signaling activity is initiated by TLR9 receptor on bovine monocytes. However, it is possible that CpG ODN triggers these cellular responses through other TLR signaling pathways. In this investigation we have assumed that CpG ODN modulates responses through the TLR9 receptor. Therefore, it is necessary to study expression of TLR9 on these cells through gene expression or marker expression techniques. An alternative would be to study kinome responses of TLR9-/- bovine monocytes and compare kinome responses to present investigation.

Peptide arrays for kinome analysis allow for characterization of global patterns of signal transduction activity as well as dissection of dynamic patterns of phosphorylation which can occur at distinct sites of the same protein to regulate unique biological objectives allowing for much more descriptive analysis of how particular pathways, and individual proteins, and being post-translationally adapted to different stimuli. We anticipate these arrays will be of considerable utility to rapidly screen specific cell populations to determine if they can respond to an agonist and whether this response is conserved when compared to the same cell type from other species or different cell types within the same species.

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7.0 Appendices

Appendix A

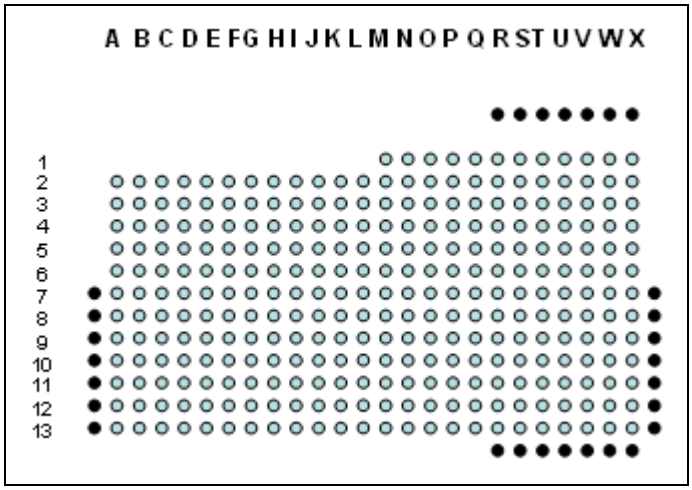

Figure A1. Mapping of the Bovine Peptide Array. An illustration of the positioning of the 300 peptides (including the negative controls) and the positive controls (black spots) on a block; an array holds three replicate blocks of the same configuration. Positive controls (Histon 1-4, MBP bovine, and alpha/beta casein) proteins are printed around the borders of each block for visualization purposes.

Table A1. Protein names and target amino acids for peptides on the bovine array. Positioning of peptide identifiers in the table is according to the map in Appendix A- Figure A1.

	1	2	3	4	5	6	7	8
A	Empty	Cdc42(Y64)	DAPK1(S308)	TRAF6(T83)	Src(S96)	Rb(S780)	PLCG1(Y783)	eNOS(S1176)
B	Empty	iNOS(S909)	Bid(T59)	TRAF6(T317)	Src(S74)	BCAP(Y459)	PLCG1(Y771)	Grb10(S150)
C	Empty	iNOS(S739)	Control(-)	TRAF6(T486)	Lyn(Y507)	BCAP(Y195)	PLCG1(Y472)	IFNGR1(Y479)
D	Empty	iNOS(Y151)	Control(-)	TRAF6(S48)	cyclin E1(T395)	RAC1(S71)	PKR(T451)	IFNGR1(Y457)
E	Empty	IL-8R B(S351\2\3)	SRF(S77\9)	TRAF6(S97)	Cdc25A(S88)	SEK1(S257\61)	FLT3(Y597\9)	Jun(S73)
F	Empty	IL-8R B(S347)	CREB(S129)	TRAF6(S12)	Cdc25A(S82)	SEK1(S80)	FLT3(Y589\91)	Jun(S63)
G	Empty	CCR7(T372\3\4)	CREB(S111\4)	MEK5(S311)	LRRFIP1(S690)	4E-BP1(T45)	gp130(Y814)	STMN1(S62)
H	Empty	CCR7(S356\7)	MSK2(S347)	JNK3(T131)	LRRFIP1(S497)	4E-BP1(T36)	gp130(S782)	STMN1(S15\Y15)
I	PKCT(T219)	MSK2(S196)	JNK2(T404\7)	TNIK(T987)	CD45(Y1216)	gp130(Y767)	CDK2(T160)	
J	PKCT(Y90)	MSK1(T581)	JNK2(S129)	TNIK(S764)	CD45(S999\02\03)	MEK2(Y216)	CDK2(T14)	
K	PKCA(T637)	PPARG(S112)	ASK1(S966)	Shc1(Y349\50)	Bad(S118)	PIK3R1(S607\8)	Pyk2(Y402)	
L	Empty	Raf1(S259)	MEKK1(T1383)	IGF1R(Y1166)	Etk(Y40)	Kit(S821)	CDK7(T170)	
M	FADD(S194)	PKCE(S729)	Raf1(S499)	P38 G(T183\5)	FRS2(Y435)	CTLA-4(Y201)	Kit(Y721)	cyclin D1(T286\8)
N	p53(S215)	PKCE(T710)	H-Ras-1(T35)	DAPK3(T225)	FRS2(Y195)	PXN(Y31)	RIPK1(Y384\7\9)	p300(S1834)
O	p53(S6\9)	PKCB(T323)	RasGAP(Y460)	DAPK3(T180)	FGFR3(Y760)	CSK(Y184)	ROCK2(S1133\4)	p300(S893)
P	IFNAR1(S535)	PKCB(S15\T16)	Fes(Y713)	MLK3(T278)	FGFR3(Y724)	Crk(Y221)	IKK-α(T23)	CD28(Y206\9)
Q	IFNAR1(Y466)	ASK1(S1033)	PKG1(T58)	MEK1(S217)	FGFR4(Y754)	NGFR(Y336)	mucin 1(T1227\9)	HSP70(Y525)
R	JNK1(Y185)	ASK1(S83)	TPH1(S58)	Mos(S25)	FGFR1(Y154)	FRS3(Y416)	PDGFRb(Y740)	HSP27(S82\83)
S	Akt1(S173)	Daxx(S668\71)	IkB-epsilon(S18)	B-Raf(T598)	FGFR1(Y653\4)	TrkC(Y516)	PDGFRb(Y579\81)	EphA2(Y772)
T	Akt1(T310)	Fas(Y291)	IkB-β(T19)	A-Raf(Y301)	FGFR1(Y463)	TrkB(Y706\7)	CSFR(Y809)	EphA2(Y594)
U	smad2(S405)	Fas(Y232)	IkB-β(S313\5)	JIP1(T103)	Met(Y1349)	VEGFR3(Y1265)	CSFR(Y561)	EphA2(Y588)
V	IKKA(T180)	TNFRSF5(T254)	IkB-α(Y42)	Ezrin(T566)	Met(Y1234)	VEGFR3(Y130\1)	PPP2CA(T304)	EphA1(Y781)
W	MKK3(S98)	TNF-R1(S274)	IkB-α(S32\6)	VASP(S156)	RhoA(S188)	VEGFR-2(Y1214)	PTP1B(S50)	p47phox(S370)
X	PKCA(T323)	DAPK1(S735)	TRAF6(T429)	Calmodulin(Y99)	Rb(S795)	VEGFR-1(Y1169)	Nik(T559)	p40phox(T154)

13	12	11	10	9
IRF-3(S385\6)	IRAK-1(T209)	Akt1(S129)	IL2RB(Y536)	WASP(Y291)
CTNNB1(S675)	IRAK-1(T100)	ERK3(S189)	IL2RB(Y364)	Syk(Y525/6)
CTNNB1(Y142)	FADD(S194)	ERK2(Y205)	IL-10R-A(Y496)	Cot(S400)
CTNNB1(T41)	HIF2A(T840)	ERK1(T202/4)	IKK-β(Y199)	CaMK2-α(T286)
CTNNB1(S33)	HIF1A(T796)	TBK1(S172)	IKK-β(Y188)	smMLCK(S1773)
STAT6(Y641)	XIAP(S87)	Jak3(Y981)	HMGA1(T52)	MKP-1(S359)
STAT5B(Y699)	BATF(T48)	Jak2(Y1007\8)	LEF-1(S166)	MKP-1(S323)
STAT5B(S731)	NFAT4(S163\5)	Jak1(Y1022\3)	LEF-1(T155)	MKP-1(S296)
STAT4(S721)	NFAT2(S294)	JNK1(S377)	DOCK2(Y209\212)	PLCB1(S887)
STAT4(Y693)	NFAT2(S245)	p38-α(Y323)	CBP(S2063)	PLCB1(T333\4\6)
STAT3(S727)	NFAT1(S326)	p38-α(T179\Y181)	DNA-PK(T2638)	BLNK(Y96)
STAT3(Y705)	NFAT1(S168)	axin-1(S486)	DNA-PK(T2609)	BLNK(Y72)
STAT2(Y690)	Smad4(T277)	Notch 2(S2070)	PKR(T446)	DAPP1(Y139)
STAT1(S727)	Smad3(S422\3\5)	Notch 2(T1808)	Btk(Y550)	RelB(S573)
STAT1(Y701)	Smad3(S208)	GSK3-β(Y216)	Btk(Y222)	APE1(S289)
IKK-G(S43)	Smad2(S255)	GSK3-β(S9)	ITK(Y512)	Tyk2(Y1054\5)
IKK-G(S31)	Smad2(T8)	Elk-1(T417)	FAK(Y397)	p70S6Kb(S473)
NFkB-p105(S907)	Smad1(S462\3\5)	Elk-1(S389)	TAK1(S192)	CXCR4(Y157)
NFkB-p105(S337)	ATF-4(S245)	Fos(T232)	TAK1(T184)	CCR5(S336\7)
NFkB-p65(S536)	ATF-2(T51\3\5)	Casp8(S347)	PKACa(S338)	CCR2(Y139)
NFkB-p65(S311)	ATF-2(S44)	Casp3(S150)	PKACa(T195\7)	IL7R(Y449)
NFkB-p65(S276)	IRF-5(S437)	IRAK-1(S568)	Akt3(T447)	IL1A(S87)
NFkB-p100(S870\2)	IRF-3(S402\T404)	IRAK-1(T387)	Akt3(S120)	IL-16(S143\4)
NFkB-p100(S866\Y868)	IRF-3(S396)	IRAK-1(S376)	Akt1(Y326)	IL4R(Y713)

Table A2. Peptide sequences printed on the bovine peptide array. A list of all the peptide sequences that were printed on the array is presented. For each sequence in a cell, its protein name and target amino acid position is available in Appendix A- Table A1.

6	5	4	3	2	1	
STRPPTLSPIPHIPR	VALYDYESRTETDLS	ALREAVQTPCGHRFC	ARKKWKQSVRLISLC	DTAGQEDYDRLRPLS	Empty	A
PAATEDLYVEMLQAS	NSSDTVTSPQRAGPL	DHQIRELTAKMETQS	FHDDELQTDGNRCSH	PILKPRYYSISSSRD	Empty	B
FQAETTVYVIVRCKL	DDFYTATEGQYQQQP	LEALRQRTFIKDDTL	NIANSATYGFKSGTA	VFTMRLKSRQNLQSP	Empty	C
YDRLRPLSYPQTDVF	PLPTGVLTPPQSSKK	TVTLSSSFMEEIQGY	KEKLGYTGESSVQAA	IEFVNQYYGSFKEAK	Empty	D
GQLVDSIAKTRDAGC	DSGFCLDSPGPLDSK	CKACIIKSIRDAGHK	PTAGALYSGSEGDSE	PSFVGSSSGNTSTTL	Empty	E
IERLRTHSIESSGKL	GSSESTDSGFCLDSP	HCENSCGSSQSESDC	QKRREILSRRPSYRK	LAKDGRPSFVGSSSG	Empty	F
PGGTLFSTTPGGERR	IDGATQSSPAEPKSE	TQLVNSIAKTYVGTN	TIAESEDSQESVDSV	SSMSVEAETTTTFSP	Empty	G
PPGDYSTTPGGTLFS	IKTEVPGSPAGTEGN	ISLLNVFTPQKTLEE	PVYSPPGSPPPGDPR	EQLRQWSSCRHIRRS	Empty	H
MVPTFEQYQFLYDVI	RVYQTSPTDEDEEDE	MSTEQTLASDTDSSL	EEKERTFSFCGTIEY	SAINSRETMFHKERF	Empty	I
SEQDSDESSDDDSDL	RTRVRANSKSEGSPV	ELDHERMSYLLYQML	PDNQPLKTPCFTLHY	SETTVELYSLAERCR	Empty	J
GRELRRMSDEFHVSF	EEPPDHQYYNDFPGK	NEYLRSISLPVPVLV	AIKVEPVSPPYYSEK	TRGQPVLTPPDQLVI	Empty	K
LTKTNLSYYEYDKMK	DIYETDYYRKGGKGL	ARLASKGTGAGEFQG	SQRQRSTSTPNVHMV	QSDFEGFSYVNPQFV	Empty	L
SPLTTGVYVKMPPTE	LEHRQLNYIQVDLEG	RQADSEMTGYVVTRW	VKSRWSGSQQVEQPT	QEEFKGFSYFGEDLM	QNRSGAMSPMSWNSD	M
FLSEETPYSYPTGNH	AEEQVHTYVNTTGVQ	LGETKQETLTNISAV	FVDEYDPTIEDSYRK	TREEPVLTLVDEAIV	DRNTFRHSVVVPYES	N
VAAQDEFYRSGWALN	TVTSTDEYLDLSVPF	EFKNIFGTPEFVAPE	AVDGKEIYNTIRRKT	PEEKTTNTISKFDNN	MEEPQSDPSVEPPEE	O
GGPEPGPYAQPSVNT	ANCTHDLYMIMRECW	REWHKTTQMSAAGTY	REEADGIYAASGGLR	PSEGEESTVRFARKG	SSQTSQDSGNYSNED	P
LKGDGGLYSSLPLAK	LLAVSEEYLDLRLTF	GQLIDSMANSFVGTR	THIGPRTTRAQGISA	EDHSAPPSPEEKDSG	VFLRCVKYVFFPSSK	Q
EPPRQLNYIQVELKG	NRMPVAPYWTSPEKM	SGDSRPCSSPCELLG	RKSKRRSSEFEIFVD	ATRCRGNSGGGGGRR	TSFMMTPYVVTRYYR	R
PVIENPQYFRQGHNC	DIHHIDYYKKTTNGR	IGDFGLATVKSRWSG	ESQYDSGIESLRSLR	HTLPSPPSPLASMAP	RPHFPQFSYSASATA	S
RDVYSTDYYRVGGHT	MLAGVSEYELPEDPR	LGYRDSGYYWEVPPS	DEWCDTGLGSLGPDA	SHGKKNAYCTLTKSL	KDGATMKTFCGTPEY	T
FPMTPTTYKGSVDNQ	STFIGEHYVHVNATY	LIDAAGDTPGAEDDE	SGPCSSSSDSDGGDE	TDVDLGKYIPSIAEQ	LTQMGSPSVRCSSMS	U
RHSLAARYYNWVSFP	RDVYDKEYYSVHNKT	QGRDKYKTLRQIRQG	DSMKDEEYEQMVKEL	PPPPVQETLCWCQPV	LDQGSLCTSFVGTLQ	V
VCDPKFHYDNTAGIS	LQARRGKKKSGCLVL	MERERRASNAGGPPA	DDRHDSGLDSMKDEE	LAPNPSFSPTPGFTP	GYLVDSVAKTMDAGC	W
VQQDGKDYIPLNAIL	SPYKFSSSPLRIPGG	FDKDGNGYISAAELR	LPWPFQGTIRLTILD	NSTRFPPSPLASKPA	DGVTTKTFCGTPDYI	X

13	12	11	10	9	8	7
IARQGGASSLENTVD	LHEICQGTHDFSEEL	SGSPGENSGAEEMEV	LTLNTDAYLSLQELQ	RETSKVIYDFIEKTG	TSRIRTQSFSLQERH	EGRNPGFYVEANPMP
QDYKKRLSVELTSSL	LRARDIITAWHPPAP	YSHKGHLSEGLVTKW	SCFTNQGYFFFHLPD	ALRADENYYKAQTHG	PELCGPGSPPALTPG	IGTAEPDYGALYEGR
AVVNLINYQDDAELA	QSGSANPGSFTAWDS	IMLNSKGYTKSIDIW	LPALAKGYVQQDPPE	EDQPRCQSLDSALFE	GKESLIGYRLTADSR	KLAEGSAYEEVPTSV
HSGATTTAPSLSGKG	YLLPELTRYDCEVNV	HTGFLTEYVATRWYR	ELLEQQKYTVTVDYW	SCMHRQETVDCLKKF	NTTTSFGYDKPHVLV	RTSKKGTLRYMSPEQ
QSYLDSGIHSGATTT	ESGLPQLTSYDCEVN	EDDEQFVSLYGTEEY	SFVGTLQYLAPELLE	GLSGRKSSTGSPTSP	VGLLKLASPELERLI	YIDFREYEYDLKWEF
MGKDGRGYVPATIKM	IGRHRRVSPNCRFIN	LPLDKEYYVVREPGQ	KEPSEVPTPKRPRGR	SALSYLQSPITTSPS	KNSDLLTSPDVGLLK	TGSLDNEYLYIDFRE
TAKAVDGYVKPQIKQ	QKSRQRQTQKADTLH	VLPQDKEYYKVKEPG	TYSDEHFSPGSHPSH	HCSAEAGSPAMAVLD	AAEERRKSHEAEVLK	GILPRQRYFKQNCSQ
TYMDQAPSPAVCPQP	SYRESSLSPSPASSI	AIETDKEYYTVKDDR	SHAVHPLTPLITYSD	KQRRSIISPNFSFMG	KELEKRASGQAFELI	QVFSRSESTQPLLDS
PSDLLPMSPSVYAVL	PQGSPRVSVTDDTWL	GVIRGQPSPLGAAVI	MSKDQPDYGMYSRIS	HSQPAPGSVKAPAKT	GVPVRTYTHEVVTLW	STVVHSGYRHQVPSV
TEKGDKGYVPSVFIP	PSTSPRTSVTEESWL	DEPVADPYDQSFESR	PPRSISPGALQDLLR	INSSHNTYLTAGQLA	EKIGEGTYGVVYKAR	KLCDFGVSGQLIDSM
NTIDLPMSPRTLDSL	PPKMWKTSPDPSPVS	RHTDDEMTGYVATRW	MAGQVRATQQQLDFT	EEAGDDSYEPPPVEQ	CSIESDIYAEIPDET	ENTEDQYSLVEDDED
DPGSAAPYLKTKFIC	YREPLCLSPASSGSS	MRTPGCQSPGPGHRS	LTPMFVETQASQSTL	SDDFDSDYENPDEHS	GSPSRAYTHQVVTRW	ARDIKNDSNYVVKGN
NPEERKKYLKHKLIF	GSRTAPYTPNLPHHQ	DEYNVTPSPPGTVLT	LKNDETRTSKKGTLR	KVEEPSIYESVRVHT	VDLACTPTDVRDVNI	CNDSTNEYMDMKPGV
TDNLLPMSPEEFDEV	LTQMGSPSIRCSSVS	RTPSLALTPPQAEQE	RYVLDDEYTSSVGSK	AGFGGGLLSPGPEAT	MLRRRMASMQRTGVV	EEANYHLYGSRMDRR
DGPKGTGYIKTELIS	DAGSPNLSPNPMSPT	RGEPNVSYICSRYYR	LKKVVALYDYMPMNA	DYFLLSHSLLPALCD	SELLRSGSSPNLNMG	HIGLDSSSIGSGPGD
PAMLHVPSEQGTPET	ELSPTTLSPVNHSLD	SGRPRTTSFAESCKP	RFVLDDQYTSSTGTK	AVPEGHEYYCVREDG	PGPTRRHYQPYAPAR	EMRERLGTGGFGNVC
QDMLGEESSLGKPAM	MSSILPFTPPVVKRL	ISVDGFSTPVVLSPG	TTPETDDYAEIIDEE	PPSGTKKSKRGRGRP	MVQEAEKYKAEDEVQ	PPGSTKRSPYEEVSA
QAHSLPLSPASTRQQ	LTQMGSPHNPISSVS	LSPIAPRSLAKLSFQ	HMTNNKGSAAWMAPE	LLAEKVVYVGVWLPA	RALSRQLSSGVSEIQ	PGESDGGYMDMSKDE
FVQLRRKSDLETSEP	SRGSPNKSLLSPGAL	GGLPEAATPESEEAF	GTACDIQTHMTNNKG	EAPERVSSVYTRSTG	EDDPEATYTTSGGKI	VSSDGHEYIYVDPMQ
SGDEDFSSIADVDFS	IVADQTPTPTRFLKN	FTGLKCPSLIGKPKI	EEEEIRVSINEKCGK	ILLTIDRYLAIVHAV	TYVDPHTYEDPNQAV	DIMNDSNYIVKGNAR
RTYETFKSIMKKSPF	FGPARNDSVIVADQT	FRGDYCRSLTGKPKL	RVKGRTWTLCGTPEY	GSSQEEAYVTMSSFY	QLKPLKTYVDPHTYE	ESYEGNSYTFIDPTQ
SMQLRRPSDRELSEP	FSGELSWSADSIRLQ	WQPLAAPSGASAQAA	TAQTITITPPEKYDE	ILKKRRLSLNQFITD	LDNFDGTYETQGGKI	RGEPHVTRRTPDYFL
DSAYGSQSVEQEAEK	NSDPLSLTPDQYMAC	RTRTVRGTLAYLPEE	EEERMNCSPTSQIDN	SSIKQRISSFETFSS	PAVPPRPSADLILHR	RNRYRDVSPFDHSRI
STEVKEDSAYGSQSV	NTVDLHISNSDPLSL	GSSPSQSSMVARTQT	EVLEDNDYGRAVDWW	LGNGIIYSALTCHLC	LRRLRPRTRRVKSES	TGDYIPGTETHMAPE

Table A3. Complete set of data for the peptide arrays with CpG and LPS treated monocytes.

Protein Name	Target	Fold Change		p value		Upstream kinase	Phosphorylation effects
		LPS	CpG	LPS	CpG		
FADD (human)	S194	0.8	0.6	0.01	0.01		
p53	S215	0.9	0.9	0.01	0.01		
p53	S6/9	1.0	0.9	0.01	0.01	ATM(S6/9)	Activation (S6), regulates apoptosis (S6/9), and more (S6/9)
IFNAR1	S535	1.8	8.5	0.01	0.01		Regulates association with cellular proteins , protein degradation
IFNAR1	Y466	0.9	0.9	0.01	0.01	Tyk2	Regulates association with cellular proteins
JNK1	Y185	0.7	0.7	0.01	0.01		
Akt1	S173	0.7	0.9	0.15	0.12		
Akt1	T310	1.1	1.0	0.01	0.01		
smad2	S405	1.3	1.3	0.01	0.01		
IKKA	T180	0.9	0.4	0.06	0.49		
MKK3	S98	0.9	0.7	0.01	0.01		
PKCA	T323	1.8	1.7	0.01	0.01		
Cdc42	Y64	0.2	0.4	0.03	0.09	Src	Regulates cell growth
iNOS	S909	0.8	0.7	0.01	0.01		
iNOS	S739	0.4	1.0	0.50	0.01		
iNOS	Y151	0.5	1.0	0.73	0.01	Src	Altered intracellular location , enzymatic inhibition
IL-8R-B	S351/2/3	1.2	1.6	0.01	0.01		
IL-8R-B	S347	0.4	1.2	0.01	0.01		Receptor internalization , protein degradation , altered receptor desensitization
CCR7	T372/3/4	0.2	0.1	0.01	0.01		
CCR7	S356/7	0.3	0.3	0.18	0.16		
PKCT	T219	0.8	0.7	0.01	0.01	PKCT	Altered intracellular location
PKCT	Y90	1.1	1.1	0.01	0.01	Lck	Regulates transcription, activation
PKCA	T637	1.2	0.7	0.74	0.17		
PKCA	S657	1.1	1.0	0.01	0.01		Altered intracellular location
PKCE	S729	0.9	0.7	0.01	0.01	PKCE	Activation
PKCE	T710	0.9	1.0	0.01	0.01		
PKCB	T323	0.9	0.8	0.01	0.02		
PKCB	S15/T16	0.9	1.1	0.30	0.01		
ASK1	S1033	1.1	1.1	0.29	0.18		
ASK1	S83	1.0	1.0	0.01	0.01	Akt	Inhibition
Daxx	S668/71	0.9	0.7	0.57	0.18		Altered intracellular location(S668), regulates association with cellular proteins(S668)
Fas	Y291	1.0	1.0	0.01	0.01		Regulates apoptosis , altered

							intracellular location , regulates association with cellular proteins
Fas	Y232	0.8	0.7	0.01	0.01		Regulates apoptosis , altered intracellular location , regulates association with cellular proteins
TNFRSF5	T254	0.2	0.4	0.08	0.26		Regulates association with cellular proteins , activation
TNF-R1	S274	0.6	0.7	0.48	0.46		
DAPK1	S735	0.3	0.3	0.06	0.07	ERK2	Enzymatic activation
DAPK1	S308	1.3	1.0	0.01	0.01	DAPK1	Regulates cell growth, regulates apoptosis , regulates association with cellular proteins, enzymatic inhibition
Bid	T59	1.8	3.4	0.01	0.01	CK2-A1	
Cntrll(-)		0.4	1.1	0.05	0.33		
Cntrl(-)		0.0	0.0	0.01	0.01		
SRF	S77/79	0.9	1.1	0.31	0.94	CK2-A1(S77/79)	Regulates transcription(S77)
CREB	S129	0.4	2.3	0.01	0.05	GSK3B	Regulates transcription, transcription
CREB	S111/4	0.6	5.1	0.01	0.01	ATM, CK2-A1	Inhibition(S111), phosphorylation(S114), regulates transcription and more(S111)
MSK2	S347	0.9	0.8	0.01	0.01		Activation
MSK2	S196	0.8	0.2	0.31	0.03	p38-α	Enzymatic activation , activation
MSK1	T581	0.9	0.9	0.01	0.01	p38-α, ERK2, ERK1	Enzymatic activation
PPARG	S112	0.3	0.9	0.02	0.05	ERK2	Regulates transcription, protein degradation
Raf1	S259	1.0	1.0	0.01	0.01	PKACa	Inhibition
Raf1	S499	1.0	2.3	0.01	0.01	PKACa	Activation
H-Ras-1	T35	0.9	1.0	0.01	0.01		
RasGAP	Y460	0.4	0.8	0.01	0.01		
Fes	Y713	1.0	1.5	0.01	0.01	Fes	Enzymatic activation, regulates association with cellular proteins
PKG1	T58	1.0	1.0	0.01	0.01	PKCA	Enzymatic activation
TPH1	S58	0.8	0.7	0.01	0.01		
IkB-epsilon	S18	0.5	0.6	0.01	0.01		Protein degradation
IkB-β	T19	0.8	0.8	0.01	0.01		Protein degradation
IkB-β	S313/15	0.7	0.8	0.67	0.82		Regulates transcription(S313/5)
IkB-α	Y42	1.1	0.5	0.80	0.21	IKK-epsilon, IKK-β	Activation, protein degradation, transcription
IkB-α	S32/36	0.8	0.5	0.06	0.62	CK2-A1 (S32/6),IKK-α (S32/6), Nik (S32/6)	Activation(S32), protein degradation and more(S32/6)
TRAF6	T429	0.6	0.1	0.01	0.01		
TRAF6	T83	0.1	0.1	0.01	0.01		
TRAF6	T317	0.4	0.3	0.08	0.01		
TRAF6	T489	0.9	0.8	0.01	0.01		
TRAF6	S48	0.8	0.4	0.01	0.01		

TRAF6	S97	0.9	0.9	0.01	0.01		
TRAF6	S12	1.1	1.2	0.02	0.01		
MEK5	S311	0.9	1.1	0.01	0.01		
JNK3	T131	1.0	1.0	0.01	0.01	CDK5	
JNK2	T404/7	0.7	1.2	0.01	0.01	CK2-A1 (T404/7), MKK7 (T404/7)	Activation(T404/7)
JNK2	S129	2.3	1.5	0.11	0.02		
ASK1	S966	0.2	0.4	0.01	0.01	PKD	Inhibition
MEKK1	T1383	0.8	0.7	0.01	0.01	MEKK1	Activation
P38 γ	T183/5	0.4	1.1	0.16	0.32		
DAPK3	T225	0.9	0.9	0.01	0.01	DAPK4	Activation
DAPK3	T180	1.0	0.8	0.01	0.01	DAPK3	Activation
MLK3	T278	1.2	1.1	0.01	0.01		
MEK1	S217	1.3	1.4	0.01	0.01	Raf1 and Cot	Activation
Mos	S25	0.5	1.2	0.32	0.29		
B-Raf	T598	6.0	2.3	0.54	0.05	Ras	Activation
A-Raf	Y301	0.9	2.2	0.01	0.06		
JIP1	T103	1.1	1.8	0.92	0.01	JNK2, JNK1	Regulates association with cellular proteins
Ezrin	T566	1.0	1.2	0.01	0.03	GRK2, Akt2, ROCK1	Cytoskeletal reorganization, altered intracellular location, regulates association with cellular proteins , activation
VASP	S156	1.0	0.7	0.01	0.01	PKG1, PKACa	regulates transcription
Calmodulin	Y99	2.2	3.4	0.01	0.01		
Src	S96	0.9	0.9	0.71	0.93		
Src	S74	1.0	1.0	0.01	0.01		
Lyn	Y507	0.6	0.6	0.03	0.02	CSK	
cyclin E1	T395	1.6	1.3	0.01	0.01	GSK3B, CDK2, GSK3A	Regulates cell cycle, regulates association with cellular proteins, protein degradation, enzymatic inhibition
Cdc25A	S88	0.9	1.1	0.01	0.01		regulates cell cycle, protein degradation
Cdc25A	S82	1.4	1.3	0.01	0.01		Regulates cell cycle, association with cellular proteins, protein degradation
LRRFIP1	S690	1.1	2.1	0.01	0.01		
LRRFIP1	S497	1.1	1.0	0.01	0.01		
TNIK	T987	1.1	1.1	0.13	0.08		
TNIK	S764	0.6	0.8	0.03	0.01		
Shc1	Y349/0	0.9	0.8	0.01	0.01	Src(Y349/50)	Regulates apoptosis(Y349/50), regulates association with cellular proteins(Y349/50)
IGF1R	Y1166	0.7	0.6	0.01	0.01		Enzymatic activation, regulates association with cellular proteins.
FRS2	Y435	0.6	0.5	0.33	0.21		Regulates association with cellular proteins.
FRS2	Y195	0.9	0.8	0.03	0.05		Regulates association with

							cellular proteins (mouse)
FGFR3	Y760	1.4	0.9	0.01	0.01		Enzymatic activation, regulates association with cellular proteins
FGFR3	Y724	1.4	1.4	0.04	0.02	FGFR3	enzymatic activation, regulates association with cellular proteins, not reported
FGFR4	Y754	0.9	0.7	0.01	0.01	FGFR4	
FGFR1	Y154	0.7	1.4	0.34	0.72	FGFR1	Cytoskeletal reorganization , receptor internalization , protein degradation , altered receptor desensitization
FGFR1	Y653/4	5.7	6.4	0.05	0.01	FGFR1(Y653/4)	Activation(Y653/4)
FGFR1	Y463	1.0	1.0	0.01	0.01		Regulates cell cycle , regulates transcription
Met	Y1349	1.0	0.9	0.01	0.01	Met, Abl	Regulates cell growth , regulates association with cellular proteins
Met	Y1234/5	1.0	1.0	0.01	0.01	Met(Y1234/5)	Enzymatic activation (Y1234/5), phosphorylation (Y1234/5), activation (Y1234/5)
RhoA	S188	1.1	0.9	0.02	0.26		
Rb	S795	1.3	0.9	0.02	0.07	CDK6, CDK5	Regulates cell cycle, inhibition , regulates association with cellular proteins
Rb	S780	0.8	0.7	0.01	0.01	CDK6, CDK4	Regulates cell cycle , regulates apoptosis , inhibition , regulates association with cellular proteins
BCAP	y459	0.9	0.9	0.01	0.01		
BCAP	Y195	0.9	0.8	0.81	0.57		
RAC1	S71	1.0	1.0	0.01	0.01	Akt1	Inhibition
MKK4	S257/61	4.1	2.9	0.01	0.01	ASK1(S261), MLK3(S261)	Enzymatic activation(S261)
MKK4	S80	1.1	0.8	0.01	0.11	Akt	Inhibition
4E-BP1	T45	0.6	0.9	0.04	0.23	mTOR	Inhibition, regulates association with cellular proteins, regulates translation
4E-BP1	T36	2.5	1.6	0.01	0.01	mTOR	Inhibition, regulation translation
CD45	Y1216	1.0	1.0	0.67	0.69	CSK	Activation, regulates association with cellular proteins
CD45	S999/02/03	0.9	1.0	0.05	0.01	CK2-A1 (S999/02/03)	Enzymatic activation(S999/02/03)
Bad	S118	1.9	0.3	0.54	0.03	PPP2CB	Regulates apoptosis, altered intracellular location, association with cellular proteins
Etk	Y40	7.2	1.6	0.01	0.01	FAK	Enzymatic activation, cytoskeletal reorganization, altered intracellular location
CTLA-4	Y201	0.4	2.8	0.01	0.01	jak2, fyn, lck	Regulates association with cellular proteins, altered intracellular location
PXN	Y31	3.0	3.7	0.01	0.01	Brk	Regulates association with cellular proteins
CSK	Y184	1.0	1.1	0.01	0.01		
Crk	Y221	1.0	1.0	0.01	0.01	Abl	Regulates cell adhesion and interaction with other cellular

							proteins
NGFR	Y336	0.8	0.9	0.01	0.01		
FRS3	Y416	0.9	0.9	0.01	0.01		
TrkC	Y516	0.6	1.0	0.41	0.66		
TrkB	Y705/6	0.8	0.3	0.01	0.82		
VEGFR3	Y1265	0.4	0.4	0.01	0.01		
VEGFR3	Y130/1	0.7	0.9	0.98	0.19		
VEGFR2	Y1214	1.0	1.0	0.01	0.01	VEGFR2	Enzymatic activation
VEGFR1	Y1169	1.3	0.7	0.01	0.11		Regulates association with cellular proteins
PLCG1	Y783	2.8	5.7	0.01	0.01	Syk, EGFR	Regulates association with cellular proteins, regulates cell motility
PLCG1	Y771	1.0	0.9	0.01	0.01	Syk, EGFR	
PLCG1	Y472	1.5	1.3	0.17	0.05		
PKR	T451	0.6	0.2	0.24	0.03	PKR	Activation
FLT3	Y597/9	1.2	0.8	0.01	0.42	FLT3(Y599)	Regulates association with cellular proteins(Y599), activation(Y599)
FLT3	Y589/91	0.9	1.1	0.78	0.14	FLT3(Y589)	Inhibition(Y589), regulates association with cellular proteins(Y589), activation(Y591)
gp130	Y814	1.1	1.4	0.02	0.03		Regulates cell cycle , regulates transcription
gp130	S782	1.1	0.7	0.02	0.01		Receptor internalization , protein degradation
gp130	Y767	0.4	1.3	0.01	0.04		Regulates cell cycle , regulates transcription
MEK2	Y216	0.4	0.7	0.11	0.29		Inhibition
PIK3R1	S607/8	1.1	0.5	0.27	0.05	InsR (S607), PIK3CA (S608)	Activation(S608)
Kit	S821	1.2	1.4	0.06	0.01	kit	Phosphorylation , activation
Kit	Y721	0.9	0.9	0.01	0.01		Regulates cell growth , regulates apoptosis
RIPK1	Y384/7/9	0.2	1.1	0.04	0.83		
ROCK2	S1133/4/7	0.9	0.4	0.03	0.63		
IKK-α	T23	1.0	1.0	0.01	0.01	Akt2	Regulate apoptosis, enzymatic activation
mucin 1	T1227/9	0.5	0.4	0.19	0.07	GSK3B (T1227), Src(T1229)	Regulates association with cellular proteins(T1227/9)
PDGFRb	Y740	0.9	0.9	0.01	0.01	PDGFRb	Regulates association with cellular proteins , activation
PDGFRb	Y579/81	0.9	0.8	0.01	0.01	PDGFRb (Y579/81)	Enzymatic activation(Y579/81), regulates association with cellular proteins (Y579/81)
CSFR	Y809	0.7	0.7	0.01	0.01	CSFR	Regulates cell cycle, activation
CSFR	Y561	0.8	0.7	0.01	0.04	CSFR	Activation, regulates association with cellular proteins
PPP2CA	T304	0.7	0.8	0.01	0.01		Regulates cell growth , inhibition

PTP1B	S50	1.3	1.1	0.01	0.01	CLK1, Akt1	Enzymatic activation , inhibition , regulates association with cellular proteins
Nik	T559	1.0	0.9	0.01	0.01		
eNOS	S1176	0.9	1.3	0.46	0.71	AMPK1, Akt1	Enzymatic activation, activation
Grb10	S150	0.4	0.5	0.01	0.01	ERK2, ERK1	Inhibition
IFNGR1	Y479	0.9	0.6	0.85	0.06		
IFNGR1	Y457	6.3	3.4	0.01	0.01		Regulates association with cellular proteins
Jun	S73	0.9	1.1	0.04	0.01	JNK1	
Jun	S63	1.9	1.6	0.01	0.01	JNK1 and JNK2	
STMN1	S62	1.0	1.0	0.01	0.01	PKACa	Regulates cell cycle, regulates apoptosis, cytoskeletal reorganization, inhibition, regulates association with cellular proteins
STMN1	S15	1.1	0.9	0.01	0.02	PAK1, PKACa, CaMK4	Regulates cell cycle, regulates apoptosis , cytoskeletal reorganization , inhibition, regulates association with cellular proteins
CDK2	T160	0.9	0.6	0.15	0.01	CCRK	Regulates cell cycle, enzymatic activation, activation
CDK2	T14/Y15	1.0	0.9	0.01	0.01	wee1(Y15)	Inhibition (T14/Y15), regulates cell cycle(Y15)
Pyk2	Y402	1.0	0.9	0.01	0.01		Regulates cell cycle, enzymatic activation, altered intracellular location. Induce interaction with: Src
CDK7	T170	0.7	0.9	0.10	0.01	CDK2, Cdc2	Activation
cyclin D1	T286/8	1.0	0.9	0.01	0.01	IKK-α(T286)	Protein degradation(T286)
p300	S1834	0.9	1.1	0.01	0.01	Akt1	Regulates transcription , regulates association with cellular proteins
p300	S89	0.6	4.1	0.01	0.36	PKCA, AMPK1, PKCD	Regulates cell growth, inhibition, regulates transcription, regulates association with cellular proteins, activation
CD28	Y206/9	0.7	0.4	0.82	0.37		
HSP70	Y525	1.1	0.4	0.01	0.39		Altered intracellular location
HSP27	S82/83	0.5	0.9	0.39	0.34	MAPKAPK2 (S82), Akt1(S82), PKD1(S82)	Activation, altered intracellular location, and more(S82/83)
EphA2	Y772	0.9	1.8	0.28	0.45		
EphA2	Y594	8.6	7.6	0.01	0.01		
EphA2	Y588	0.9	1.3	0.50	0.39		
EphA1	Y781	1.7	1.4	0.08	0.04		
p47phox	S370	4.6	3.1	0.01	0.01	PKCA, PKACa	Enzymatic activation , altered intracellular location
p40phox	T154	1.7	0.4	0.01	0.01	PKCA	
WASP	Y291	1.2	0.3	0.45	0.05	Hck, Lyn, Abl, Lck, Ack	Enzymatic activation , regulates association with cellular protein

Syk	Y525/6	0.9	0.5	0.62	0.24	Syk(Y525/6)	Enzymatic activation(Y525/6)
Cot	S400	0.6	3.1	0.01	0.19	Akt1	regulates transcription
CaMK2α	T286	1.2	1.5	0.32	0.78	CaMK2-α	Enzymatic activation
smMLCK	S1773	0.9	0.9	0.01	0.01		
MKP-1	S359	0.3	0.7	0.01	0.01		Protein stabilization
MKP-1	S323	0.3	0.5	0.06	0.32	ERK3	Regulates association with cellular proteins , protein degradation
MKP-1	S296	0.9	1.3	0.40	0.02	ERK2	Regulates association with cellular proteins , protein degradation
PLCB1	S887	1.0	1.1	0.01	0.01	PKCA(rat)	Inhibition(rat)
PLCB1	T333/4/6	0.8	1.2	0.01	0.01		
BLNK	Y96	0.9	0.9	0.01	0.01	Syk	Regulates transcription, regulates association with cellular proteins.
BLNK	Y72	1.0	1.0	0.01	0.01	Syk	Regulates transcription, regulates association with cellular proteins.
DAPP1	Y139	1.1	4.0	0.01	0.01		Altered intracellular location, regulates association with cellular proteins, activation.
RelB	S573	1.0	1.5	0.01	0.01		
APE1	S289	1.2	0.8	0.01	0.24		
Tyk2	Y1054/5	1.2	0.5	0.10	0.07	Tyk2 (Y1054/5)	Enzymatic activation(Y1054/5)
p70S6Kb	S473	1.1	0.9	0.02	0.11	PKCB, PKCA, PKCI, PKCG, PKCE, PKCZ, PKCD	altered intracellular location
CXCR4	Y157	6.1	2.3	0.01	0.01		Regulates transcription, phosphorylation
CCR5	S336/7	1.7	9.4	0.01	0.01	GRK3(S336/7)	receptor internalization(S336/7)
CCR2	Y139	1.4	1.9	0.01	0.01	jak2	Regulates association with cellular proteins.
IL7R	Y449	1.0	0.8	0.63	0.26		Regulates association with cellular proteins
IL1A	S87	1.2	0.9	0.01	0.01		
IL-16	S143/4	1.2	0.4	0.66	0.06	ERK2(S144), ERK1(S144)	
IL4R	Y713	0.7	0.8	0.24	0.03		Regulates cell growth , regulates association with cellular proteins
IL2RB	Y536	0.9	1.0	0.28	0.02	Lck	
IL2RB	Y364	0.8	0.9	0.01	0.01		Regulates association with cellular proteins
IL-10R-A	Y496	0.2	0.7	0.01	0.01		
IKK-β	Y199	0.7	0.7	0.27	0.44	Src	Regulates transcription and activation
IKK-β	Y188	0.4	1.1	0.56	0.01	Src	Regulates transcription and activation
HMGA1	T52	0.8	0.9	0.01	0.01		Regulates apoptosis
Lef1	S166	1.3	1.0	0.01	0.01		

Lef1	T155	1.0	1.2	0.01	0.01		
DOCK2	Y209/212	1.0	1.0	0.01	0.01		
CBP	S2063	1.0	1.0	0.01	0.01		
DNA-PK	T2638	1.0	0.7	0.07	0.11	ATM , DNA-pk	Enzymatic inhibition
DNA-PK	T2609	1.3	1.1	0.01	0.01	ATM , DNA-pk	enzymatic inhibition
PKR	T446	1.4	0.7	0.01	0.71	PKR	Regulates cell growth, regulates cell cycle, enzymatic activation, activation
Btk	Y550	2.7	1.1	0.54	0.10	syk, lyn, btk	Activation
Btk	Y222	1.0	1.8	0.01	0.01	syk, lyn, btk	Activation
ITK	Y512	0.8	0.4	0.01	0.01	Lck	Activation
FAK	Y397	2.9	2.5	0.14	0.01	FAK	Regulates apoptosis, cytoskeletal reorganization, altered intracellular location, regulates cell adhesion, regulates association with cellular proteins , protein degradation, activation
TAK1	S192	15.0	22.9	0.15	0.09	TAK1	Activation
TAK1	T184	1.3	1.0	0.01	0.01	TAB1	Activation
PKACa	S338	3.9	2.5	0.02	0.02		
PKACa	T195/7	7.7	1.9	0.02	0.01		
Akt3	T447	1.0	1.0	0.01	0.01		
Akt3	S120	1.4	1.2	0.82	0.74		
Akt1	Y326	0.5	1.3	0.01	0.06	Src	Regulates apoptosis, transcription. Activation
Akt1	S129	1.1	0.5	0.60	0.25	CK2-A1	Enzymatic activation
ERK3	S189	1.3	2.1	0.05	0.21	ERK3	
ERK2	Y204	1.2	1.0	0.01	0.01	MEK1	
ERK1	T202/4	1.0	1.1	0.01	0.01	Cot(T202/4), MEK1/2 (T202/4), Lck(T204), ERK1(T204)	Activation (T202/4), enzymatic activation (T202/4)
TBK1	S172	1.0	1.1	0.01	0.01		Enzymatic activation
Jak3	Y981	1.0	3.5	0.01	0.01	Jak3	Enzymatic inhibition
Jak2	Y1007/8	1.5	2.0	0.78	0.04	Jak2	Activation(Y1007/8)
Jak1	Y1022/3	0.3	0.6	0.01	0.01	Jak3 (Y1022/3)	
JNK1	S377	1.1	1.2	0.01	0.01		
p38-α	Y323	1.2	1.0	0.01	0.01	Fyn, Lck, ZAP70	Enzymatic activation, phosphorylation
p38-α	T179/Y181	1.3	1.4	0.88	0.76	MKK3/6(T179/Y181)	Activation, Enzymatic activation, and more (T179/Y181)
axin-1	S486	3.9	3.8	0.02	0.01	GSK3B (mouse)	Activation(mouse)
Notch 2	S2070	1.3	1.6	0.01	0.01		
Notch 2	T1808	0.8	1.0	0.02	0.01		
GSK3-β	Y216	1.1	1.0	0.01	0.01	MEK1, GSK3B	
GSK3-β	S9	0.8	0.6	0.01	0.14	PKACa	Regulates apoptosis, inhibition,

							regulates cell adhesion, enzymatic inhibition
Elk-1	T417	0.9	0.9	0.01	0.01	PRP4	transcription
Elk-1	S389	1.2	1.2	0.01	0.01	ERK1	Activation, regulates transcription
Fos	T232	0.2	0.2	0.05	0.01	ERK5, ERK2	Altered intracellular location , activation
Casp8	S347	3.4	2.8	0.01	0.01	p38-α	Regulates apoptosis, inhibition
Casp3	S150	1.2	1.0	0.01	0.01	p38-α	Regulates apoptosis, inhibition
IRAK-1	S568	0.2	0.2	0.01	0.01		
IRAK-1	T387	1.0	1.1	0.01	0.01		
IRAK-1	S376	1.1	7.3	0.01	0.22	IRAK-4	Activation and results after phosphorylation of T209
IRAK-1	T209	3.2	3.7	0.01	0.01	IRAK-1	Required for S376 phosphorylation
IRAK-1	T100	1.3	1.2	0.01	0.01	Akt1	regulates transcription
FADD	S194	10.6	5.9	0.08	0.01	CK1-A, MEKK1, MKK7	Altered intracellular location, regulates apoptosis, regulates cell cycle
HIF2A	T840	1.0	1.0	0.01	0.01		Regulates transcription, activation(mouse)
HIF1A	T796	3.0	9.2	0.01	0.01		
XIAP	S87	0.9	0.8	0.03	0.18	Akt2, Akt1	Regulates apoptosis , protein stabilization
BATF	T48	1.0	2.0	0.01	0.01		
NFAT4	S163/5	0.2	0.5	0.01	0.01	JNK2(S163/5)	Altered intracellular location(S163/5), regulates transcription(S163/5)
NFAT2	S294	2.4	1.9	0.01	0.01	PKACa	Inhibition , altered intracellular location
NFAT2	S245	0.9	1.1	0.01	0.01	PKACa	Inhibition , altered intracellular location
NFAT1	S326	0.9	0.8	0.01	0.01		
NFAT1	S168	0.9	0.8	0.01	0.01		
Smad4	T277	1.6	1.0	0.01	0.01		
Smad3	S422/3/5	11.3	8.2	0.01	0.01		Regulates transcription(S422/3/5)
Smad3	S208	2.8	4.2	0.22	0.38	ERK2	Inhibition , altered intracellular location , regulates transcription
Smad2	S255	1.2	1.1	0.88	0.75	ERK2, ERK1	Regulates transcription, protein stabilization, regulates association with cellular proteins, activation. Induce interaction with: Smad4
Smad2	T8	2.9	1.4	0.01	0.01	ERK1	Regulates transcription , protein stabilization , regulates association with cellular proteins , activation .Induce interaction with:Smad4
Smad1	S462/3/5	7.5	1.0	0.01	0.01	BMPR1B (S462/3/5)	Activation, altered intracellular location, and more(S462/3/5)
ATF-4	S245	1.0	0.8	0.01	0.01	RSK2	activation, regulates cell growth
ATF-2	T51/3/5	0.7	1.0	0.29	0.01	VRK1(T55), p38-α(T51/3), JNK1(T51/3)	Regulates transcription(T55), protein stabilization (T51/3/5), activation(T55)