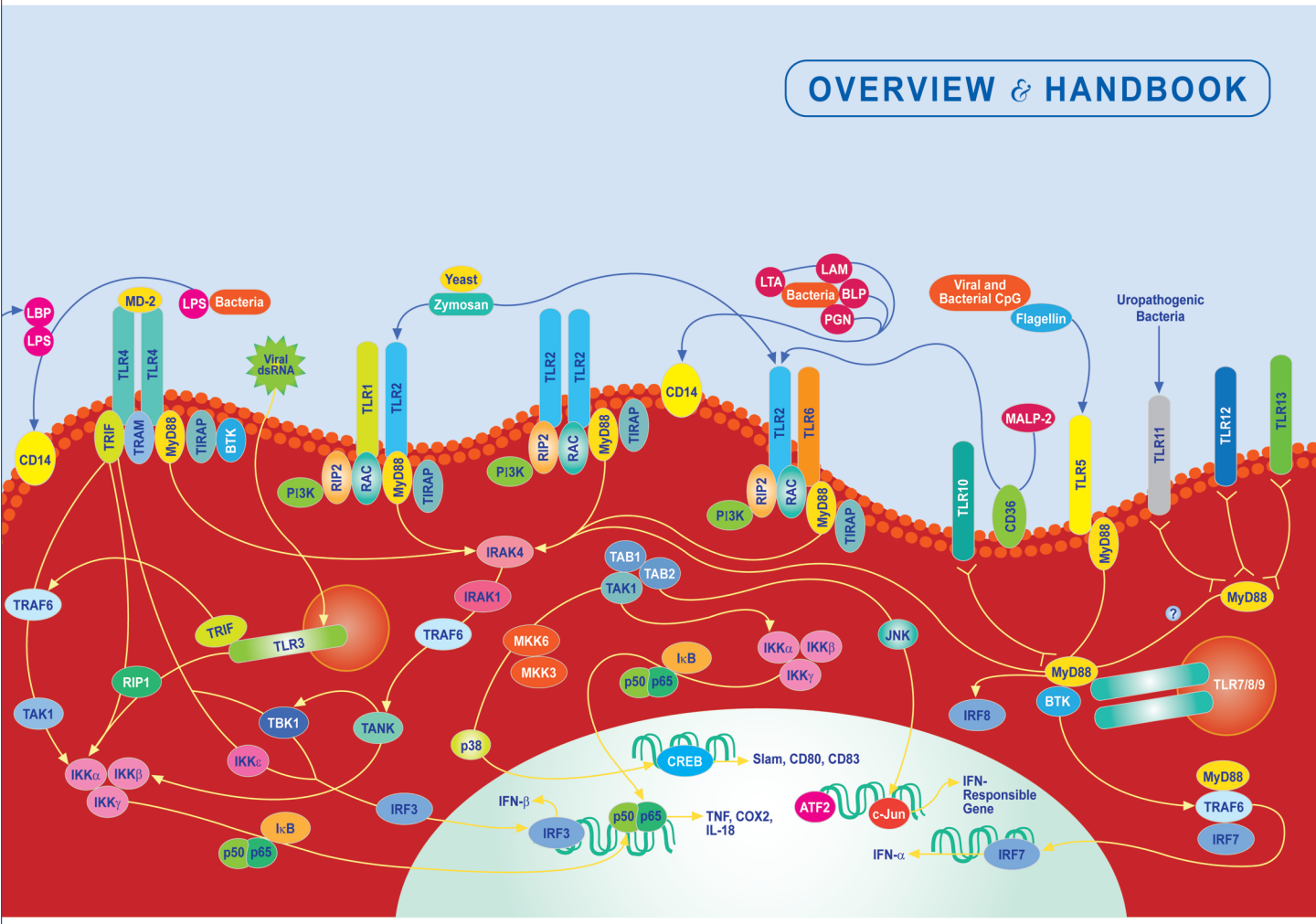
TOLL-LIKE RECEPTORS

Novus Biologicals & Innate Immunity: **The Story Toll'd**

OVERVIEW & HANDBOOK



NOVUS BIOLOGICALS

HANDBOOK & OVERVIEW

Toll-like Receptors

Novus Biologicals & Innate Immunity: **The Story Toll'd**

UPDATED EDITION • 2014

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We dedicate this book to TLR researchers worldwide, including our customers, collaborators, colleagues and heretofore unmet scientists. We stand in awe of the researchers who came before us and set the groundwork for the present day knowledge of TLRs and immunity overall. We thank present day researchers for their ongoing contributions to the expanding knowledge of the TLR field; their presence and publications are invaluable to moving TLR understanding forward.

We hope that our book will be an inspiration and useful guide for established and emerging TLR researchers alike. Likewise, we hope that researchers in related and distant fields will gain insight into how TLRs might be linked to their area of research.

We wish for students to use our book as a foundation for gaining additional information about how they, too, might become involved in the exciting and promising arena of TLR research.

In closing, we hope that all of our readers will provide us with feedback, new publications, and suggestions for our next edition of the TLR Handbook.

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Abbreviations

a.k.a.	also known as
aa	amino acid(s)
Ab	antibody
AD	Alzheimer's disease
APC	antigen presenting cells
Aβ	Amyloid β peptides
B-CLL	B-cell chronic lymphocytic leukemia
BCG	Mycobacterium bovis bacillus Calmette-Mycobacterium bovis bacill Guérin
CAGR	compound annual growth
CLL	chronic lymphocytic leukemia
CTL	cytotoxic T lymphocyte
DAMP	damage associated molecular pattern
DC	dendritic cell
dsDNA	double stranded DNA
ECD	extracellular domain, a.k.a ectodomain
ER	endoplasmic reticulum
FADD	Fas/Apo-1 associated death domain protein
hBD	human β-defensin
HDP	host-defense peptides
HMGB1	high-mobility-group box 1
H. pylori	Helicobacter pylori
HPA	hypothalamic-pituitary-adrenal
ICAM1	intercellular adhesion molecule 1
iDC	immature dendritic cell
IFN	interferon
IL-1R	interleukin-1 receptor
ISS-ODN	immunostimulatory sequence oligodeoxynucleotide
IP	intraperitoneal injection
IRAK	IL-1R associated kinase
IRF	interferon regulatory factor
LPS	lipopolysaccaride
LRR	leucine rich repeats
mAb	monoclonal antibody
Mature DC	highly efficient professional APC
MSC	mesenchymal stem/progenitor cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex

Abbreviations

mDC	myeloid DC
MyD88	myeloid differentiation factor 88
NLR	Nod-like receptor
NO	Nitric Oxide
pAb	polyclonal antibody
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PMN	polymorphonuclear neutrophils
pDC	plasmacytoid DC
PRR	pattern recognition receptor
RLR	RIG-1 like receptor
S. aureus	Staphylococcus aureus
SD	standard deviation
SEAP	secreted alkaline phosphatase
SLE	systemic lupus erythematosus
SNP	Single nuclear polymorphism
TAB1/TAB2	Tak binding proteins
TAK1	TGF-β activated kinase
TIR	Toll/IL-1 receptor
TLR	Toll like receptor
Tc2	T cytotoxic 2 cell, a.k.a. CD8+ T cytotoxic 2 cell
Teff	effector T cell
Th	T helper cell, a.k.a CD4+ T helper cell
Th1	T helper type 1 cell
Th2	T helper type 2 cell
Th3	Type 3 regulatory T cell, i.e. , Type 3 Treg, a.k.a T helper type 3 cell
Th17	T helper type 17 cell
Treg	T regulatory cell, a.k.a. suppressor T cell
TID	Type I diabetes
TIRAP	TIR domain-containing adaptor protein, a.k.a. MAL (MyD88 adaptor like)
Tr1	Type 1 regulatory T cell, i..e., Type 1 Treg
TRAM	a.k.a. TICAM2, TIRP
TRIF	a.k.a. TICAM1
WB	western blot
WT	wildtype

Note: some of these abbreviations are defined in the text upon their first use. Other more common ones are defined only in this list.

Contents

TLRSystem™

A system of CD antibodies, kits & reagents to study Toll-like Receptors (TLRs), Dendritic Cells (DCs) and T Cell pathways shaping Innate and Adaptive Immune Responses

Fluorochrome Conjugates for TLR Phenotyping of DCs & T Cell Subsets by Flow Cytometry
Additional Applications: Immunohistochemistry • Western Blotting • ELISA

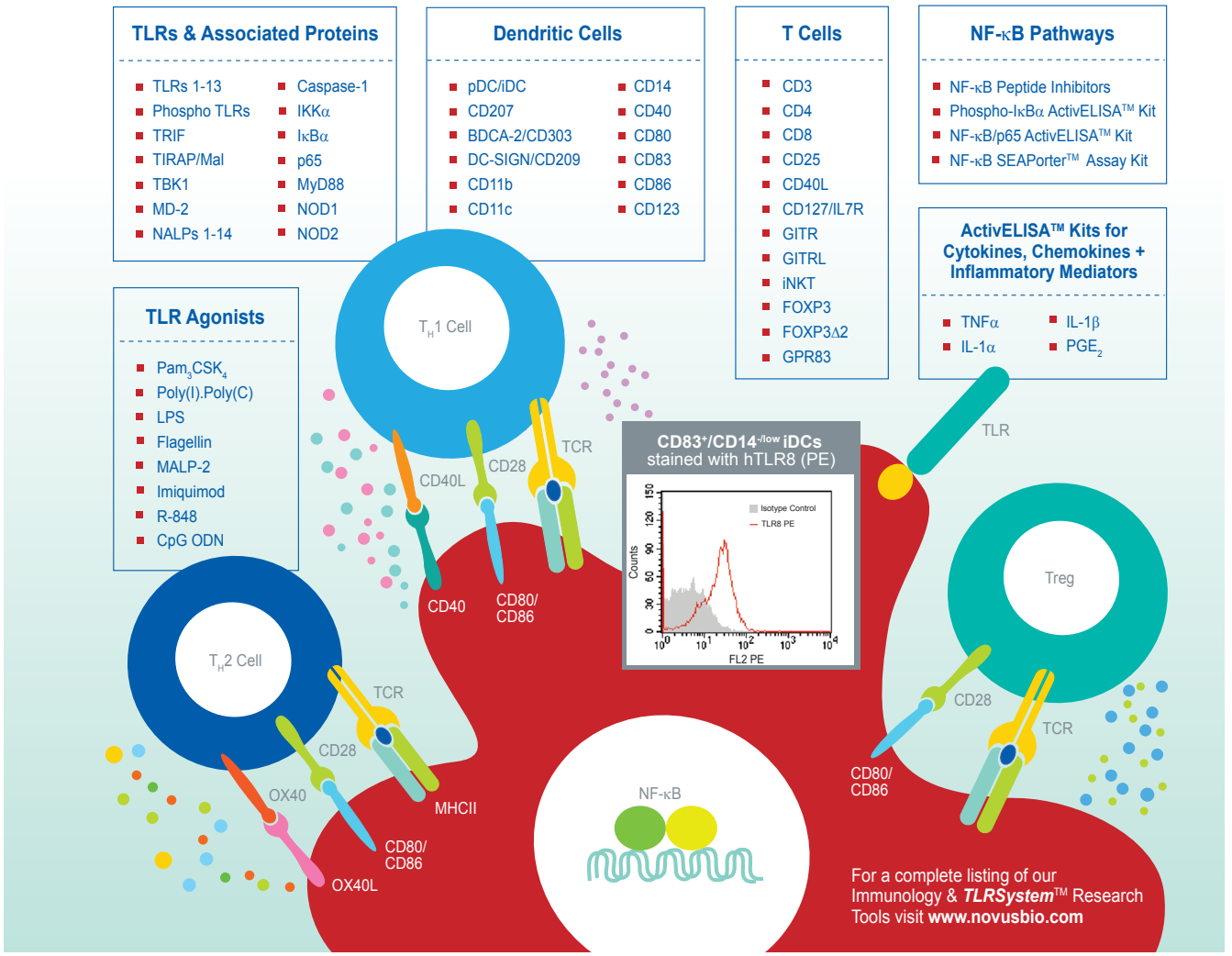


Figure 1. TLRSystem™ portfolio of antibodies and related reagents for studying TLRs and signaling pathways in innate and adaptive immunity. Novus offers researchers a comprehensive TLR product line and a wide range of supporting products through its TLRSystem™ portfolio.

Preface - Update

The Handbook grew out of researcher's requests for scientific, protocol, and technical information for the rapidly evolving TLR field. We have responded by developing this Handbook as a comprehensive resource that will be useful for researchers, students and educators as well as the lay person or business professional who is interested in learning more about TLR signaling.

Novus is a biotechnology company which develops and commercializes leading edge research reagents for the worldwide Life Sciences community. Novus' TLR portfolio of reagents includes antibodies, assays kits, peptide inhibitors, protein expression systems and tissue microarrays. Our major areas of focus are immunology, stem cell biology, cell signaling, cell death, phosphorylation, cancer, and infectious diseases.

Novus' TLRSystem™ is the most comprehensive portfolio of TLR, innate immunity, inflammation, and related immunology reagents in the world. Many of the TLRSystem™ products are highly cited in scientific publications, enabling researchers to easily tap information about how these products are used by the scientific community. Representative TLRSystem™ products are shown in Figure 1. For a complete listing and additional information about TLRSystem™, Screening Services, and the entire Novus product portfolio, please visit our website at www.novusbio.com.

This updated edition, reflects the rapidly developing TLR field and the emergence of Novus as the leading innovator and source of TLR products and technology.

Overview of TLR Related Scientific & Market Sectors

The Toll-like receptors (TLRs) belong to a family of innate immune receptors which also includes Nod-like receptors (NLRs) and RIG-I like receptors (RLRs) (reviewed in Takeuchi and Akira, 2009; and Kawai and Akira, 2009). TLRs have taken center stage as scientists have recognized that these receptors have far-reaching effects beyond their well-known role of pathogen recognition and activation of the innate immune response.

Specifically, a large body of evidence has accumulated indicating that TLRs have key roles in the development, direction, and modulation of immune responses overall (reviewed in Medzhitov, 2009 and Sabroe et al, 2008). The extensive interplay between TLRs and immune/inflammatory signaling networks modulates not only immune responses, but is also tied to other fundamental biological processes such as homeostasis and cell survival/cell death.

TLRs are expressed in a variety of cell types, many within the immune system where they have been linked to different cellular activation states, immune defense, maintenance of homeostasis, and various diseases (reviewed in Mogensen, 2009 and Sabroe et al, 2008). As sensors and shapers of immune responses, TLRs and related immunological pathways are being aggressively studied across research, diagnostic and therapeutic market sectors. In the therapeutics sector, the applications spectrum spans potential therapeutics and vaccines based on the numerous TLR/immune signaling networks and heretofore uncharted intervention points for drug and vaccine development. Hence the therapeutic market alone represents significant grant opportunity and revenue for TLR related products (Makkouk and Abdelnoor, 2009).

Table I represents some of the significant commercial opportunity or overall areas for TLR related products in each of the therapeutic, diagnostic and research market sectors. From Table I, the term 'addressable market' is defined as the total potential market measured in billions of dollars of revenue per year. Hence the addressable research market including the major areas in the Table (bead arrays, ELISA, protein arrays, cell signaling, flow

Table I. Key addressable commercial markets for TLRs

Market Sector	Therapeutic	Diagnostic	Research
Addressable Market Estimate	$120 Billion	$45 Billion	$1.5 Billion
Key Market Areas by Sector	Vaccines	Immunodiagnostics	Bead Arrays
	Allergy	Microbiology	ELISA
	Atherosclerosis	Molecular Diagnostics	Protein Arrays
	Autoimmunity	Hematology	Cell Signaling
	Sepsis	Coagulation	Flow Cytometry

Source: Data compiled by Redmont Marketing Associates (2009).

cytometry) is estimated to be $1.5 billion. The direct potential TLR and innate immunity reagents market is estimated to be $315 miliion or ~21% of the overall $1.5 billion research market (Fig 2).

Surveys, reports and journals were utilized as sources of information to help generate the information in Table I and Figure 2, including Biocompare (2007 and 2009), Brown et al (2007), Business Insights (2006), Frost & Sullivan (2005), and Terradaily (2007). Estimations of the overall direct potential market for the research areas are also based on surveys and polls at immunology venues where approximately 15-20% of individuals indicate interest or use of TLRs or related reagent tools.

As indicated in Table 1 and Figure 2, the therapeutic, diagnostic and research sectors are large and growing markets, measuring in the multi-billion dollar range with the therapeutic area being the largest. Therapeutics is estimated to be $120 billion for tools and reagents that are used in the pre-clinical pre-human drug discovery areas. Tools and reagents represent ~27% of total R&D expenditures in the therapeutic market sector according to PhRMA's 2009 Annual Report (PhRMA, 2009). The direct potential market for TLRs and innate immunity tools and reagents is estimated to be ~$35 billion, which is ~29% (35/120) of the total tools and reagents expenditures in the therapeutic sector.

Direct Potential Research Market $315M

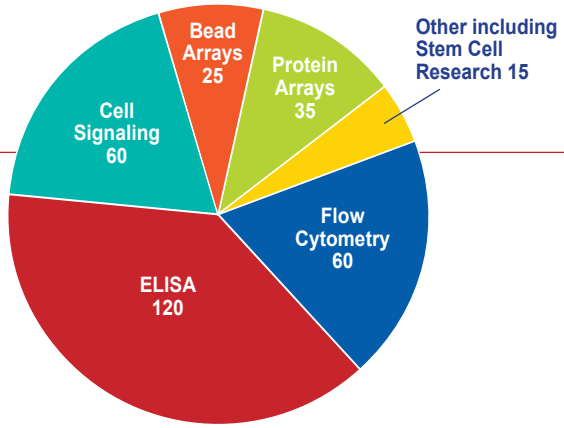


Figure 2. Direct potential research market of TLR & innate immunity reagents. *Areas of biology and platforms representing major uses of TLR and related reagents in the market are calculated as percentages of their respective overall addressable research market sizes shown in Table I.*

TLR signaling is a complex process and defining the nuances is key to the success and growth of all three market sectors. Li et al (2009) developed a conceptual model system which provides a useful framework for elucidating nodes in the TLR signaling network (Fig 3). For example, probes and assays from the research sector are essential for defining the molecules and underlying processes comprising the signaling nodes. Likewise, the knowledge gained from researchers is key for developing biomarker screening systems in the diagnostic sector as well as for identifying targets for intervention in the therapeutic sector.

Overview of TLR Related Scientific & Market Sectors

Key TLR Signaling Pathways

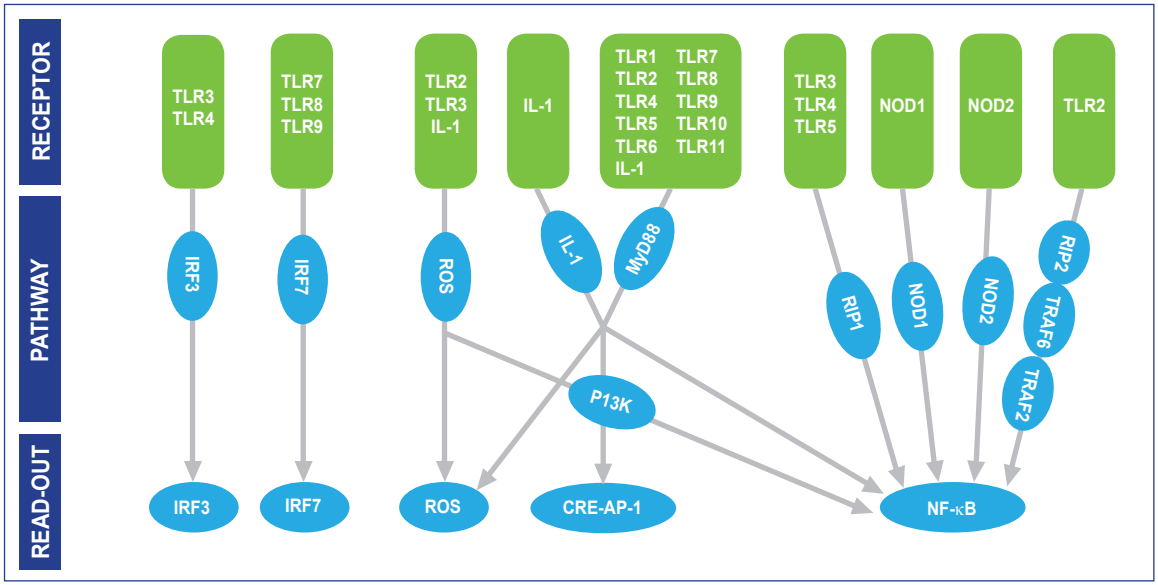


Figure 3. Key TLR signaling pathways. *The TLR signaling network can be divided into 14 input receptors (TLRs 1-11, IL-1, NODs 1 and 2*) which collectively signal through ten pathways (IRF3, IRF7, ROS, PI3K, IL-1 and MyD88, RIP1, NODs 1 and 2 and RIP2) to five output transcription objectives (IRF3, IRF7, ROS, CRE/AP-1 and NF-κB). NF-κB is the most redundant transcriptional objective where it is a target for the majority of the pathways. Four of the pathways (RIP1, NOD1, NOD2, and RIP2/TRIP6/TRAF2) are thought to signal only to NF-κB. Adapted from Li et al (2009).*NODs belong to the Nod-like receptor (NRL) family of innate immune receptors. Whereas TLRs detect ligands exposed either in the extracellular milieu or in the lumen of endocytic vesicles, NLRs detect analogous ligands in the cytosol (reviewed in Benko et al, 2008).*

The mounting interest in TLRs is underscored by the rise in the number of publications in PubMed [Fig 4 (Keywords: Toll-like receptors or TLR)] over the past 15 years. For example, there were 13 citations in 1996, the year when *Drosophilia* Toll was identified as key to fly immunity. There were just 14 citations the next year, 1997, when the first human Toll homologue (TLR) was discovered. However, the publication rate jumped every year thereafter and there were over 2700 citations in 2008. Growth is very robust, with a three year publication (2006-2009) compound annual growth rate (CAGR) of over 20%.

In summary, the importance of studying Toll-like receptors is clearly indicated and interest is expected to continue to grow in each of the three market sectors: research, diagnostics and therapeutics.

TLR publications

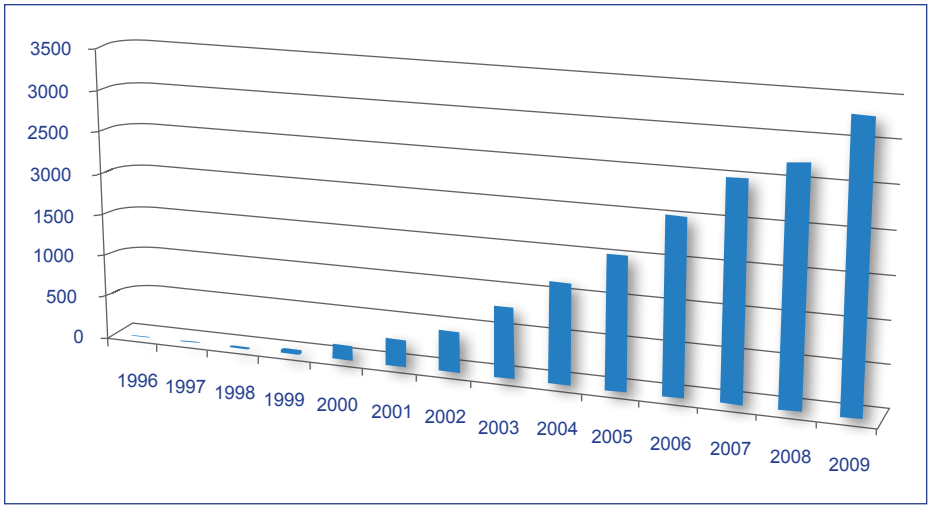


Figure 4. TLR publications. This figure was generated using Keywords: *Toll-like receptors or TLR as key words in a PubMed search (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed).*

Concepts of Host Immunity Against Pathogens

Organisms are continuously exposed to infectious agents, yet in most cases are able to avoid infections and survive. How is this possible? It is possible because organisms descriminate between between self and nonself, enabling them to detect and protect themselves against invading pathogens, which are recognized as nonself. Self/nonself descrimination is also essential for eliminating tumor and other abnormal cells.

Self/nonself descrimination is an integral function of the immune system (reviewed in Beutler, 2009). Two main subsystems developed during evolution that act in cooperation to protect against infection in vertebrates (Table II and Fig 5). The most ancient is the “non-specific” innate system found in all multicellular organisms. The evolutionarily newer “antigen-specific” adaptive system is present in all vertebrates except jawless fish. This feature enables the vertebrate immune system to recognize and remember specific pathogens, i.e., vertebrates aquire immunity through pathogen exposure. Acquired immunity, in turn, enables the organism to generate a more rapid and finely tuned immune response each time the pathogen is encountered. The adaptive system is activated by the evolutionarily older innate immune system. Whereas innate immunity is the sole mode of pathogen defense in invertebrates, it is the first line of defense in vertebrates.

Table II. Classical Comparison of Innate and Adaptive Immunity

	Innate	Adaptive
Response	Immediate (hours): critical to adaptive immune response	Delayed (days): clones of responding cells need 3-5 days to develop
Cells	Dendritic cells, granulocytes, macrophages, monocytes, neutrophils, natural killer cells	B and T cells
Receptors	Germ-line encoded: pattern recognition receptors (PRRs) including TLRs, NLRs, and RLRs	Randomly somatically generated: B cell (BCR) and T cell (TCR) antigen receptors Gene rearrangement necessary for receptor generation
Receptor specificity	Broad: recognizes many conserved pathogen associated molecular patterns (PAMPs)	Narrow: each recognizes a unique epitope
Receptor ligands	PAMPs PAMPs are essential pathogen entities that are highly conserved PAMPs are not found in hosts	Unique epitopes Epitopes reflect pathogen individuality Collectively epitopes recognize a wide number (~10^{18}) and variety (proteins, peptides) of molecular structures
Memory	No memory	Memory of prior exposure
Evolution	Conserved: plants-animals	Vertebrates: jawed fish –human

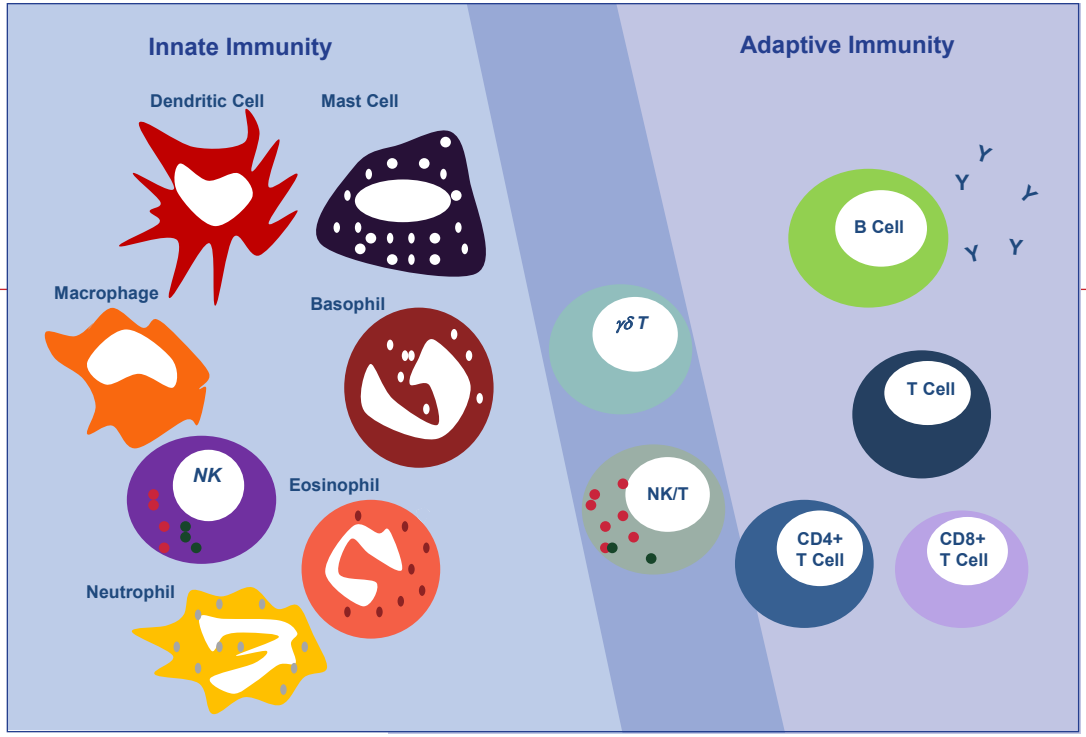


Figure 5. Cells of the innate and adaptive immune systems.

The innate immune system expresses a series of conserved germ line encoded transmembrane receptors called pattern recognition receptors (PRRs). PRRs identify potential pathogens by recognizing microbial motifs as ligands (reviewed in Kawai and Akira, 2009 and Mogensen, 2009). The PRR ligand motifs are commonly referred to as pathogen associated molecular patterns (PAMPs). The original definition states that PAMPs have the following characteristics:

I. Expressed by microbes but not host cells
II. Expression is essential for microbe survival (integrity, function, or replication)
III. Structure is conserved among microbes of a given class
IV. Ability to stimulate innate immunity

The classical PAMP-PRR dogma states that

I. PAMPs are recognized by the PRRs as "molecular signatures" of infection. This is because PAMPs are expressed only by microbes and not by host self.
II. PAMP-PRR binding is an integral mechanism used by the innate immune system to distinguish microbial (PAMP) nonself from self.
III. PAMP-PRR binding activates PRR signaling pathways resulting in antimicrobial responses, expression of genes involved in inflammation, and pathogen eliminaton.
IV. Microbial pathogens should theoretically not be able to escape the strict surveillance of the innate immune system. This is because their PAMPs bind to PRRs, and pathogens are eliminated through downstream PRR signaling responses.

Concepts of Host Immunity Against Pathogens

It should be noted that the term 'PAMP' is widely used and has been accepted into the complex language of immunology, a.k.a immunology lingo! However, it is important to note that PAMPs are not unique to pathogens because they are also expressed by microbes that do not cause disease. Hence, there has been some controversy about the use of the term 'PAMP' for referring to PRR ligands. Some researchers use the more recently coined more general term 'MAMP' (microbial associated molecular patterns) to refer to TLR and other PRR ligands in lieu of, or along with PAMP for this reason [see Palazzo et al (2008) as an example]. We will continue to use the term PAMP as it is more commonly used than MAMP. However over time the terminology may shift towards MAMP.

In addition to PAMPs, the current PAMP-PRR dogma also recognizes the existence of danger or damage associated molecular patterns (DAMPs) as PRR ligands. DAMPs arise from molecules of non-microbial origin that already exist in the host and are released from cells during tissue injury or necrosis. DAMPs are also referred to as "hidden-self" and are thought to activate TLRs in an analogous manner to PAMPs. However, for various reasons, much less is known about DAMPs than PAMPs (reviewed in Cambi and Figdor, 2009). The overall concept of the current DAMP/PAMP-PRR dogma is shown in Fig 6. Both PAMPs and DAMPs will be referred to in more depth throughout this handbook.

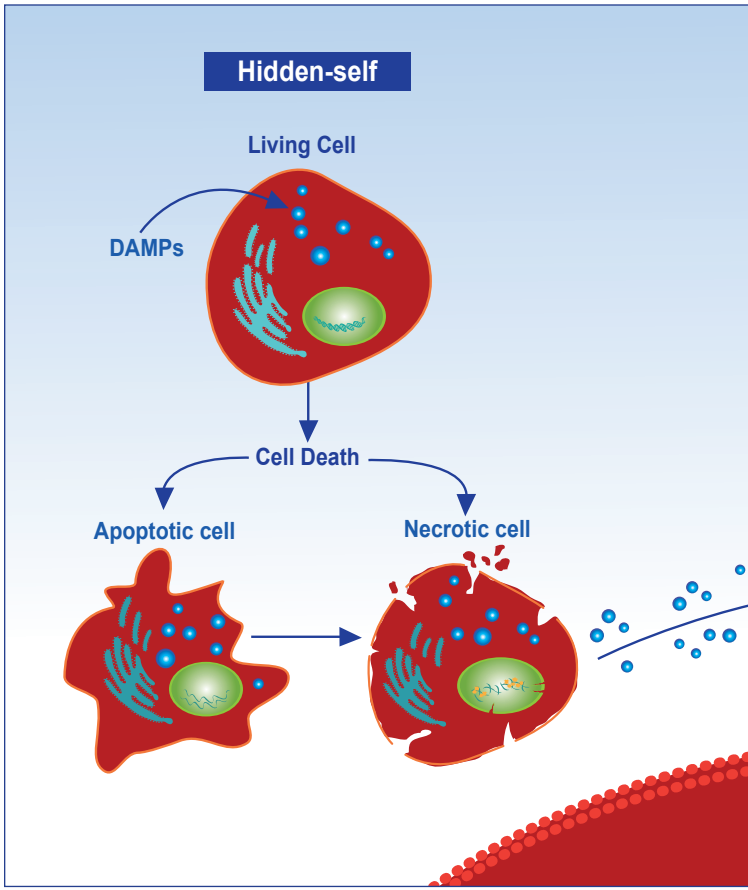
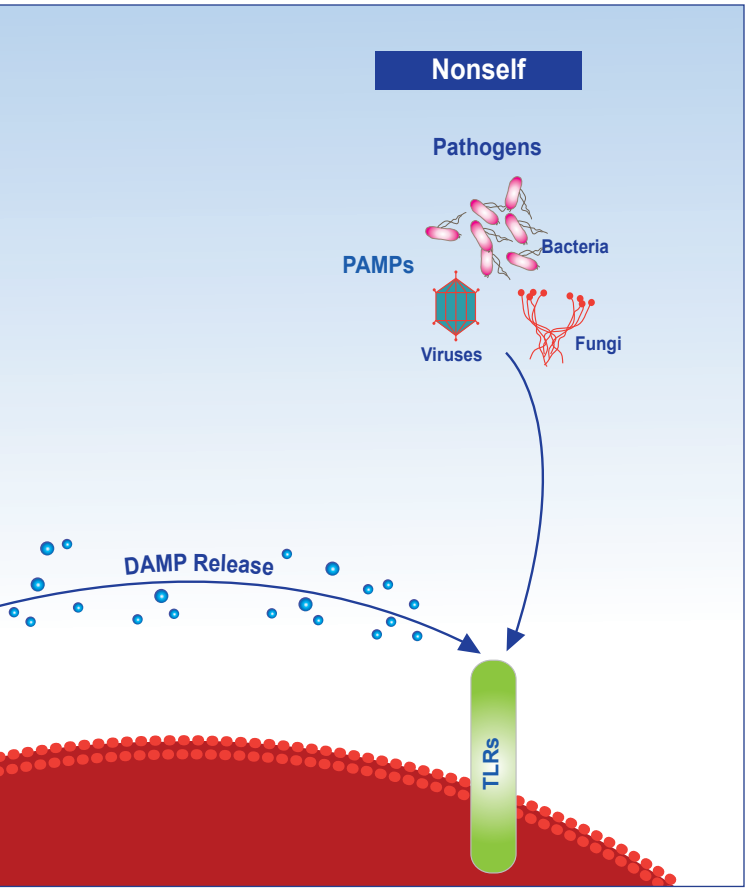


Figure 6. Hidden–self (DAMPs) and microbials nonself (PAMPs) in PRR Activation. *Initially, TLR ligands were thought to be exclusively of pathogen or non-self origin and were coined PAMPs for pathogen associated molecular patterns. Hence TLRs provided a built in mechanism for the host innate immune system to distinguish non-self (PAMPs) from self, eg they could bind to PAMPs but not self. Later it was discovered that TLRs could recognize self, but under normal conditions this recognizable self was hidden. Hidden self emerges during tissue injury or cell death and components thought to be ligands have been coined DAMPs for damage associated molecular patterns (reviewed in Bianchi, 2007). Source: Cambi and Figdor, 2009.*



Several distinct classes of PRRs developed during evolution, recognizing different PAMP classes and inducing various host defense signaling pathways. Toll-like receptors (TLRs) are ancient, phylogenetically conserved PRRs that play a crucial role in defending the host against pathogenic microbes through the induction of inflammatory cytokines and type I interferons (reviewed in Kumar et al, 2009). Whereas it is well established that TLRs are essential for innate immunity, it is increasingly being recognized that TLRs are also important for developing B and T cell mediated, pathogen-specific adaptive immunity. However, the role of TLRs in adaptive immunity is much more elusive and less well defined than their essential function in innate immunity.

It is without question though, that TLRs are the most well known and studied PRRs (Mogensen, 2009). Interest in TLRs is expected to reach new heights as scientists from diverse scientific disciplines seek to understand their roles in both health and disease, and to leverage their TLR signaling pathways for basic diagnostics and therapeutics.

The remaining chapters of this handbook provide a comprehensive overview of TLRs and the research tools available for studying them. Examples of how Novus' TLRSystem™ (Tools, Ligands, Antibodies, and other Reagents) products are used for advancing scientific understanding of innate and adaptive immune mechanisms are shown throughout the handbook. Detailed protocols are also provided in the Appendix.

Milestones in the TLR Field

The TLR story as it is "Toll'd" could be said to begin in 1985 when proteins inducing dorsal-ventral polarity in fruit fly (*Drosophila melanogaster*) embryos were discovered by Christiane Nusslein-Volhard, et al., and termed "Toll." She coined the term Toll from the German for 'weird' because flies lacking Toll develop in a weird way. Christiane Nusslein-Volhard and her collaborators later won a Nobel Prize for discovering the "Toll" signaling pathway and other components of Drosophila embryogenesis.

However, the discovery of "Toll" turned out to have far reaching implications beyond *Drosophila* development, becoming a catalyst that sparked a new age in immunology (reviewed in Beutler, 2009; Lemaitre, 2004; and O'Neil, 2004). By the early 1990's multiple labs had identified similarity between Toll and the mammalian interleukin-1 receptor (IL-1R). Their cytoplasmic domains [now termed Toll-IL-1R (TIR) domains] and signaling pathways were found to be highly conserved and homologous. This was the merging point of the Toll field with more conventional innate immunity research. Indeed, in 1996 Toll was found to be integral to *Drosophilia* immunity against fungal infections. This pivotal discovery tied Toll to immunity and launched a furious quest for homologous mammalian proteins.

Within one year, the first human homologue of the *Drosophila* Toll was described and termed hToll. hToll, now known as TLR4, was shown to activate immune signaling pathways. Four additional Toll homologues were reported in 1998, and hTolls became known as human Toll-like receptors [hTLRs (the "h" was later dropped from the name)]. Over the next decade tremendous progress was made as TLRs were characterized in many vertebrate species where they were found to detect and defend against a variety of bacterial, viral, fungal, and protozoal ligands. Even plants are now known to express Toll-like molecules, called R proteins that are also involved in disease resistance (reviewed in Rafiqi et al, 2009).

Figure 7 shows a historical time line, and the overall history of the TLR field can be said to trace back to Mechnikov's first description of phagoyctes in the late 1800's. Mechnikov was one of immunology's founding fathers and won the Nobel prize in 1908 for his discovery of cellular immunity. In his Nobel Prize lecture, Mechnikov mentions microbial endotoxins and nucleic acids as activators of phagocytes. Of course we now know that microbe PAMPs bind to TLRs and activate immune responses. However, Mechnikov would have had to wait 90 years for the discovery of TLRs to incorporate them into his lecture!

The discovery of TLRs revolutionized our understanding of innate immunity and its relationship to adaptive immunity. A renaissance of interest in innate immunity was sparked, and TLRs have been pegged as a prime contender for the most important discovery in immunology over the past three decades (O'Neil, 2004). The current dogma indicates that TLRs, as well as Toll and plant homologs, evolved as sensing molecules (PRRs) to provide defense against pathogens by

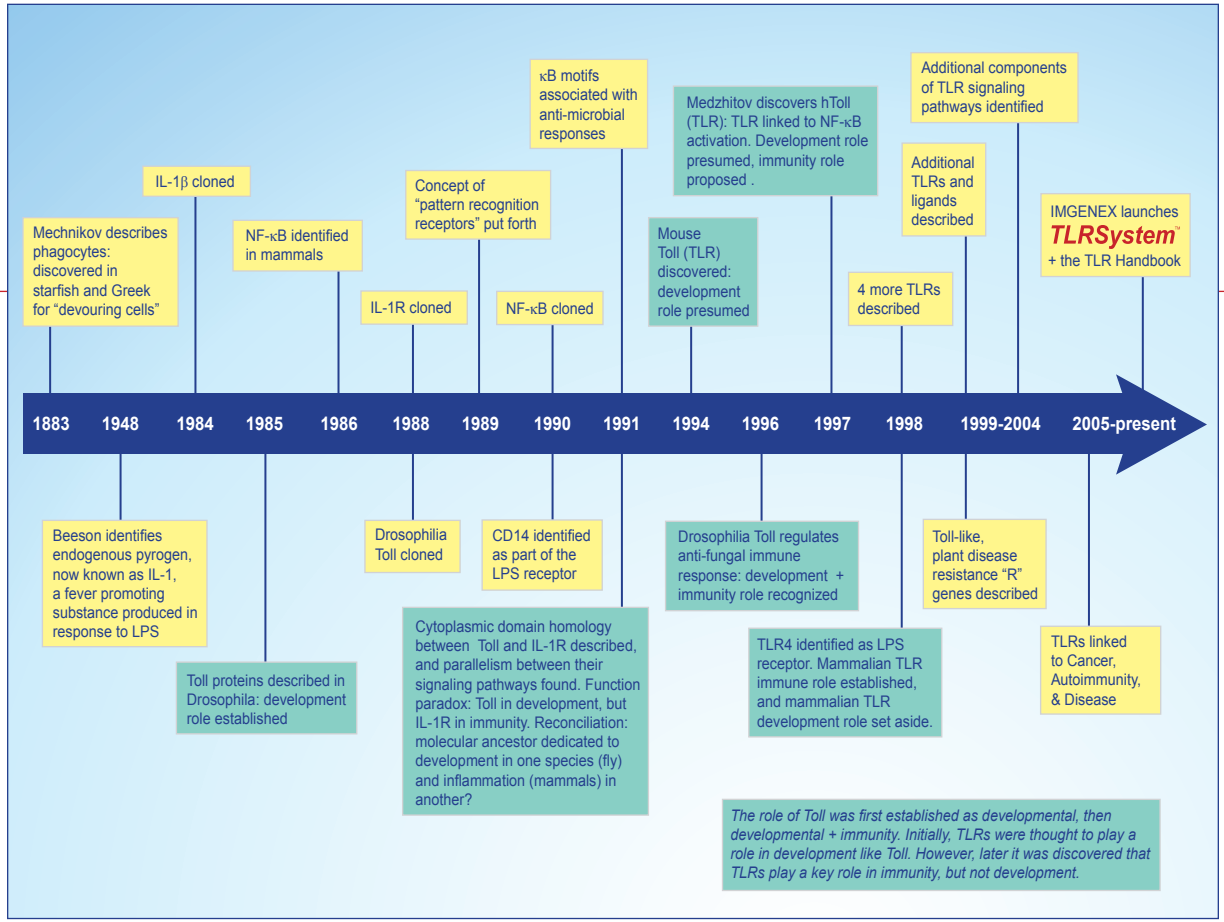


Figure 7. Milestones in TLR research

recognizing PAMPs. PAMP-PRR interaction results in activation of innate immune signaling pathways, leading to pathogen elimination. Furthermore, TLR activation in vertebrates also results in dynamic extensive interplay between innate and adaptive immunity that shapes and modulates adaptive immune responses.

However, the role of TLRs and other PRRs in immune defense and immunity remains to be fully elucidated. Of particular interest is defining the obligate links of PRRs to the initiation of adaptive immunity, and developing a comprehensive map of the signaling pathway interplay between innate and adaptive immunity.

Translational medicine also ranks high on the TLR goals list. The recognition that infectious diseases are more readily and rapidly exhibiting global spread than ever before in history is fueling the renaissance of interest in innate immunity. For example, the World Health Organization (WHO) declared H1N1 (swine flu) as the

Milestones in the TLR Field

first influenza pandemic of the 21st century on June 11, 2009, only two months after the first identified outbreak (synopsis in CNN, 2009). Global travel facilitated the rapid H1N1 spread which resulted in world wide fears about the virus. Since global travel has become common place, understanding the relationships between pathogens and immune defense mechanisms is critical. The need for translational research to develop the knowledge, technology and therapeutics to maintain and improve world health has never been more urgent.

A case in point is VaxInnate's (www.vaxinate.com) recombinant H1N1 flu vaccine which was developed in less than 3 weeks and reported positive preclinical results on June 17, 2009, only 6 days after the WHO announcement (Skidmore J, 2009). This vaccine is based on a TLR-mediated immune enchancement mechanism; it contains sequences from the TLR5 agonist flagellin which interact with host TLRs to enhance immunological potency.

In this context, Novus has recently launched their TLRSystem™ portfolio of high quality and frequently published research reagents for TLRs and other aspects of innate immunity. The availability of highly specific and well-validated research reagents is key for facilitating leading edge research and for translating research findings into clinical strategies and applications. Examples of published results using Novus' TLRSystem™ research reagents are shown in Figs 8 and 9. The figures illustrate specificity validations of monoclonal (Fig 8) and polyclonal (Fig 9) TLR9 Abs in WT and TLR9-/- mice.

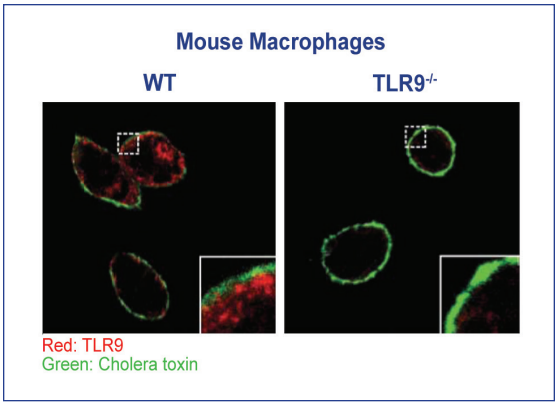


Figure 8. Immunofluorescence analysis of TLR9 in peritoneal macrophages from WT and TLR9-/- mice using TLR9 mAb, clone 26C593.2 (NBP2-24729). *Cells were counterstained with cholera toxin (green) which labels membranes. The data shows that the TLR9 antibody (red) labeled the WT but not the TLR9-/- macrophages. In the WT cells, TLR9 was detected as punctate staining in sub-plasmalemmal vesicular compartments.*

This data is an example of specificity validation of the TLR9 Ab for TLR9 because the Ab labeled the WT but not the TLR9-/- macrophages. Source: Tabeta et al (2006).

Publications are valuable tools for reserachers to gain additional information not only about scientific advancements, but also about the properties of antibodies and other research reagents as well as technical approaches. For example, both Figs 8 and 9 are good examples of how researchers can conduct antibody specificity validations to evaluate and optimize antibodies for their own model systems and techniques.

Additional examples of published TLRSystem™ products will be presented throughout this manual. We encourage researchers to notify Novus with their publications and we will add them to our technical literature. This gives exposure to the research of individual scientists, and enables the scientific community to stay up to date with both relevant scientific and product application information.

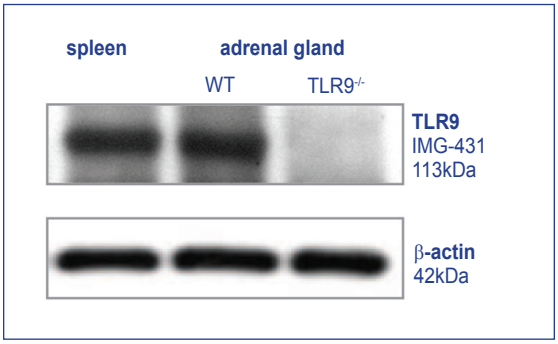


Figure 9. Western blot analysis of TLR9 in adrenal gland tissue lysate from WT and TLR9-/- mice using TLR9 pAb (IMG-431). *WT mouse spleen was used as a positive control for TLR9 expression. The data shows that the antibody detected TLR9 in the WT but not the TLR9-/- tissue. This data is an example of specificity validation of the TLR9 antibody for TLR9 because the antibody detected TLR9 in the WT but not the TLR9-/- tissue. Source: Tran et al 2007.*

A Beta Actin antibody was used to standardize protein loading. The Western Blot Loading Control Kit (NBP2-25090) is recommended and includes a Beta Actin polyclonal antibody (NB100-56874) and other polyclonal antibodies [GAPDH (NB100-56875), Beta Tubulin (IMG-5810), and Histone H2B (NB100-56347)] are widely used as protein loading controls. The Kit also contains an HRP-conjugated secondary antibody.

Mammalian TLRs

TLR4, identified through Toll homology searches and initially called hToll, was the first recognized human Toll homologue (reviewed in Beutler, 2009). TLR4 was also the first TLR to have its specificity elucidated, the Beutler laboratory identified it as the LPS endotoxin receptor in 1998. Subsequent studies revealed multiple structurally and functionally related proteins. Collectively, these hToll/TLR proteins were found to recognize a broad array of microbial structures and became known as TLR family members.

It is interesting to note that despite the homology between Drosophila Toll and TLRs, an orthologous relationship does not exist between mammals and fruit flies. Hence, it is thought that the TLR and Toll families developed independently during evolution.

Collectively they recognized a broad array of microbial structures and became known as TLR family members. Despite the similarity between Toll and TLRs, an orthologous relationship does not exist between mammalian and fruit flies. Hence, it is thought that TLR and Toll families developed independently during evolution.

Most mammalian species have between ten and fifteen types of TLRs. Ten functional TLRs (TLR1-10) have been identified in human (Fig 10) (Kaisho T, 2006). Human TLRs can be divided into five subfamilies based on chromosomal localization, genomic and amino acid sequence, and intracellular signaling adaptor molecules:

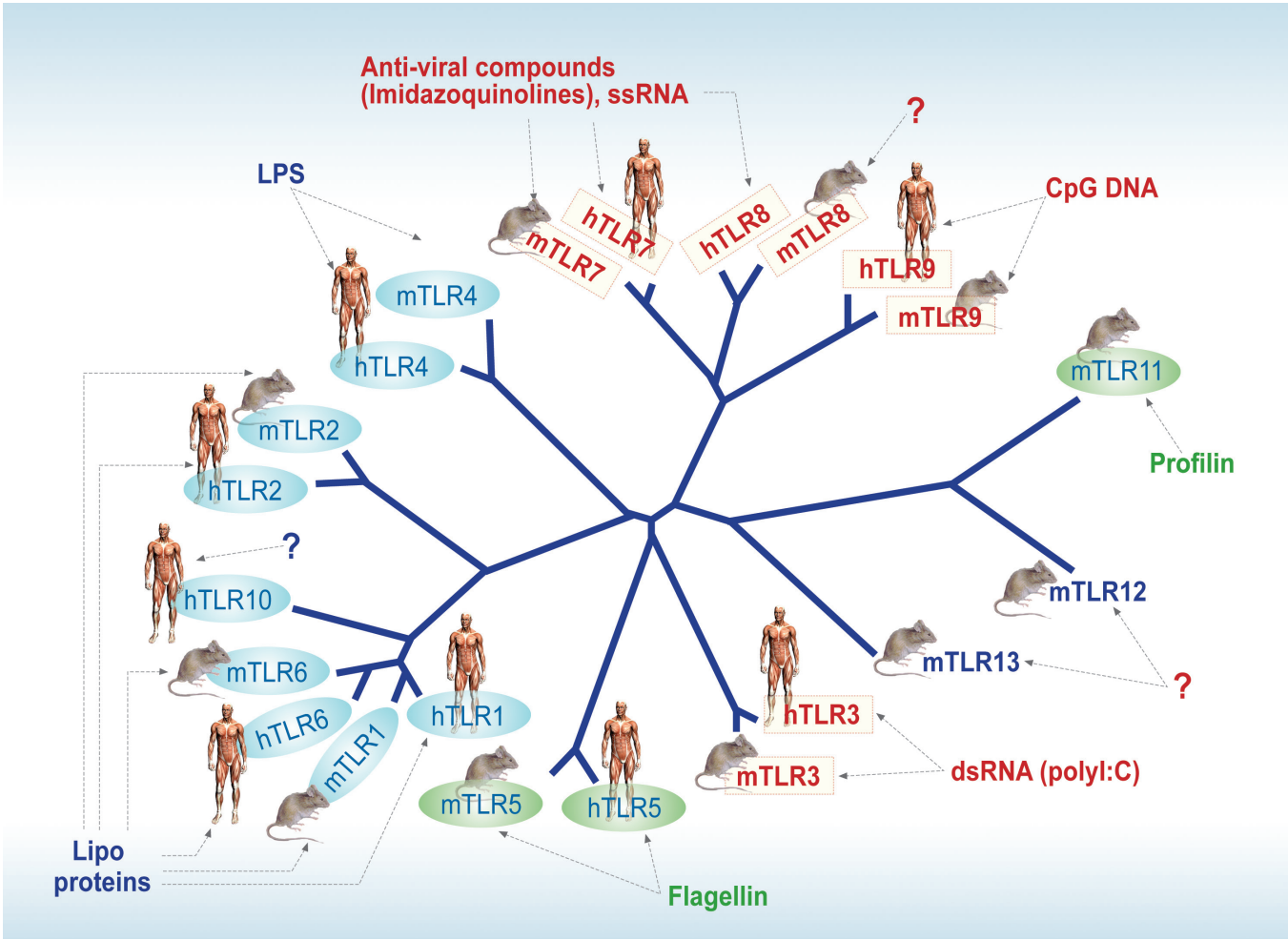
1. **TLR2 (TLRs 1, 2, 6 and 10)**
2. **TLR3**
3. **TLR4**
4. **TLR5**
5. **TLR9 (TLRs 7-9)**

Humans also encode a TLR11 gene, but it contains several stop codons and protein is not expressed. Mouse and rat TLR11 are functional, and it is thought that human TLR11 function was lost during evolution (Reviewed in Lauw et al, 2005).

Homologous forms of human TLRs have been found in most other mammalian species as well as many other vertebrate species (Reviewed in Roach et al, 2005). However, certain TLRs are not expressed in all mammals. For example, the C-terminal half of the mouse TLR10 gene is substituted with a retroviral insertion sequence which is unrelated to the human TLR10 sequence and is non-functional (Hasan et al, 2005). The rat genome, however, encodes a complete TLR10 sequence.

Additionally, both mouse (Fig 10) and rat express TLR12 and TLR13 which are not encoded for in the human genome (Roach et al, 2005). The fact other mammals may express TLRs which are not found in humans and vice versa can make it challenging to generalize findings about innate immunity between humans and different animal model systems.

Figure 10. Phylogenetic tree of human and murine TLRs. *Solid lines: A phylogenetic analysis of amino acid structures was used to establish connections between human (h) and murine (m) TLRs. Dotted arrows: representative ligands. Murine TLR8 does not appear to recognize individual ligands known to activate hTLR8. Murine TLR8 may recognize TLR8 ligands in combination. However, this remains to be fully elucidated (adapted from Kaisho and Akiro, 2006).*



TLRs: Structure, Ligands & Expression

STRUCTURE

The TLR family is a member of interleukin-1 receptor (IL-1R)/TLR superfamily. This superfamily was delineated in 1998 as a family of type I transmembrane proteins that contain a TIR intracellular domain (Fig 11). All TLRs have a common basic structure (reviewed in Akira and Takeda, 2004):

1. N-terminal extracellular or ecto domain (ECD) containing leucine rich repeat (LRR) modules and a 60 aa domain rich in cysteines. LRRs make up the majority of the ECD where there is 19-25 repeated tandem modules. LRRs contain the highly conserved segment LxxLxLxxNxL, in which "L" is Leu, Ile, Val, or Phe; "N" is Asn, Thr, Ser, or Cys; and "x" is any amino acid. Each LRR module is 20-30 aa long, and the modules are sandwiched between the LRRNT (cysteine clusters on the N-terminal side of LRRs) and LRRCT (cysteine clusters on the C-terminal side of LRRs) modules. As far as is known, it is just the ECD that is directly involved in pathogen (ligand) recognition.

2. Transmembrane domain

3. C-terminal intracellular globular domain containing a conserved region which has high homology with mamallian IL-1R family members. This region is called the Toll/IL-1 receptor (TIR) domain. The TIR domain contains 200 aa including three conserved regions (Boxes 1-3) that are essential for signal transduction. Although highly conserved, the TLR TIR domains are not identical.

The LRR modules and the TIR domain are the two core domains of the TLRs. The LRRs are involved in ligand binding where they play an integral role in ligand specificity and TLR dimerization. Both TLR dimerization and the TIR domains are thought to be required for signal transduction.

Both extracellular and intracellular membrane-bound (endosomal) PAMPs bind to LRR domains and activate downstream TLR signaling pathways via the interaction of TIR domains with adaptor molecules. This leads to NF-κB pathway activation, which in turn induces inflammatory responses such as the production of pro-inflammatory cytokines.

It is interesting to note that the TLR signaling cascades are much more well defined than the structural mechanisms that confer LRR pathogen recognition specificity. This is because, in part, obtaining the crystral structure of the LRRs for the TLRs has proven to be very difficult to achieve. However, recent advances, including the hybrid LRR technique, are helping to elucidate TLR-ligand structures. The following crystal structures have now been described (reviewed in Carpenter and O'Neill, 2009) and are illustrated in Figure 12:

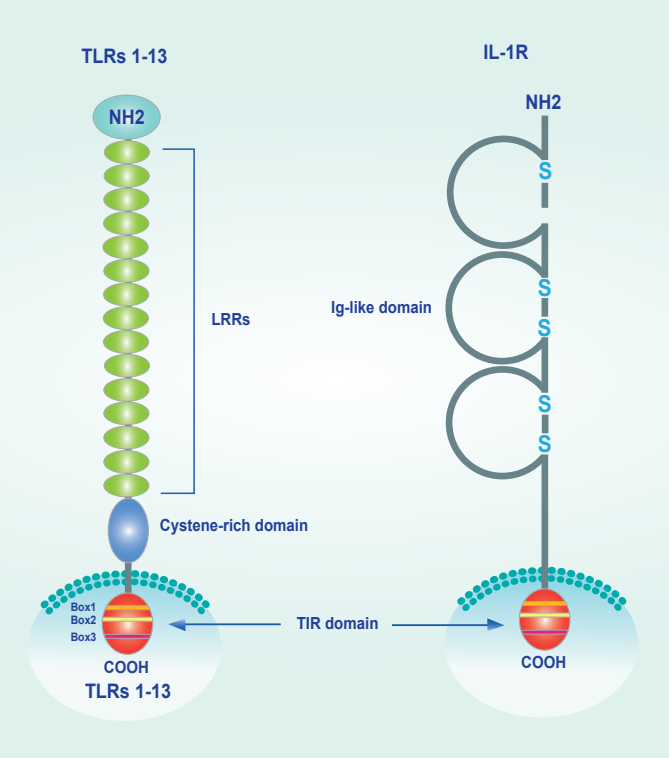


Figure 11. Structural comparison of TLR and interleukin-1 receptor (IL-1R). *Adapted from Akira and Takeda (2004). TLRs and IL-1R have highly homologous cytoplasmic TIR domains, both containing three regions known as boxes 1,2 and 3. However, their extracellular regions vary significantly. TLRs have tandem LRR repeats whereas IL-1R has three immunoglobulin-like (Ig-like) extracellular domains.*

1. **TLR1/2:** The crystal structure of the extracellular domains of TLR1 and TLR2 bound to the Pam3CSK4 ligand was described in 2007 by Jin et al. This group found that TLR1 and TLR2 resolved as monomers in the absence of bound ligand. However, the TLRs formed stable heterodimers when Pam3CSK4 was added. PAM3CSK4 consists of three lipid chains; two inserted within a hydophobic pocket in TLR2, and one inserted into a narrow hydrophobic channel in TLR1. Hydrophobic, hydrogen-bonding and ionic interactions between TLR1 and TLR2 added stability to the TLR1-TLR2 heterodimer/Pam3CSK4 complex.

2. **TLR3:** The first structure of TLR3 was described in 2005 by two independent groups, Bell et al and Choe et al. Both groups showed that TLR3 has a heavily glycosylated horseshoe-shaped structure. However, the groups differed in their prediction of where the dsRNA ligand would bind. The crystal structure of TLR3 bound to dsRNA was subsequently solved by Lui et al in 2008. This group found that TLR3 ECDs are monomeric in solution and that TLR3 dimerization occurs upon dsRNA ligand binding. Binding to dsRNA occurs on N-terminal (LRR1-LRR3) and C-terminal (LRR19-LRR21) sites of the

LRR modules. These are convex surfaces of the ECD which lie on opposite sites of the dsRNA. The binding between the two C-terminal TLR3 domains brings stability to the TLR3 dimer, and also brings the TIR domains into contact with one another. These features are thought to allow the downstream signaling cascade to occur.

3. **TLR4:** The crystal structure of the TLR4-MD2 complex binding to the LPS antagonist Eritoran was described in 2007 by Kim et al. Eritoran is a structural analog of the lipid A portion of LPS. TLR4 requires its co-receptor MD2 to recognize LPS. The crystal structure showed that all four acyl chains of Eritoran bound to a hydrophobic pocket in MD2, but that there was no apparent direct interaction of Eritoran with TLR4.

   The lack of direct TLR4 interaction remained a puzzle until 2009, when Park et al solved the crystal structure of TLR4-MD2 binding to LPS. This group showed that TLR4 and MD2 associate without LPS. However, dimerization of the TLR4-MD2 complex with another TLR4-MD2 complex only occurs following binding of LPS. The structure showed that LPS bound to two symmetrically arranged copies of TLR4 and MD2. TLR4 forms hydrophobic and hydrophilic interactions with LPS which is required for TLR4 dimerization to occur.

   It is interesting to note that LPS associates with the Phe126 and Leu86 loops of MD2. This association has helped to understand the mechanisms underlying Eritoran's function as a TLR4 antagonist. When Eritoran binds to MD2, the Phe126 loop of MD2 is exposed. This inhibits TLR4 dimerization, thereby preventing the activation of TLR4 downstream signaling pathways. Hence, knowledge of TLR/ligand structures is critical for understanding inhibitor mechanisms, like Eritoran, which block ligand binding and thereby inhibit TLR activation.

Solving TLR/ligand crystal structures is key to elucidating the molecular basis of dimerization, and has significantly advanced our knowledge about how TLR signaling is initiated. This knowledge is important for drug and vaccine design, designing TLR inhibitors, as well as for elucidating autoimmune and other disease mechanisms. See the chapter 'TLR Signaling Pathways' which illustrates how the TLR3 Novus antibody (IMG-315A) was instrumental in identifying a deletion in the ECD that caused structural changes and abolished TLR3 function, but did not prevent protein expression.

Likewise, solving TLR/adaptor structures will be critical for advancing our understanding of TLR signaling. TIR-TIR domain interactions also occur between TLRs and their adaptor proteins. Therefore, elucidating the crystal structures of TLR/adaptor TIR-TIR domain interactions will be particularly important for defining the mechanisms linking TLR ligand binding to downstream signaling events. This knowledge will provide additional points in the TLR pathway which can be potentially manipulated by inhibitors or other compounds.

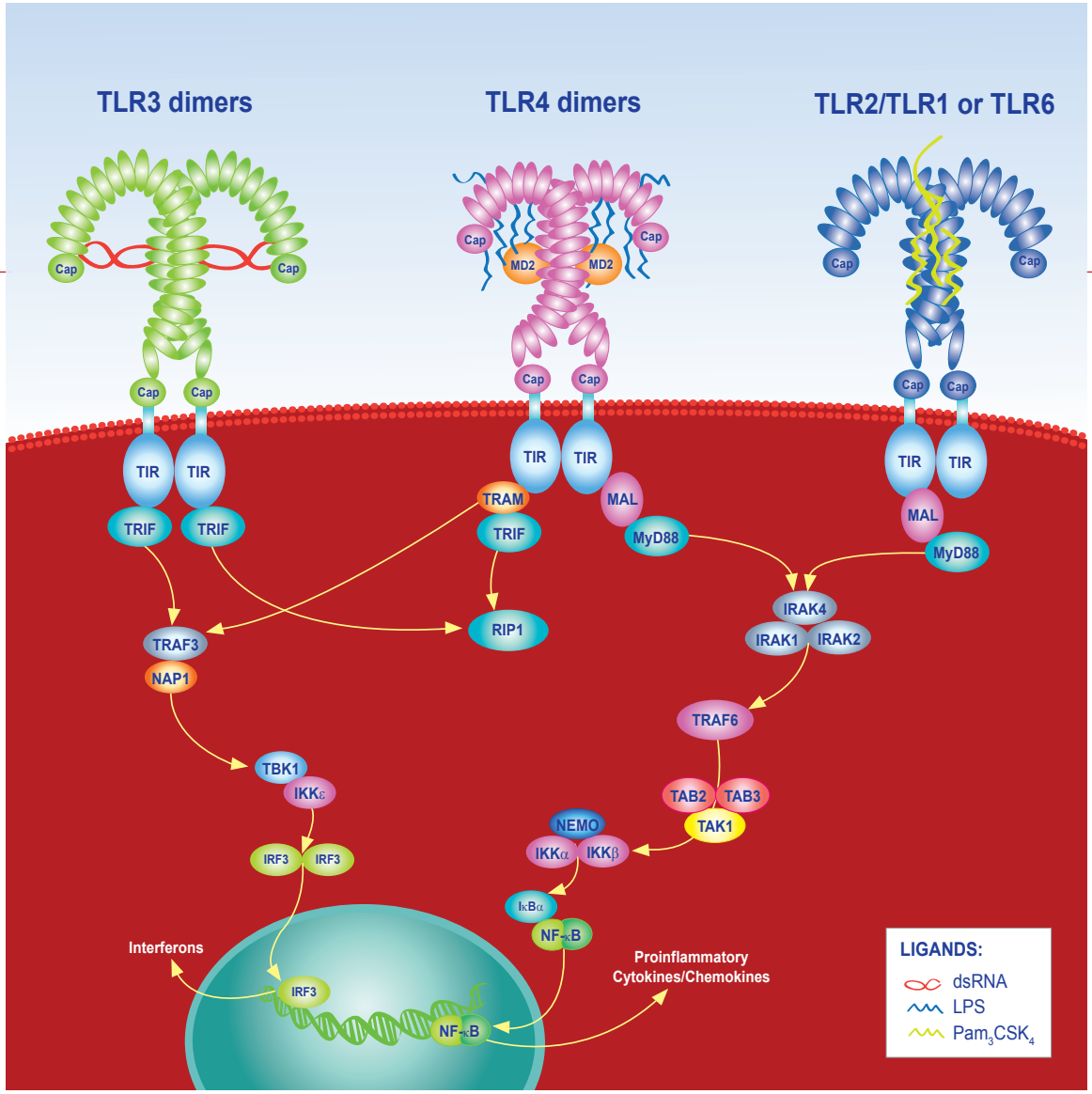


Fig 12. Structural interactions between TLRs and their ligands. *Crystral structures of TLRs bound to their ligands indicate TLR-TLR homo or heterodimer configuration with the formation of a common 'M' shaped architecture. The C-termini of the TLR ECDs converge between the TLRs in the dimer. This is thought to enable interactions between TIR domains to occur and initiate downstream signaling events. Adapted from Carpenter and O'Neil, 2009.*

TLRs: Structure, Ligands & Expression

LIGANDS

TLRs are essential for allerting the innate immune system to the presence of a myriad of pathogens, including protozoa, bacteria, fungi, and viruses, as well as to synthetic analogs of natural products (reviewed in Kumar, 2009). They accomplish this through recognizing the PAMPs of pathogens as ligands. PAMPs then activate TLRs, setting the stage for pathogen elimination through downstream TLR signaling pathways and subsequent immune system responses. PAMPs, referred to as exogenous TLR ligands, are molecularly diverse and include nucleic acids, protein, lipids and polysaccharides (Table III).

Table III. Exogenous (PAMP) TLR Ligands

TLR	LIGAND	SOURCE	REFERENCE
TLR1 (associated with TLR2)	Glycosylphosphatidylinositol (GPI)-anchored proteins	Parasite	JI 167:416-423 (2001)
	Lipoarabinomannan	Mycobacterial cell wall	JI 163:3920-3927 (1999)
	Outer surface lipoprotein	Borrelia burgdorferi	Nat Med 8:878–884 (2002)
	Pam3CSK4	Synthetic lipopeptide	JI 172:2739-2743 (2004)
	Soluble factors	Neisseria meningitidis cell wall	JI 165:7125–7132 (2000)
	Triacylated lipopeptides	Gram positive and gram negative bacteria	JI 169:10–14 (2002)
TLR2 (associated with TLR2)	Atypical LPS	L. interrogans	Nat Immunol 2:346-352 (2001)
		P. gingivalis	Int Immunol 14:1325-1332 (2002)
	CMV envelope proteins	Human cytomegalovirus	J Virol 77:4588-4596 (2003)
	Diacylated and triacylated lipopeptides, lipoproteins	Many pathogens	JI 169:10–14 (2002)
	Glycoinositolphospholipids	Trypanosoma cruzi	JI 163:6748–6755 (1999)
	Glycolipids	Spirochetes	JI 167:987–994 (2001)
	GPI anchors	Trypanosoma cruzi	JI 167:416–423 (2001)
	HSV-1	Herpes simplex virus 1	PNAS 101:1315-1320 (2004)
	Lipoarabinomannan	Mycobacteria	PNAS 96:14459–14463 (1999)

Table III. Exogenous (PAMP) TLR Ligands

TLR	LIGAND	SOURCE	REFERENCE
TLR2 (associated with TLR2)	LPS	Gram negative bacteria	Science 282:2085–2088 (1998)
		H. pylori	JBC 278:32552–32560 (2003)
		Spirochetes	J Am Soc Nephrol 15:854–867 (2004)
	Lipoteichoic acid	Gram positive bacteria	J Biol Chem 274:17406–17409 (1999)
	Mannuronic acid polymers	Pseudomonas aeruginosa	JI 164:2064–2069 (2000)
	Outer membrane porins	N. gonorrhoeae, H. pylori	JI 168:1533-1537 (2002)
	Outer membrane protein A	Klebsiella	Nat Med 8:878–884 (2002)
	Outer surface lipoprotein	Borrelia burgdorferi	Nat Med 8:878–884 (2002)
	Soluble factors	Neisseria meningitides	JI 165:7125–7132 (2000)
	Wild-type H protein	Measles virus	J Virol 76:8729-8736 (2002)
	Zymosan	Yeast	JI 171:417–425 (2003)
TLR3	dsRNA	Viruses	Nature 413:732–738 (2001)
	ssRNA	Viruses	J Clin Invest 110:1175–1184 (2002)
	Poly(I).Poly(C)	Synthetic analogue of double stranded viral RNA (West Nile virus)	JI 176:1348 (2006)
TLR4	Fusion protein	RSV (respiratory syncytial virus)	Nat Immunol1:398–401 (2000)
	LPS	Gram negative bacteria	Science 282:2085–2088 (1998)
	Lipoteichoic acids	Gram positive bacteria	Gene 231:59–65 (1999)
	Mannuronic acid polymers	Pseudomonas aeruginosa	JI 164:2064–2069 (2000)
	Murine retroviral envelope protein	Murine mammary tumor virus (MMTV)	PNAS 99:2281–2286 (2002)
	Taxol	Plants (antitumor agent)	JBC 275:2251–2254 (2000)
TLR5	Flagellin	Motile bacteria	JI 170:5165–5175 (2003)
TLR6	Diacetylated lipopeptides	Mycoplasma fermentans	JI 169:10–14 (2002)
	Lipoteichoic acid	Bacteria	PNAS 97:13766–13771 (2000)
	Modulin	Staphylococcus	JI 166:15–19 (2001)
	Outer surface protein A	Borrelia burgdorferi	JI 167:987–994 (2001)
	Zymosan	Yeast	PNAS 97:13766–13771 (2000)
TLR7	Imidazoquinolines (imiquimod, resiquimod, loxoribine (a guanosine analogue), bropirimine), antiviral compounds	Synthetic compounds	PNAS 100:6646–6651 (2003) Hemmi H et al Nat Immunol 3:196–200 (2002)
	ssRNA	Viruses	PNAS 101:5598–5603 (2004)
TLR8 (inactive in mice)	Imidazoquinolines (imiquimod, resiquimod, loxoribine, bropirimine), antiviral compounds	Synthetic ligands	JI 36:1815–1826 (2006)
	ssRNA	Influenza virus, vesicular stomatitis virus	Science 303:1526–1529 (2004)
	Guanosine and uridine-rich ssRNA oligonucleotides	HIV-1	PNAS 100:6646–6651 (2003)

TLRs: Structure, Ligands & Expression

TLRs are also activated by several nonpathogenic organisms as well as by self (host) proteins and nucleic acids, which are referred to as endogenous TLR ligands or DAMPs (Table IV). Under normal physiological conditions, DAMPs are typically not produced by host cells or are otherwise concealed from the immune system. This avoids spurious activation of TLRs by self.

However, molecules with TLR stimulatory activity (i.e., DAMPs) are thought to be produced and released by dying cells during tissue inflammation, oxidative stress, necrosis or other conditions that disrupt homeostasis. The emerging concept is that TLR DAMP ligands act as signals to the host that an immediate immune response is required, akin to PAMP signals (Fig 6). Damaged tissue and necrotic cell components, immune complexes, heat shock proteins, uric acid crystals, surfactant Protein A, mammalian genomic DNA, and extracellular matrix breakdown products have all been proposed to act as DAMPs (Table IV). The degree to which "self' or DAMPs can act as TLR ligands remains to be fully elucidated, in part because defining their role can be compounded by technical challenges (reviewed in Tsan and Gao, 2007):

Caveats in endogenous ligand evaluation

1. There is a significant possibility of mycoplasma or endotoxin contamination, particularly LPS in ligand preparations. Hence, observed effects can potentially be from the putative DAMP, from PAMP contamination or a combination of both.

2. Low levels of endotoxin can be difficult to detect by conventional approaches, and can therefore be a major problem in interpreting results. Thus, it is important for researchers to assess whether LPS or other PAMP contamination could be wholly or partially responsible for observed effects of putative DAMPs.

3. The exact amount of LPS in ligand preparations is not always rigorously quantified or even reported in publications. Thus, researchers should take this into consideration when evaluating results in the literature regarding DAMPs. This is because observed results could have been due to or tainted by the presence of PAMPs. That is, DAMP preparations can contain elusive PAMP contamination.

Table III. Exogenous (PAMP) TLR Ligands

TLR	LIGAND	SOURCE	REFERENCE
TLR9	Unmethylated CpG oligode-oxynucleotides	Viruses, bacteria, protozoa	Nature 408:740–745 (2000)
	Viral genomic DNA	HSV-2	J Exp Med 198:513-520 (2003)
TLR10 (found in humans but not mice)	Unknown		
TLR11 (found in mice; human form is truncated and thought to be inactive)	Uropathogenic bacteria	Toxoplasma gondii, E. coli, Stapy-lococcus, Enterococcus, Proteus, Psedomas	Science 303:1522–1526 (2004)
	Profilin		Science 308:1626–1629 (2005)
TLR12 (found in mice but not humans)	Unknown		
TLR13 (found in mice but not humans)	Unknown		

Table IV. Endogenous (DAMP) TLR Ligands

TLR	LIGAND	REFERENCES
TLR1 (associated with TLR2)	Unknown	
TLR2 (associated with TLR2)	Heat shock proteins	Curr Top Microbiol Immunol 270:169–184 (2002)
	HMGB1	J BiolChem 279:7370–7377 (2004)
	Necrotic cells	JI 166:7128–7135 (2001)
	Oxygen radicals	JBC 276:5197–5203 (2001)
	Urate crystals	Arthritis Rheum 52:2936–2946 (2005)
TLR3	mRNA	JBC 279:12542–12550 (2004)
TLR4	Beta-defensin 2	Science 298:1025–1029 (2002)
	Extravascular fibrinogen/fibrin	JI 167:2887–2894 (2001)
	Fibronectin extra domain A	JBC 276:10229–10233 (2001)
	Gp96	JBC 277:20847–20853 (2002)
	Heat shock proteins	Curr Top Microbiol Immunol 270:169–184 (2002)
	HMGB1	JBC 279:7370–7377 (2004)
	Hsp22	JI 176:7021–7027 (2006)
	Hsp60	JI 164:558–561 (2000)
	Hsp70	JBC 277, 15028–15034 (2002)

Table IV. Endogenous (DAMP) TLR Ligands

TLR	LIGAND	REFERENCES
TLR4	Lung surfactant protein A	JI 168:5989–5992 (2002)
	Minimally modified (oxidized) low density lipoproteins (mmLDL)	JBC 278:1561–1568 (2003)
	Pancreatic elastase	BBRC 323:192–196 (2004)
	Polysaccharide fragments of heparan sulfate	JI 168:5233–5239 (2002)
	Soluble hyaluronan	J Exp Med 195:99–111 (2002)
	Alpha A-crystallin	JI 176:7021–7027 (2006)
TLR5	Unknown	
TLR6	Unknown	
TLR7	RNA immune complexes	J Exp Med 202:1131–1139 (2005)
TLR8 (inactive in mice)	Unknown	
TLR9	CpG chromatin-IgG complexes	Nature 416:603–607 (2002)
	DNA immune complexes	J Exp Med 202:1131–1139 (2005)
TLR10 (found in humans but not mice)	Unknown	
TLR11 (found in mice; human form is truncated and thought to be inactive)	Unknown	
TLR12 (found in mice but not humans)	Unknown	
TLR13 (found in mice but not humans)	Unknown	

It is well recognized that ligand-induced TLR activation is a double-edge sword. On the one hand, exogenous threats initiate a quick anti-microbial innate immune response through the PAMP-TLR system which signals adaptive immunity to mount a protective response. On the other hand, excessive TLR activation can result in too much inflammation leading to tissue damage and cell death. The injured and dying cells release DAMPs which in turn can activate TLR pathways, causing more tissue damage and DAMP release, thereby perpetuating cycles of inflammation.

Nevertheless, DAMP-TLR activation is thought to be important in restoring homeostasis after cell death. For example, when the immune system is activated by necrosis it leads to necessary clean up or necrotic cell removal (reviewed in Rock and Kono, 2008). This process is orchestrated by the release of danger signals from injured or dying cells that alert the host to cell death. Some of the released signals are DAMPs that activate TLRs on dendritic cells, thereby promoting the generation of immune responses leading to elimination of cellular debris. This can be considered akin to pathogen elimination, eg DAMP + TLR leads to cellular debris elimination whereas PAMP + TLR leads to pathogen elimination.

Excessive activation of TLR signaling pathways by either PAMPs or DAMPs is thought to lead to significant tissue injury, sepsis, chronic inflammation, and inappropriate autoimmune responses (reviewed in Rock and Kono, 2008). This process contributes to the pathogenesis of many chronic diseases, including:

1. Asthma (reviewed in Willart and Lambrecht, 2008)
2. Autoimmune diseases (reviewed in McGonagle et al, 2007 and Foell et al, 2008)
3. Alzheimer's disease (reviewed in Salminen et al, 2009)
4. Cancer (reviewed in Huang et al, 2008)

Elucidating the mechanisms and consequences of aberrent recognition of DAMPs or "self" is key to developing therapeutics targeting TLR pathways. TLR pathway targeted therapeutics based on management of DAMP activation will be potentially useful for treating pathological autoimmune responses, allergies, and diseases.

EXPRESSION

Historically TLR expression has been most extensively studied in the immune system. In general, TLRs are highly expressed in immune competent cells, including macrophages, dendritic cells, neutrophils, mucosal epithelial cells and dermal endothelial cells. However, TLRs have also now been identified in numerous other cell types and anatomical tissue locations, where they are expressed either constitutively or induced during infection.

Tables V-VIII provide an overview of TLR expression in various cells [Tables V (protein) and VI (mRNA)] and tissues [Tables VII (protein) and VIII (mRNA)], compiled from the published literature. Protein and mRNA expression are displayed in separate Tables for clarity. This is because publications often report either protein or mRNA expression, and compiling information together would add a level of ambiguity to understanding what is known about TLR expression patterns.

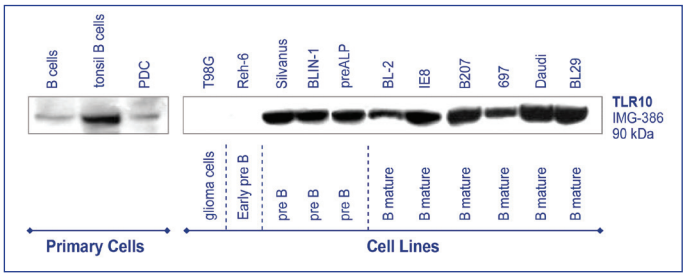


Figure 13. Western blot analysis of TLR10 using IMG-386 mAb (clone 158C1114) in human ex vivo cells and cell lines. *B cells were purified from whole blood or tonsil. pDCs (plasmacytoid DC) were purified from tonsil and matured (activated) overnight with IL-3 and CD40L-expressing L cells. TLR10 expression was detected in all samples except the T98G gliobastoma cell line and the Reh-6 early pre B cell line. Source: Hasan et al, 2005.*

Although TLR expression patterns and their underlying regulating mechanisms likely have profound biological significance, many aspects of TLR expression and regulation remain poorly characterized (Mogensen, 2009). This is in part because the expression patterns of TLRs are complex both within and outside of the immune system. For example, little may be known in a given model system about how TLR expression changes in response to infection, inflammation or other mediators, or even during the course of normal cell culture conditions.

It is increasingly being recognized that donor variability, and the plasticity of TLR expression during cell differentiation, maturation, and various physiological, pathological, and infectious states are among the factors contributing to the complexity of TLR expression. For example, Figure 13 shows that TLR10 was detected in pre B and mature B cell lines, but not in an early pre B cell line (Reh-6).

Table V. TLR Protein Expression in Cells and Cell Lines

TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR1	B cells (Tonsillar B lymphocytes)		FACS, IF	JI 173:7426–7434 (2004) Infect Immunol 72:7202–7211 (2004) J Leukoc Biol 69:474–481 (2001)
	Eosinophils	NB100-56563	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	Immature DCs		FACS	JI 166:249–255 (2001)
	Mature DCs		FACS	Infect Immunol 72:7202–7211 (2004)
	Monocytes		FACS	JI 166:249–255 (2001)
	Myeloid DCs		FACS	JI 31:3388–3393 (2001)
	Neutrophils	NB100-56563	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	Plasmacytoid DCs		FACS	Infect Immunol 72:7202–7211 (2004)
	T cells		FACS	PNAS 101:3029–3034 (2004)
TLR2	293/TLR2 transfected cells	NB100-56722	FACS	Immunol, 114:83 (2005)
	B cells (Tonsillar B lymphocytes)	NB100-56058	FACS	Immunol Lett 117:26-34 (2008) JI 173:7426–7434 (2004) Infect Immunol 72:7202–7211 (2004)
	Basophils		FACS	JI 168:4701–4710 (2002)
	CD16+56+cells	NB100-56058	FACS	Immunol Lett 117:26-34 (2008)
	CD4+T cells	NB100-56058	FACS	Immunol Lett 117:26-34 (2008)
	CD8+T cells	NB100-56058	FACS	Immunol Lett 117:26-34 (2008)
	Eosinophils	NB100-56058	FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	Granulocytes	NB100-56722	FACS	J Leukoc Biol 69:474-481 (2001)
	HeLa cells	NB100-56728	WB	JBC 277:17263-17270 (2002)
	HMEC(LPS-treated, LPS-stimulated)	NB100-56722	WB, IHC	JI 166: 2018-2024 (2001)
	HOK-16B	NB100-56058	FACS	Infection Immunity 77: 1044-1052 (2009)
	Human airway epithelial cell lines	NB100-56720	WB	JI 177:1330-1337 (2006)
	Immature DCs		FACS	JI 166:249–255 (2001)
	Macrophages	NB100-56722	IF/ICC	J Leukoc Biol 69:474-481 (2001)
	Mast cells		WB	JI 167:2250–2256 (2001) J Leukoc Biol 70:977–984 (2001)

Table V. TLR Protein Expression in Cells and Cell Lines

TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR2	Monocytes	NB100-56728, NB100-56722	WB, FACS, IP	JI 171:6680-6689 (2003) Surgery 140:170-178 (2006) Immunol, 114:83 (2005)
	Myeloid DCs		FACS	Eur J Immunol 31:3388–3393 (2001)
	Neutrophils	NB100-56058, NB100-56726	FACS	J Respir Cell Mol Biol 7:85-96 (2007) J Leukoc Biol 69:474–481 (2001) JI 168:4701–4710 (2002)
	PBMC	NB100-56722, NB100-56058	FACS	Vet Immunol Immunopathol 124:184-191 (2008)
	Platelets	NBP2-24909	FACS	Blood 107:637–641 (2006) Immunol Cell Biol 83: 196-198 (2005)
	RAW 2647. cells	NB100-56573	WB	JBC 279:55127-55136 (2004)
	SRIK-NKL cells	NB100-56722	IF/ICC	Leukemia Res 29:813-820 (2005)
	T cells	NB100-56058	FACS	PNAS 101:3029–3034 (2004) J Immunol 184: 2814-2824 (2010)
	THP-1 cells	NB100-56726	FA	JI 175:4641-4646 (2005)
	Treg cells	NB100-56058	FACS	Immunol Lett 117:26-34 (2008)
TLR3	Eosinophils	NBP2-24899	FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	HT29 human colon adenocarcinoma cell line	NBP2-24875	IF	JI 175:376-384 (2005)
	Immature DCs		FACS	JI 166:249–255 (2001)
	J774 macrophages	NB100-56571	FACS	J Am Soc Nephrol 17:141-149 (2006)
	Mature DCs		FACS	JI 164:5998–6004 (2000)

Table V. TLR Protein Expression in Cells and Cell Lines

TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR3	Mesangial cell lines	NB100-56571	FACS	J Am Soc Nephrol 16:1326-1338 (2005)
	Monocytes	NBP2-24875	FACS	Surgery 140:170-178 (2006)
	Neutrophils	NBP2-24875, NBP2-24899	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) FJI 174:3633-3642 (2005)
	PBMC	NBP2-24875	FACS	Vet Immunol Immunopathol 124:184-191 (2008)
	T cells		FACS	PNAS 101:3029–3034 (2004)
	THP-1	NBP2-24902	FACS	Infection Immunity 78: 1364-1375 (2010)
TLR4	B cells (Tonsillar B lymphocytes)	NB100-56059	FACS	Immunol Lett 117:26-34 (2008) JI 173:7426–7434 (2004) Infect Immunol 72:7202–7211 (2004)
	Basophils		FACS	JI 168:4701–4710 (2002)
	B-CLL cells from a leukemia patient	NB100-56727	FACS	Nitric Oxide 19:138-145 (2008)
	Caco-2 cells	NB100-56566	FACS	Infect Immun doi: 10.1128/IAI.01406-09 (2010)
	CD16+56+cells	NB100-56059	FACS	Immunol Lett 117:26-34 (2008)
	CD4+T cells	NB100-56059	FACS	Immunol Lett 117:26-34 (2008)
	CD8+T cells	NB100-56059	FACS	Immunol Lett 117:26-34 (2008)
	Cholangiocyte cell lines	NB100-56723	IF	JBC 282:28929-28938 (2007)
	Endothelial cells	NBP2-24821	FACS	Am J Pathol 166: 185-196 (2007)
	Eosinophils	NB100-56059	FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	TLR4 transfected HEK 293T cells	NB100-56723	WB, IP	JI 177:322-332 (2006)
	HT29 human colon adenocarcinoma cell line	NB100-56723	IF	JI 175:376-384 (2005)
	Immature DCs		FACS	JI 166:249–255 (2001)
	Leukocytes	NBP2-24450	FACS	JI 179:4444-4450(2007)
	Macrophages	NBP2-24450, NBP2-24865	FACS, Fluorescent microscopy	Med Mycol 45:708-719 (2007) Blood 99:3427–3431 (2002) Gastroenterol 122:1987–2000 (2002)
	Mast cells		WB	JI 167:2250–2256 (2001) J Leukoc Biol 70:977–984 (2001)

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Table V. TLR Protein Expression in Cells and Cell Lines

TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR4	Monocytes	NB100-56723, NB100-56566	FACS	Surgery 140:170-178 (2006) JI 177:5077-5087 (2006) PLoS Med 2006 Sep;3(9):e358
	Myeloid DCs		FACS	Eur J Immunol 31:3388–3393 (2001)
	Neutrophils	NB100-56059	FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) J Leukoc Biol 69:474–481 (2001) JI 168:4701–4710 (2002)
	PBMC	NB100-56723, NB100-56059	FACS	JBC 282:1039-1050 (2007) Int Immunol 20: 829-840 (2008)
	Platelets	NB100-56062	FACS	Immunol Cell Biol 83: 196-198 (2005); Blood 107:637–641 (2006)
	Splenocytes	NBP2-24821	FACS	Am J Pathol 166: 185-196 (2007)
	SRIK-NKL cells	NB100-56723	IF/ICC	Leukemia Res 29:813-820 (2005)
	T cells		FACS	PNAS 101:3029–3034 (2004)
	THP-1 cells	NB100-56727, NB100-56566	Functional Assay, FACS	JBC 280:4279-4288 (2005) Infection Immunity 78: 1364-1375 (2010)
	Treg cells	NB100-56059	FACS	Immunol Lett 117:26-34 (2008)
TLR5	Caco-2 cells	NBP1-97728	FACS	Infect Immun doi: 10.1128/IAI.01406-09 (2010)
	CD14+ monocytes	NB200-571	FACS	JI 175:8051-8059 (2005)
	CD4+ T cells	NB200-571, NBP2-24787	FACS, WB	JI 175:8051-8059 (2005)
	Dendritic cells	NB200-571	FACS	JI 180:3399-3405 (2008)
	Eosinophils	NBP2-24787, NB200-571	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	Immature DCs		FACS	JI 166:249–255 (2001)

Table V. TLR Protein Expression in Cells and Cell Lines

TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR5	Lymphatic endothelial cells	NB200-571	FACS	JI 180:3399-3405 (2008)
	Macrophages	NBP2-24784	FACS	Med Mycol 45:708-719 (2007); Blood 99:3427–3431 (2002); Gastroenterol 122:1987–2000 (2002)
	Monocyte-derived dendritic cells	NB200-571	FACS	JI 175:8051-8059 (2005)
	Monocytes		FACS	JI 166:249–255 (2001)
	Myeloid DCs		FACS	Eur J Immunol 31:3388–3393 (2001)
	Neutrophils	NBP2-24787, NB200-571	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) JI 174:3633-3642 (2005)
	PBMC	NBP2-24787, NBP1-97728	FACS	Veterinary Immunol Immunopathol 124:184-191 (2008)
	RAW 264.7 cells	NB200-571	FACS	JI 177:7122-7130 (2006)
	T cells		FACS	PNAS 101:3029–3034 (2004)
TLR6	B cells (Tonsillar B lymphocytes)		FACS, IF	JI 173:7426–7434 (2004) Infect Immunol 72:7202–7211 (2004) J Leukoc Biol 69:474–481 (2001)
	Dendritic cells	NBP2-24971	FACS	JI 180:3399-3405 (2008)
	Eosinophils	IMG-304A, NBP2-24971	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	Immature DCs		FACS	JI 166:249–255 (2001)
	Langerhans cells	NB100-56536	FACS	J Leukoc Biol 83:1118-1127 (2008)
	Lymphatic endothelial cells	NBP2-24971	FACS	JI 180:3399-3405 (2008)
	Mast cells		WB	JI 167:2250–2256 (2001) J Leukoc Biol 70:977–984 (2001)
	Mature DCs		FACS	Infect Immunol 72:7202–7211 (2004)
	Monocytes	NB100-56536	FACS	Rheumatol Int 28(5):401-406 (2008)
	Neutrophils	NB100-56536, NBP2-24971	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) J Leukoc Biol 69:474–481 (2001) JI 168:4701–4710 (2002)
	Plasmacytoid DCs		FACS	Infect Immunol 72:7202–7211 (2004)

Table V. TLR Protein Expression in Cells and Cell Lines

TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR6	Platelets	NB100-56536	FACS	Immunol Cell Biol 83: 196-198 (2005)
	T cells		FACS	PNAS 101:3029–3034 (2004)
TLR7	B cells (Tonsillar B lymphocytes)		FACS, Fluorescent microscopy	JI 173:7426–7434 (2004) Infect Immunol 72:7202–7211 (2004) J Leukoc Biol 69:474–481 (2001)
	Bone marrow-derived dendritic cells	NBP2-24906	IP	JCB 177:265-275 (2007)
	Eosinophils	NB100-56682, NB100-56588, NBP2-24760	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) J Leukocyte Bio 85: 1-9 (2009)
	Fetal Cardiac Fibroblasts	NBP2-24906	IHC-P	J Immunol 184: 2148-2155 (2010)
	HCV-infected liver cells	NB100-56588	IHC-P	PNAS 103:1828-1833 (2006)
	Huh-7 hepatoma cells	NB100-56588	FACS	PNAS 103:1828-1833 (2006)
	J774 macrophages	NBP2-24906	FACS	J Am Soc Nephrol 17:141-149 (2006)
	Liver carcinoma cells	NB100-56588	IHC-P	PNAS 103:1828-1833 (2006)
	Macrophages	NB100-56588	FACS	J Immunol 184: 2148-2155 (2010)
	MBT-2, TCCSUP, T24, and J82 cancer cell lines	NBP2-24906	WB	J Urol 177:2347-2351 (2007)
	Monocytes		FACS, Fluorescent microscopy	JI 166:249–255 (2001)
	Myeloid DCs		FACS	Eur JI 31:3388–3393 (2001) J Exp Med 195:1507–1512 (2002)

Table V. TLR Protein Expression in Cells and Cell Lines

TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR7	Neutrophils	NBP2-24906, NB100-56682, NB100-56588	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) JI 174:3633-3642 (2005)
	NKL human leukemia cell line	NBP2-24906	WB	JI 175:1636-1642 (2005)
	Normal fibroblasts	NB100-56588	IHC-P	PNAS 103:1828-1833 (2006)
	Plasmacytoid DCs		FACS	Infect Immunol 72:7202–7211 (2004)
	Squamous cell carcinoma tumor cells	NBP2-24906	IF/ICC	J Exp Med 205:2221-2234 (2008)
	SRIK-NKL cells	NBP2-24906	IF/ICC	Leukemia Res 29:813-820 (2005)
TLR8	B cells (Tonsillar B lymphocytes)		FACS, IF	Infect Immunol 72:7202–7211 (2004) J Leukoc Biol 69:474–481 (2001)
	Eosinophils	NBP2-24917, NBP2-24972	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	Huh-7 hepatoma cells	NBP2-24972	FACS	PNAS 103:1828-1833 (2006)
	Neonatal cord blood CD25+ Tregs	NBP2-24917, NBP2-24972, NBP2-24818, NBP2-24817	FACS	J Immunol Methods 319:1-5 (2007)
	Macrophages	NBP2-24972	FACS	J Immunol 184: 2148-2155 (2010)
	Mast cells		WB	JI 167:2250–2256 (2001) J Leukoc Biol 70:977–984 (2001)
	Monocytes	NBP2-24917	FACS, IF	Surgery 140:170-178 (2006) JI 166:249–255 (2001) J Leukoc Biol 69:474–481 (2001)
	Myeloid DCs	NBP2-24818	FACS	Eur J Immunol 31:3388–3393 (2001) J Exp Med 195:1507–1512 (2002) J Immunol 184: 2518-2527 (2010)
	Neonatal cord or adult peripheral blood cells	NBP2-24817	FACS	Blood 108:1284-1290 (2006)
	Neutrophils	NBP2-24917, NBP2-24972	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) J Leukoc Biol 69:474–481 (2001) JI 168:4701–4710 (2002)
	NK92 human lymphoma cell line	NBP2-24917	WB	JI 175:1636-1642 (2005)
	NKL human leukemia cell line	NBP2-24917	WB	JI 175:1636-1642 (2005)
	PBMC	NBP2-24972	FACS	J Immunol 184: 2710-2717 (2010)

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TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR8	Platelets	NBP2-24817	FACS	Immunol Cell Biol 83: 196-198 (2005)
	Squamous cell carcinoma tumor cells	NBP2-24917	IF/ICC	J Exp Med 205:2221-2234 (2008)
	T cells		FACS	PNAS 101:3029–3034 (2004)
TLR9	TLR9 transfected cells	NBP2-24908, NBP2-24907	FACS	Immunol 114:83 (2005) Vet Immunol Immunopathol 124: 209-219 (2008)
	B cells (Tonsillar B lymphocytes)		FACS, Fluorescent microscopy	JI 173:7426–7434 (2004) Infect Immunol 72:7202–7211 (2004) J Leukoc Biol 69:474–481 (2001)
	B-CLL cells from a leukemia patient	NBP2-24908	FACS	Nitric Oxide 19:138-145 (2008)
	Eosinophils	NBP2-24729, NBP2-24908	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007)
	Huh-7 hepatoma cells	NBP2-24907	FACS	PNAS 103:1828-1833 (2006)
	J774 macrophages		FACS	Nephrol Dial Transplant 21:3062-3073 (2006)
	Monocytes	NBP2-24729	FACS, IF	Immunol 114:83 (2005) JI 173:7426–7434 (2004)
	Myeloid DC's	NBP2-24908	FACS, IF/ICC	J Immunol 184: 2518-2527 (2010) J Immunol 180: 4067-4074 (2008)
	Neutrophils	NBP2-24729, NBP2-24908	WB, FACS	Am J Respir Cell Mol Biol 7:85-96 (2007) J Leukoc Biol 69:474–481 (2001) JI 168:4701–4710 (2002)
	PBMC	NBP2-24729	FACS	Vet Immunol and Immunopathol 124:184-191 (2008)

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TLR	CELL TYPE	NOVUS ANTIBODIES USED	APPLICATION	REFERENCES
TLR9	Plasmacytoid DCs	NBP2-24908	FACS	Infect Immunol 72:7202–7211 (2004) J Virol 83: 1952-1961 (2008)
	Platelets	NBP2-24907	FACS	Immunol Cell Biol 83: 196-198 (2005)
	SRIK-NKL cells	NBP2-24729	IF/ICC	Leukemia Res 29:813-820 (2005)
	T cells	NBP2-24729	WB, FACS	PNAS 101:3029–3034 (2004) Clin Vaccine Immunol 17: 342-353 (2010)
TLR10	TLR10 transfected cells		WB	JI 174:2942-2950 (2005)
	B cells (Tonsillar B lymphocytes)		FACS, IF	JI 173:7426–7434 (2004) Infect Immunol 72:7202–7211 (2004) J Leukoc Biol 69:474–481 (2001)
	Human Cell Differentiation Molecules		FACS	J Immunol Methods 319:1-5 (2007)
	Neutrophils		FACS	J Leukoc Biol 69:474–481 (2001) JI 168:4701–4710 (2002)
	Non-Treg (CD4+/CD25-) cells		FACS	JI 179:1893-1900 (2007)
	Plasmacytoid DCs		FACS	Infect Immunol 72:7202–7211 (2004)
	Raji		FACS	JI 179:1893-1900 (2007)
	Treg (CD4+/CD25++) cells		FACS, WB	JI 179:1893-1900 (2007)

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TLR	CELL TYPE	NOTES	REFERENCES
TLR1	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Moderate Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharmacol 9:528–533 (2009)
	CD4+CD25+ Treg	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Intl Immunopharmacol 9:528–533 (2009)
	Eosinophils	High Expression	JI 171:3977-3982 (2003)
	Hep G2 cells	No or Weak Expression	S Biol Pharm Bull 28:886–892 (2005)
	Immature DCs	High Expression	JI 166:249–255 (2001)
	Macrophages	High Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Mast cells	High Expression	JI 164:5998–6004 (2000)
	Mature DCs	Faint Expression	JI 166:249–255 (2001)
	Monocytes	High Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001) J Exp Med 194:863-869 (2001)
	Myeloid DCs	High Expression	Eur JI 31:3388–3393 (2001) Nat Immunol 5:987–995 (2004)
	Neutrophils	High Expression	Med Mycol 99999:1-12 (2009) Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)
	NK cells	High Expression	JI 168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003) JI 164:5998–6004 (2000)
	Plasmacytoid DCs	Moderate Expression	Blood 102:956–963 (2003)
	Polymorphonuclear leukocytes	High Expression	JI 164:5998–6004 (2000)

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TLR	CELL TYPE	NOTES	REFERENCES
TLR2	A549	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	Basophils	High Expression	JI 168:4701–4710 (2002)
	CD4+CD25 − Teff	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharmacol 9:528–533 (2009)
	CD4+CD25+ Treg	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharmacol 9:528–533 (2009)
	Eosinophils	High Expression	JI 171:3977-3982 (2003)
	HepG2 cells	High Expression	S Biol Pharm Bull 28:886–892 (2005)
	Immature DCs	High Expression	JI 166:249–255 (2001)
	KG-1	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
	Langerhans cells	High Expression	J Invest Dermatol 122:95–102 (2004)
	Macrophages	High Expression	Blood 99:3427–3431 (2002) JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Mast cells	High Expression	JI 164:5998–6004 (2000)
	Monocytes	High Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001) J Exp Med 194:863-869 (2001)
	Myeloid DCs	High Expression	Eur JI 31:3388–3393 (2001) R Nat Immunol 5:987–995 (2004)
	Neutrophils	High Expression	Med Mycol 99999:1-12 (2009) Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)
	NK cells	High Expression	JI168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003) JI 164:5998–6004 (2000)
	Plasmacytoid DCs	No or Weak Expression	Blood 102:956–963 (2003)
	PMLs	Moderate Expression	JI 168:4531–4537 (2002)
TLR3	A549	Very Low Expression	Mol Immunol 44:2850–2859 (2007)
	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	Moderate Expression	Immunol 122:149–156 (2007) Inl Immunopharmacol 9:528–533 (2009) Int Immunol 19:193–201 (2007)

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TLR	CELL TYPE	NOTES	REFERENCES
TLR3	CD4+CD25+ Treg	No or Weak Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharmacol 9:528–533 (2009)
	H1299	Very Low Expression	Mol Immunol 44:2850–2859 (2007)
	HepG2 cells	High Expression	S Biol Pharm Bull 28:886–892 (2005)
	KG-1	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
	Macrophages	Moderate Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Mature DCs	High Expression	JI 166:249–255 (2001) JI 164:5998–6004 (2000)
	Monocytes	No or Weak Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001) J Exp Med 194(6):863-869 (2001)
	Myeloid DCs	High Expression	JI 31:3388–3393 (2001) R Nat Immunol 5:987–995 (2004)
	Neutrophils	Weak Expression	Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)
	NK cells	High Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003)
	Plasmacytoid Dendritic cells	No or Weak Expression	J Exp Med 194(6):863-869 (2001)
	Raji	Very Low Expression	Mol Immunol 44:2850–2859 (2007)
TLR4	A549	High Expression	Mol Immunol 44:2850–2859 (2007)
	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	Basophils	High Expression	JI 168:4701–4710 (2002)
	CD4+CD25 − Teff	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Intl Immunopharmacol 9:528–533 (2009)

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TLR	CELL TYPE	NOTES	REFERENCES
TLR4	CD4+CD25+ Treg	High expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharmacol 9:528–533 (2009)
	Endothelial cells	High expression	JI 164:5998–6004 (2000)
	Eosinophils	High expression	JI 171:3977-3982 (2003)
	H1299	High Expression	Mol Immunol 44:2850–2859 (2007)
	HepG2 cells	No or Weak Expression	S Biol Pharm Bull 28:886–892 (2005)
	Immature DCs	High Expression	JI 166:249–255 (2001)
	KG-1	High Expression	Mol Immunol 44:2850–2859 (2007)
	Langerhans cells	High Expression	J Invest Dermatol 122:95–102 (2004)
	Macrophages	High Expression	Blood 99:3427–3431 (2002) JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	MCF-7	Faint Expression	Mol Immunol 44:2850–2859 (2007)
	MDA-MB-231	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
	Monocytes	High Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001) J Exp Med 194:863-869 (2001)
	Myeloid DCs	Weak Expression	Eur J Immunol 31:3388–3393 (2001) R Nat Immunol 5:987–995 (2004)
	Neutrophils	High Expression	Med Mycol 99999:1-12 (2009) Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)
	NK cells	Moderate Expression	JI 168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003)
	Plasmacytoid DCs	No or Weak Expression	Blood 102:956–963 (2003)
	Raji	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
TLR5	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Very Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Inl Immunopharmacol 9:528–533 (2009)
	CD4+CD25+ Treg	High Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharmacol 9:528–533 (2009)
	HepG2 cells	No or Weak Expression	S Biol Pharm Bull 28:886–892 (2005)
	Immature DCs	High Expression	JI 166:249–255 (2001) JI 164:5998–6004 (2000)
	Macrophages	Moderate Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)

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TLR	CELL TYPE	NOTES	REFERENCES
TLR5	Monocytes	High Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001) J Exp Med 194:863-869 (2001)
	Myeloid DCs	Moderate Expression	Eur J Immunol 31:3388–3393 (2001) R Nat Immunol 5:987–995 (2004)
	Neutrophils	High Expression	Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)
	NK Cells	High Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003)
	Plasmacytoid DCs	No or Weak Expression	Blood 102:956–963 (2003)
TLR6	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	High Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharmacology 9:528–533 (2009)
	CD4+CD25+ Treg	Moderate Expression	J Exp Med 197:403–411 (2003) Int Immunopharmacol 9:528–533 (2009)
	Eosinophils	High Expression	JI 171:3977-3982 (2003)
	HepG2 cells	High Expression	S Biol Pharm Bull 28:886–892 (2005)
	Macrophages	Moderate Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Mast cells	High Expression	JI 164:5998–6004 (2000)
	Monocytes	High Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001) J Exp Med 194(6):863-869 (2001)
	Myeloid DCs	High Expression	JI 31:3388–3393 (2001) R Nat Immunol 5:987–995 (2004)
	Neutrophils	High Expression	Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)

Table VI. TLR mRNA Expression in Cells and Cell Lines

TLR	CELL TYPE	NOTES	REFERENCES
TLR6	NK cells	Low Expression	JI 168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003)
	Plasmacytoid DCs	High Expression	Blood 102:956–963 (2003)
TLR7	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	High Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharamcol 9:528–533 (2009)
	CD4+CD25+ Treg	High Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharamcol 9:528–533 (2009)
	Eosinophils	High Expression	JI 171:3977-3982 (2003)
	HepG2 cells	No or Weak Expression	S Biol Pharm Bull 28:886–892 (2005)
	Macrophages	Moderate Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Mast cells (Skin-derived)	Moderate Expression	JI 173:531–541 (2004)
	Monocytes	Moderate Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001)
	Monocytic DCs	Low Expression	JI 168:4531–4537 (2002) R Nat Immunol 5:987–995 (2004)
	Myeloid DCs	Low Expression	JI 31:3388–3393 (2001) J Exp Med 195:1507–1512 (2002)
	Neutrophils	Moderate Expression	Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003) Blood 102:2660–2669 (2003)
	Plasmacytoid DCs	High Expression	Blood 102:956–963 (2003) Eur J Immunol 31:3388–3393 (2001)
TLR8	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Moderate Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	Moderate Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharamcol 9:528–533 (2009)
	CD4+CD25+ Treg	High Expression	J Exp Med 197:403–411 (2003) Immunol 122:149–156 (2007) Int Immunopharamcol 9:528–533 (2009)
	HepG2 cells	No or Weak Expression	S Biol Pharm Bull 28:886–892 (2005)
	Macrophages	Moderate Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Monocytes	High Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001)

Table VI. TLR mRNA Expression in Cells and Cell Lines

TLR	CELL TYPE	NOTES	REFERENCES
TLR8	Monocytic DCs	High Expression	JI 168:4531–4537 (2002) R Nat Immunol 5:987–995 (2004)
	Myeloid DCs	High Expression	Eur JI 31:3388–3393 (2001) R Nat Immunol 5:987–995 (2004)
	Neutrophils	Moderate Expression	Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)
	NK cells	Low Expression	JI 168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003)
	Plasmacytoid DCs	No or Weak Expression	Blood 102:956–963 (2003)
TLR9	A549	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Very High Expression	Curr Opin Immunol17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Low Expression	Curr Opin Immunol17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	Moderate Expression	Immunol 122:149–156 (2007) Int Immunopharamcol 9:528–533 (2009) Int Immunol 19:193–201 (2007)
	CD4+CD25+ Treg	Moderate Expression	Immunol 122:149–156 (2007) Int Immunopharamcol9:528–533 (2009) Int Immunol 19:193–201 (2007)
	CD8+ (lymphoid) cDCs	Moderate Expression	Eur JI 33:827–833 (2003)
	CD8− (myeloid) cDCs	Moderate Expression	Eur JI 33:827–833 (2003) Immunol Cell Biol 83:92–95 (2005)
	Eosinophils	High Expression	JI 171:3977-3982 (2003)
	H1299	High Expression	Mol Immunol 44:2850–2859 (2007)
	HepG2 cells	High Expression	S Biol Pharm Bull 28:886–892 (2005)
	KG-1	High Expression	Mol Immunol 44:2850–2859 (2007)
	Langerhans cells	High Expression	J Invest Dermatol 122:95–102 (2004)
	Macrophages	Moderate Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Mast cells (Skin-derived)	Moderate Expression	JI 173:531–541 (2004)

Table VI. TLR mRNA Expression in Cells and Cell Lines

TLR	CELL TYPE	NOTES	REFERENCES
TLR9	MCF-7	Faint Expression	Mol Immunol 44:2850–2859 (2007)
	MDA-MB-231	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
	Monocytes	Weak Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001)
	Myeloid DCs	Weak Expression	Eur JI 31:3388–3393 (2001) R Nat Immunol 5:987–995 (2004)
	Neutrophils	High Expression	Blood 102:2660–2669 (2003)
	NK cells	High Expression	JI 168:4531-4537 (2002) J Infect Dis 188:1562–1570 (2003)
	Plasmacytoid DCs	High Expression	Blood 102:956–963 (2003) Eur J Immunol 31:3388–3393 (2001)
	Raji	Moderate Expression	Mol Immunol 44:2850–2859 (2007)
TLR10	B cells (Activated, Memory (CD19+CD27+) Tonsillar B cells)	Very High Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	B cells (Naïve/Resting, CD19+CD27- or Tonsillar B cells)	Low Expression	Curr Opin Immunol 17:230–236 (2005) Blood 102:956–963 (2003)
	CD4+CD25 − Teff	No or Weak Expression	Immunol 122:149–156 (2007) Int Immunopharamcol9:528–533 (2009) Int Immunol 19:193–201 (2007)
	CD4+CD25+ Treg	Moderate Expression	JI 179:1893–1900 (2007) Immunol 122:149–156 (2007) Int Immunopharamco1l9:528–533 (2009)
	Eosinophils	High Expression	JI 171:3977-3982 (2003)
	HepG2 cells	No or Weak Expression	S Biol Pharm Bull 28:886–892 (2005)
	Macrophages	Moderate Expression	JI 164:5998–6004 (2000) JI 168:4531-4537 (2002)
	Monocytes	Weak Expression	JI 168:4531-4537 (2002) JI 166:249–255 (2001)
	Myeloid DCs	Moderate Expression	Eur J Immunol 31:3388–3393 (2001)
	Neutrophils	Moderate Expression	Blood 102:2660–2669 (2003) JI 171:3977-3982 (2003)
	Plasmacytoid DCs	Moderate Expression	JI 168:4531–4537 (2002) Blood 102:956–963 (2003)

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Table VII. TLR Protein Expression in Tissues

TLR	TISSUE	NOVUS Abs	APPLICATION	REFERENCES
TLR1	Female reproductive system		IHC	Hum Reprod 20:1372–1378 (2005)
	Male reproductive tract	NB100-56563	WB	Biol Reprod 76:958-964 (2007)
TLR2	Active pouchitis		IHC	Surg Today 36:287–290 (2006)
	Aortic tissue, aortic valve interstitial cells	NB100-56722, NBP2-24909	IHC, WB, FACS	J Am College Cardiol 53:491-500 (2009) Circulation 109:2801-2806 (2004)
	Colonic epithelium		IHC	Immunol 115:565–574 (2005)
	Female reproductive system		IHC	Hum Reprod 20:1372–1378 (2005)
	Ileum		IHC	J Histochem Cytochem 56(3):267-274 (2008)
	Keratinocytes	NB100-56722	IF/ICC	J Invest Dermatol 121: 1389-1396 (2003)
	Large intestinal epithelium		IHC, WB	JI 164:966–972 (2000)
	Male reproductive system	NB100-56720	WB	Endocrinol 14:4408-4412 (2008) I Biol Reprod 76:958-964 (2007)
	Ovary		IHC-P	Cancer Immunol Immunother 58(9):1375-1385 (2009)
	Pulmonary epithelium		IHC	Histochem Cell Biol 119:103–108 (2003)
	Rectum		IHC	J Histochem Cytochem 56(3):267-274 (2008)
	Renal epithelium		IF	JI 178:6252–6258 (2007)
	Small intestinal epithelium		IHC, WB	JI 164:966–972 (2000)
TLR3	Bone marrow	NBP2-24899	FACS	Immunobiol 112:1832-1843 (2008)
	Cervical epithelium		Functional Assay	Biol Reprod 74:824–831 (2006)
	Colonic epithelium	NBP2-24875	IHC-P	Immunol 115:565–574 (2005)
	Female reproductive system		IHC	Hum Reprod 20:1372–1378 (2005)
	Ileal mucosa		IHC	Surg Today 36:287–290 (2006)
	Kidney	NB100-56571, NBP2-24875	IHC-P, WB	Am Soc Nephrol 16:1326-1338 (2005) Clinical Can Res 13: 5703-5709 (2007)
	Large intestinal epithelium		IHC, WB	Infect Immun 68:7010–7017 (2000)
	Ovary		IHC-P	Immunol Immunother 58(9):1375-1385 (2009)
	Pulmonary epithelium		FACS, WB	JI 176:1733–40 (2006)
	Small intestinal epithelium		IHC	Infect Immun 68:7010–7017 (2000)
	Synovial membrane cells	NBP2-24899	FACS	JI 181:8002-8009 (2008)

Table VII. TLR Protein Expression in Tissues

TLR	TISSUE	NOVUS Abs	APPLICATION	REFERENCES
TLR3	Tonsils	NBP2-24902	FACS	Respir Res 7:36 (2006)
TLR4	Active pouchitis		IHC	Surg Today 36:287–290 (2006)
	Aortic valve interstitial cells	NB100-56062	WB, FACS	Journal Am College of Cardiol 53:491-500 (2009)
	Bone marrow	NB100-55951, NB100-56560	FACS, FA	Immunobiol 112:1832-1843 (2008), JI 180:1080-1087 (2008)
	Brain (microglia cells)	NB100-56560	FACS	JI 174:6467-6476 (2005)
	Colonic epithelium		IHC, WB	Immunol 115:565–574 (2005)
	Female reproductive system		IHC	Hum Reprod 20:1372–1378 (2005)
	Gastric epithelium		IHC	Clin Exp Immunol 136:521–526 (2004)
	Ileum		IHC	Histochem Cytochem 56:267-74 (2008)
	Keratinocytes	NB100-56723	IF/ICC	J Invest Dermatol 121: 1389-1396 (2003)
	Large intestinal epithelium		IHC, WB	Infect Immun 68:7010–7017 (2000)
	Lymph node	NBP2-24821	IHC-Fr	JI 179:4444-4450 (2007)
	Ovary		IHC-P	Cancer Immunol Immunother 58(9):1375-1385 (2009)
	Pulmonary epithelium		IHC, WB, IF	Am J Respir Cell Mol Biol 30:777–83 (2004)
	Rectum		IHC	J Histochem Cytochem 56(3):267-74 (2008)
	Renal epithelium		IHC	Infect Immun 72:3179–86 (2004)
	Respiratory epithelium		WB, FACS	Guillot, L et al JBC 279:2712–2718 (2004)
	Small intestinal epithelium		IHC	Infect Immun 68:7010–7017 (2000)
	Ureter and Bladder epithelium		IHC	Infect Immun 72:3179–86 (2004)
TLR5	Bone marrow	NBP2-24787	WB	JI 180:1382-1389 (2008)
	Female reproductive system		IHC	Hum Reprod 20:1372–1378 (2005)
	Gastric epithelium		IHC	Clin Exp Immunol 136:521–526 (2004)
	Ileal mucosa		IHC	Surg Today 36:287–290 (2006)
	Intestine	NB200-571	IHC-P	JI 174:6137-6143 (2005)
	Keratinocyte	NBP2-24787	IHC-Fr	JI 174:6137-6143 (2005)
	Male reproductive system	NBP2-24784, NBP2-24827	WB, FACS	Endocrinology 14:4408-4412 (2008) Biol Reprod 76:958-964 (2007)
	Ovary		IHC-P	Cancer Immunol Immunother 58(9):1375-1385 (2009)
	Psoriasis	NBP2-24787	IHC-Fr	JI 174:6137-6143 (2005)
	Pulmonary epithelium		IHC, WB, IF	Am J Respir Cell Mol Biol 30:777–83 (2004)
	Skin	NBP2-24787	IHC-Fr	JI 174:6137-6143 (2005)
	Temporal arteries	NBP2-24787	WB	Circulation Res 104: 488-495 (2009)
	Tonsils	NBP1-97728	FACS	Respir Res 7:36 (2006)
TLR6	Female reproductive system		IHC	Hum Reprod 20:1372–1378 (2005)
	Male reproductive system		WB	Endocrinol 14:4408-4412 (2008) Biol Reprod 76:958-964 (2007)
TLR7	Bone marrow	NB100-56588	FACS	Immunobiol 112:1832-1843 (2008)

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Table VII. TLR Protein Expression in Tissues

TLR	TISSUE	NOVUS Abs	APPLICATION	REFERENCES
TLR7	DMD muscle fibers	NB100-56588	IHC-Fr	Neurol 65:826-834 (2005)
	Kidney	NBP2-24906	IHC-Fr	J Am Soc Nephrol 17:141-149 (2006)
	Lung epithelial	NBP2-24906	IF/ICC	Am J Respir Crit Care Med doi: 10.1164/rccm.200908-125OC (2010)
	Male reproductive tract	NBP2-24906	WB	Biol Reprod 76:958-964 (2007)
	Pulmonary epithelium			Am J Respir Cell Mol Biol 30:777–83 (2004)
TLR8	Bone marrow	NBP2-24972	FACS	Immunobiol 112:1832-1843 (2008)
	Pulmonary epithelium		IHC	Respir Res 6:1 (2005)
	Synovial membrane cells	NBP2-24972	FACS	JI 181:8002-8009 (2008)
TLR9	Bone marrow	NBP2-24908	FACS	Immunobiol 112:1832-1843 (2008)
	Cervical epithelium		Functional Assay	Biol Reprod 74:824–831 (2006)
	Colonic epithelium	NBP2-24908	WB, FACS	Clin Exp Immunol 141:298–306 (2005) Int Immunol 8: 961-970 (2008)
	Gastric epithelium		IHC	Clin Exp Immunol 136:521–526 (2004)
	Kidney	NBP2-24729	IHC-Fr, IHC-P	Nephrol Dial Transplant 21:3062-3073 (2006) Nephrol Dial Transplant doi: 10.1093/ndt/gfq058 (2010)
	Lung	NBP2-24729	IHC-P	J Immunol 184: 3145-3156 (2010)
	Male reproductive tract	NBP2-24729	WB	Biol Reprod 76:958-964 (2007)
	Pulmonary epithelium		FACS	JI 173:1219–1223 (2004)
	Small intestinal epithelium		FACS, IF	Infect Immun 75:2572–2579 (2007)
	Synovial membrane cells	NBP2-24908	FACS	JI 181:8002-8009 (2008)
	Tonsils	NBP2-24908	FACS	Respir Res 7:36 (2006)
TLR10	Male reproductive tract		WB	Biol Reprod 76:958-964 (2007)
	Lymph node		FACS	JI 179:1893-1900 (2007)
TLR11	Brain		IF	J Neuroinflammation 5:53 (2008)
	Male reproductive tract		WB	Biol Reprod 76:958-964 (2007)
TLR12	Brain		IF	J Neuroinflammation 5:53 (2008)
	Male reproductive tract	NBP2-24833	WB	Biol Reprod 76:958-964 (2007)
TLR13	Brain		IF	J Neuroinflammation 5:53 (2008)

Table VIII. TLR mRNA Expression in Tissues

TLR	RNA	Reference
TLR1	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Epidermis	Immunol 114:531–541 (2005)
	Fallopian tubes, endometrium and cervix	Infect Immun 72:5799–5806 (2004)
	Large intestinal epithelium	Gastroenterol 126:1054–1070 (2004)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
	Ovary	PNAS 95: 588-593 (1998)
	Primary keratinocytes	Immunol 114:531–541 (2005)
	Renal epithelium	JI 169:2026–2033 (2002)
	Small intestinal epithelium	PNAS 95: 588-593 (1998)
	Spleen	PNAS 95: 588-593 (1998)
	Ubiquitous	PNAS 95: 588-593 (1998)
TLR2	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Epidermis	Immunol 114:531–541 (2005)
	Fallopian tubes, endometrium and cervix	Infect Immun 72:5799–5806 (2004)
	Kidney (bladder, renal, and ureter epithelium)	Immunol 169:2026–2033 (2002)
	Large intestinal epithelium	JI 167:1609–1616 (2001)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
	Primary keratinocytes	Immunol 114:531–541 (2005)
	Small intestinal epithelium	Gastroenterol Nutr 45:187–193 (2007)
	Spleen, lung, heart, brain, muscle	Blood 91:4020–4027 (1998)
TLR3	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Epidermis	Immunol 114:531–541 (2005)
	Fallopian tubes, endometrium and cervix	Infect Immun 72:5799–5806 (2004)
	Large intestinal epithelium	Gastroenterol 126:1054–1070 (2004)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
	Primary keratinocytes	Immunol 114:531–541 (2005)
	Renal epithelium	JI 169:2026–2033 (2002)
	Spleen, lung, heart, brain, muscle	Blood 91:4020–4027 (1998)
	Ureter and Bladder epithelium	Cell Microbiol 3:153–158 (2001)
TLR4	Fallopian tubes, endometrium, cervix	Infect Immun 72:5799–5806 (2004)
	Heart	Clin Invest 104:271–280 (1999)
	Kidney (bladder, renal, and ureter epithelium)	JI 169:2026–2033 (2002)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
	Large intestinal epithelium	JI 177:3273–3282 (2006)
	Placenta	PNAS USA 95:588–593 (1998)
	Small intestinal epithelium	JI 177:3273–3282 (2006)

Table VIII. TLR mRNA Expression in Tissues

TLR	RNA	Reference
TLR5	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Epidermis	Immunol 114:531–541 (2005)
	Kidney (bladder, renal, and ureter epithelium)	Cell Microbiol 3:153–158 (2001)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
	Ovary	Blood 91:4020–4027 (1998)
	Primary keratinocytes	Immunol 114:531–541 (2005)
	Prostate, testes	Blood 91:4020–4027 (1998)
TLR6	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Kidney (renal epithelium)	Immunol 169:2026–2033 (2002)
	Large intestinal epithelium	Gastroenterol 126:1054–1070 (2004)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
	Primary keratinocytes	Immunol 114:531–541 (2005)
TLR7	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Large intestinal epithelium	Gastroenterol 126:1054–1070 (2004)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
	Small intestinal epithelium	Eur Cytokine Netw 11:362–371 (2000)
TLR8	Large intestinal epithelium	Gastroenterol 126:1054–1070 (2004)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
TLR9	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Large intestinal epithelium	Gastroenterol 126:1054–1070 (2004)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
TLR10	Cervical epithelium	Biol Reprod 74:824–831 (2006)
	Epidermis	Immunol 114:531–541 (2005)
	Primary keratinocytes	Immunol 114:531–541 (2005)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)
TLR11	Kidney (bladder, renal, and ureter epithelium)	Science 303:1522–1526 (2004)
	Lung (pulmonary epithelium)	Cell Mol Biol 30:777–783 (2004)

An overview of TLR expression during normal B cell differentiation and comparison with B cell malignancies is shown in Figure 14. The conversion of TLR (1,2,5-7, 9-10)^{low} in naïve B cells to TLR (1,2,5-7, 9-10)^{pos} in germinal center B cells is an example of the plasticity of TLR expression during the cell differentiation/maturation process. The conversion of TLR2 from low (naïve B cells) to positive (germinal center B cells) and back to low (memory B cells) is a good example of how the level of TLR expression fluctuates during normal biological processes.

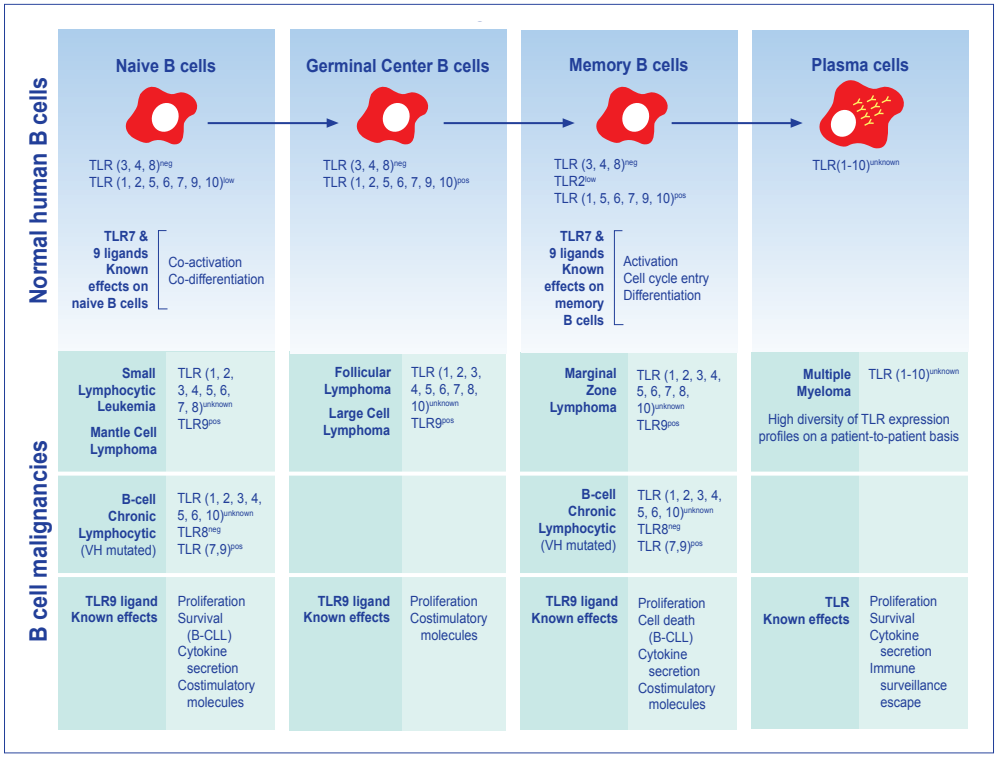

Figure 14. TLR expression and function along the human B cell differentiation process. *This schematic was developed from information compiled from a number of publications as detailed in the source: Chiron et al (2008).*

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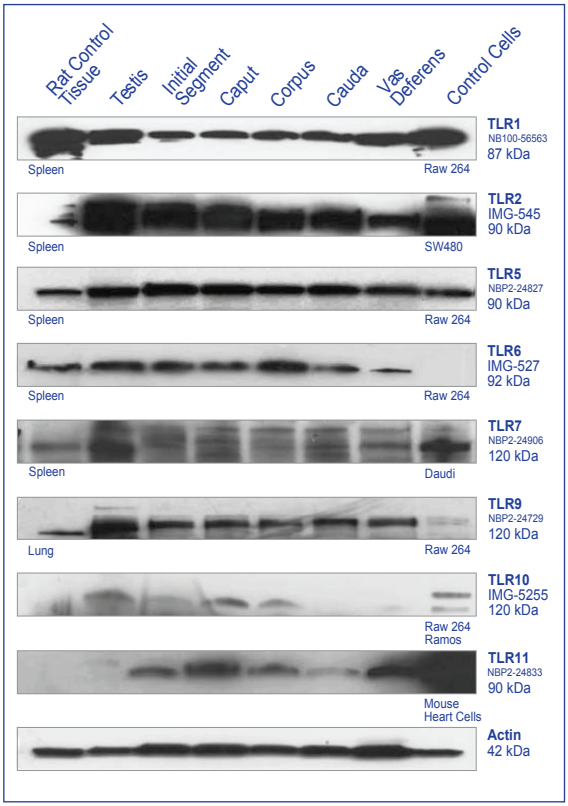
The denotation “unknown” in Figure 14 for so many of the TLRs in malignancies and normal plasma cells underscores the nascent understanding of TLR expression patterns. Additionally, the high diversity of TLR expression profiles among multiple myeloma patients underscores the challenges in making generalizations about TLR expression patterns.



As shown in Tables V-VIII, TLRs are also expressed in various cell and tissue types outside of the immune system. As an example, Figure 15 illustrates the widespread and variable expression of multiple TLRs in the male reproductive system.

Readers are encouraged to utilize the information in Tables V-VIII as a survey or starting point for learning what is known about TLR expression. However, knowledge about TLR expression patterns is relatively limited, and it is important to stay current. TLR review articles are good tools to help supplement the information in the Tables and gain more in-depth information.

Figure 15. Western blot analysis of TLRs in male rat reproductive organs. *Cytoplasmic tissue extracts (100 ug/lane) were probed for TLR expression. Blots were stripped and reprobed with actin. Representative results are shown from 3-5 rats and control cell lines.*

The Western Blot Loading Control Kit (NBP2-25090) containing a Beta Actin pAb is recommended an as internal loading control.

Cytoplasmic extraction method: Frozen tissues were homogenized on ice in three volumes of 10 mM Tris-HCL, 1.5 mM MgCl2, and 10 mM KCL containing 1 mM dithiothreitol (DTT), 1 mM Na3VO4 (phosphatase inhibitor) and protease inhibitor cocktail (P8340; Sigma-Aldrich) using a glass dounce homogenizer. The homogenate was centrifuged for 5 min at 5000 x g at 4 deg C, and the supernatant containing the cytoplasmic extract was stored at -80 deg C. Source: Palladino et al, 2007.

Researchers should be aware that there are discordant TLR expression results within the literature. This surfaces and is particularly evident when information from many sources is compiled together. For example, there are inconsistencies where TLRs are and are not expressed. This is due to a number of factors including:

1. **Model systems**
   a. There are various elusive differences between seemingly similar model systems. Hence, discordant results can be obtained even when using the same cell or tissue type. Additionally, *in vitro* and *in vivo* systems may yield different results.
   b. There can be interspecies differences in TLR expression patterns and in innate and adaptive immune responses, including responses to different TLR agonists (reviewed in Sabroe et al, 2008).

2. **Diseases**
   a. TLR expression can vary during the course of a disease and between individuals with the same disease. Thus, it can be hard to generalize about TLR expression in a particular disease
   b. Extrapolating data from different animal species to humans calls for some caution. This is because different species are susceptible to different pathogens, and both disease type and TLR expression pattern induced by a specific pathogen can be model and species dependent (reviewed in Sabroe et al, 2008).

3. **Donor variability**
   The expression level of TLRs varies widely even between normal donors. Therefore, it can be hard to generalize about TLR expression in a particular cell type or differentiation stage. There are many examples and commentary on donor-donor variability in TLR expression levels embedded within the published literature. Although it can be challenging to ferret out, this information can be quite useful in interpreting one's own research results. Hence, researchers are strongly encouraged to assess the published literature regarding donor-donor variability relevant to the TLRs and model system they are studying.

The following publications provide examples:

i. Jack et al (2005: Fig 8): Donor-donor variability is shown in flow cytometry assays of TLRs 3 and 4 in human microglia.
ii. Visintin et al (2001: page 253): Donor-donor variability in shown in flow cytometry assays of TLRs 1 and 4 in human monocytes. Monocytes from two donors did not express TLR1, whereas the other 13 donors expressed 400-5400 molecules/cell.
iii. Birmachu et al (2007:pages 1-2): Donor-donor variability is put forth as a potential explanation for differential donor expression of TLRs 4, 5, 7, 8, 9 and 10 in flow cytometry assays of human plasmacytoid dendritic cells.

iv. Wu et al (2007, results section): Donor-donor variability of mRNA expression of TLRs 1-9 is observed among human corneal epithelial cells.

4. Cell line considerations

a. Human and animal continuous cell lines are simple and representative model systems for functional studies and screening systems. They have become invaluable tools over time in both basic and translational biomedical research since 1951 when HeLa, the first cell line derived from human tissue, was isolated. For example, cells are often the starting point for basic research and for identifying new diagnositics and therapeutic targets.

However, there are a number of caveats in using cell lines that should be recognized. Passage number, culture conditions, misidentified cell lines, plasticity in cell line characteristics, and tissue/cell line storage conditions are all factors that can impact expected results, including TLR expression. These factors exist both within and between labs and can result in detection of a particular TLR in a given cell type under certain conditions but not others.

For example, the body of literature can give the impression that a given TLR is expressed in a particular cell type, and researchers will expect to find it there. If they don't, they may think that the reagents or experiment is not working. However, in reality additional investigation may need to be done to determine if a given TLR is actually expressed at detectable levels in their particular material.

It is the responsibility of the scientific community and each researcher to perform due diligence to ensure the integrity of the cell cultures they use. There are a number of excellent reviews addressing the caveats of using cell lines and providing useful tips for cell line use. These reviews include:

1. The cost of using unathenticated, over-passaged cell lines: how much more data do we need? (Hughes et al, 2007).
2. Curbing rampant cross-contamination and misidentication of cell lines (Nardone, 2008).
3. Persistent use of "false" cell lines (Lacroix, 2008).
4. Eradication of cross-contaminated cell lines: A call for action (Nardone, 2007).

5. Cell activation, maturation or differentiation state

There is plasticity in TLR expression during different states.

6. Phenomena measured

Protein and mRNA analysis may give different results.

7. Technique

a. Western blot, intracellular flow cytometry, cell surface flow cytometry, immunohistochemistry, immunoprecipitation, and mRNA analyses may give different results.

b. Researchers are particularly cautioned about the use of RT-qPCR, a gold standard mRNA expression technique, for extrapolation of protein expression levels. Interpretation of RT-qPCR results can be complex and vulnerable to artifactual conclusions (reviewed in VanGuilder et al, 2008). Additionally, mRNA levels do not always correlate with detectable protein expression level. Thus, when using antibodies probing for protein expression based on RT-qPCR results, it is critical to use a positive control known to have detectable protein expression levels.

8. Experimental reagents

There is variability in the specificity, affinity, epitopes, protein conformations recognized (native vs denatured), and applications of antibodies. Thus, a TLR or any protein for that matter may be identified by one antibody but not another. Researchers may need to screen a number of antibodies against a given specificity to identify those suitable for their particular model system and applications. Additionally, it can be good practice to use more than one antibody against a given protein to help validate results.

9. Data interpretation

Interpretation of data can be subjective. For example, terms like low, high and moderate expression are widely used, yet subjective.

Hence, the complexity and variability of TLR expression, along with experimental variation can make it hard to compare data across publications. Thus, it can be challenging to come to decisive conclusions about where TLRs are and are not expressed. However, the increasing focus on TLR research as well as the availablity of TLR antibodies and other related research tools is facilitating rapid gains in our knowledge of TLR expression and regulation.

Novus has developed the largest and most well-characterized portfolio of TLR antibodies and related reagent through its TLRSystem™ portfolio. These reagents are being used worldwide to elucidate TLR protein expression and regulation in a wide variety of model systems. Results using Novus' reagents have been widely published by reseachers worldwide in scientific journals representating diverse areas of biology, including human and veterinary medicine. There are a number of publication examples presented throughout this handbook. Additional publication examples using Novus' TLR antibodies are denoted in Table V and in the Appendix. Researchers are encouraged to consult our online data sheets (www.novusbio.com) for comprehensive publication lists. Please contact us to include your publication, citing any Novus product on our next web update.

TLRs: Structure, Ligands & Expression

SUBCELLULAR LOCALIZATION

TLRs are differentially distributed within the cell and their subcellular localization corresponds to their ligand type and role in pathogen recognition (reviewed in Kawai and Akira, 2009). As shown in Figure 16, TLRs recognizing lipid and protein PAMPs are primarily expressed on the plasma membrane (TLRs 1, 2, 4-6, 11-13). In contrast, TLRs detecting bacterial and viral nucleic acid PAMPs are expressed in intracellular comparments such as the endosome, lysosome or the endoplasmic reticulum (TLRs 3, 7, 8, and 9).

The PAMPs recognized by intracellular TLRs (TLR 3, 7, 8 and 9) are internalized and transported to the endosome for recognition before TLR signaling is initiated. Thus, it is fitting that TLRs 3 and 7-9 are intracellular, since their respective PAMPs are all internalized prior to TLR recognition.

The localization of TLRs is not static and may vary between cell types or physiological conditions (reviewed in Barton and Kagan, 2009; and Kawai and Akira, 2009). For example, TLRs 3 and 7-9 are expressed on the ER in resting cells and translocate to endosomal or endolysomal compartments in response to PAMP-mediated stimulation. The features of the compartment may differ depending on both the composition of the internalized PAMP cargo and the cell type. However, the exact nature or type of compartment where intracellular TLRs meet internalized PAMPs is still poorly defined (reviewed in Barton and Kagan, 2009).

Additionally, TLRs such as 2 and 4 which are normally present at the cell surface can also be internalized and enter the endocytic pathway following activation. Likewise, there is evidence to suggest that TLR9, typically found intracellularly, can also be expressed at the cell surface. See the 'TLRs in Stem Cells' chapter for an example by Thomchuck et al (2008) where TLR4 localized from the cell surface to endosomal-like compartments following LPS treatment.

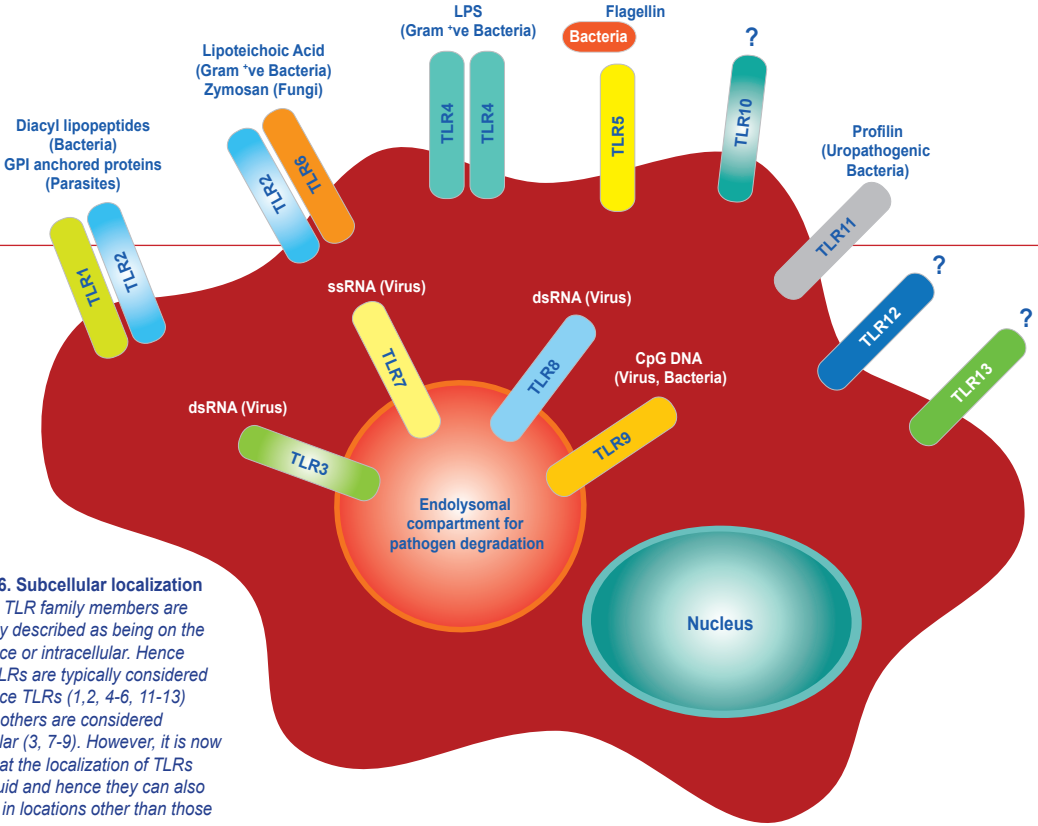


Figure 16. Subcellular localization of TLRs. *TLR family members are classically described as being on the cell surface or intracellular. Hence certain TLRs are typically considered cell surface TLRs (1,2, 4-6, 11-13) whereas others are considered intracellular (3, 7-9). However, it is now known that the localization of TLRs can be fluid and hence they can also be found in locations other than those depicted.*

As Barton and Kagon (2009) note, the mechanisms that recruit TLRs to specific cellular compartments are just now being described and many questions remain including:

1. How does the localization of TLR signaling proteins relate to the sites of adaptor-complex assembly?
2. What roles do TLR adaptors play in regulating TLR subcellular localization?
3. Can cell type specific differences in the subcellular positioning of TLRs and adaptor-complexes explain cell type specific responses to TLR ligands?
4. How do PAMPs influence the activity, expression and localization of TLRs in different cell types? Are there common underlying PAMP strategies that apply to all cell types?

The answers to these and other related questions will play an important role in shaping the direction of TLR research.

TLRs as Immune Sentinels

When TLRs were first discovered in 1994, it was assumed that they were developmental proteins akin to drosophila Toll. However, after studies showed that TLR knockout mice had normal embryonic development and that defects were limited to immunity, the focus of the TLR field shifted to immune regulation.

The immune function of mammalian TLR was first shown in 1998, and over the next 10 years it became apparent that TLRs were essential for innate immunity. TLRs are frequently referred to as 'immune sentinels' as homage to their critical role in innate immune defense. The word 'sentinel' is defined as one who keeps guard, gives a warning and indicates danger. TLRs are sentinels because they watch out for PAMPs, evoke immediate immune responses upon PAMP detection, and stimulate long-term pathogen-specific adaptive immunity. As immune sentinels, TLRs are link to have a touch to all of immunity as is shown in Figure 17.

There are a number of excellent reviews which provide overviews of the function of TLRs in immunity, many of which incorporate insightful historical or evolutionary perspectives. The rest of this chapter will focus on information distilled from some of these reviews, including Beutler (2008), Kawai and Akira (2009), Kumar (2009), Medzhitov (2009), Miller (2008), Mogenson (2009), Montero and de Andres (2008), O'Neil (2008), and Takeuchi and Akira (2009). Most of this information is common to all of these reviews unless otherwise noted.

The role of TLRs in immunity can be divided into three distinct but interconnected functions:

1. Sensor: detects the presence and type of pathogen.
2. Effector: evokes an immediate anti-pathogenic response.
3. Regulator: stimulates adaptive responses germane to pathogen type.

The three primary TLR functions are coordinated by specialized antigen presenting cells (APCs) such as dendritic cells, and T and B lymphocytes. The APCs integrate TLR signals into specific immune responses.

TLRs as Sensors. As sensors, TLRs identify and classify opportunistic pathogens that breach epithelial barriers and enter tissue sites. This pathogen sensory function is carried out on TLR expressing host cells including macrophages, mast cells, immature dendritic cells (DCs), and both endothelial and epithelial cells. These TLR expressing cells continually screen both the external (skin, mucosal surfaces) and internal environments for the presence of pathogens, i.e., pathogenic nonself. TLRs are pivotal deciders of the fate of the nonself. TLRs make a determination if the nonself can continue to be

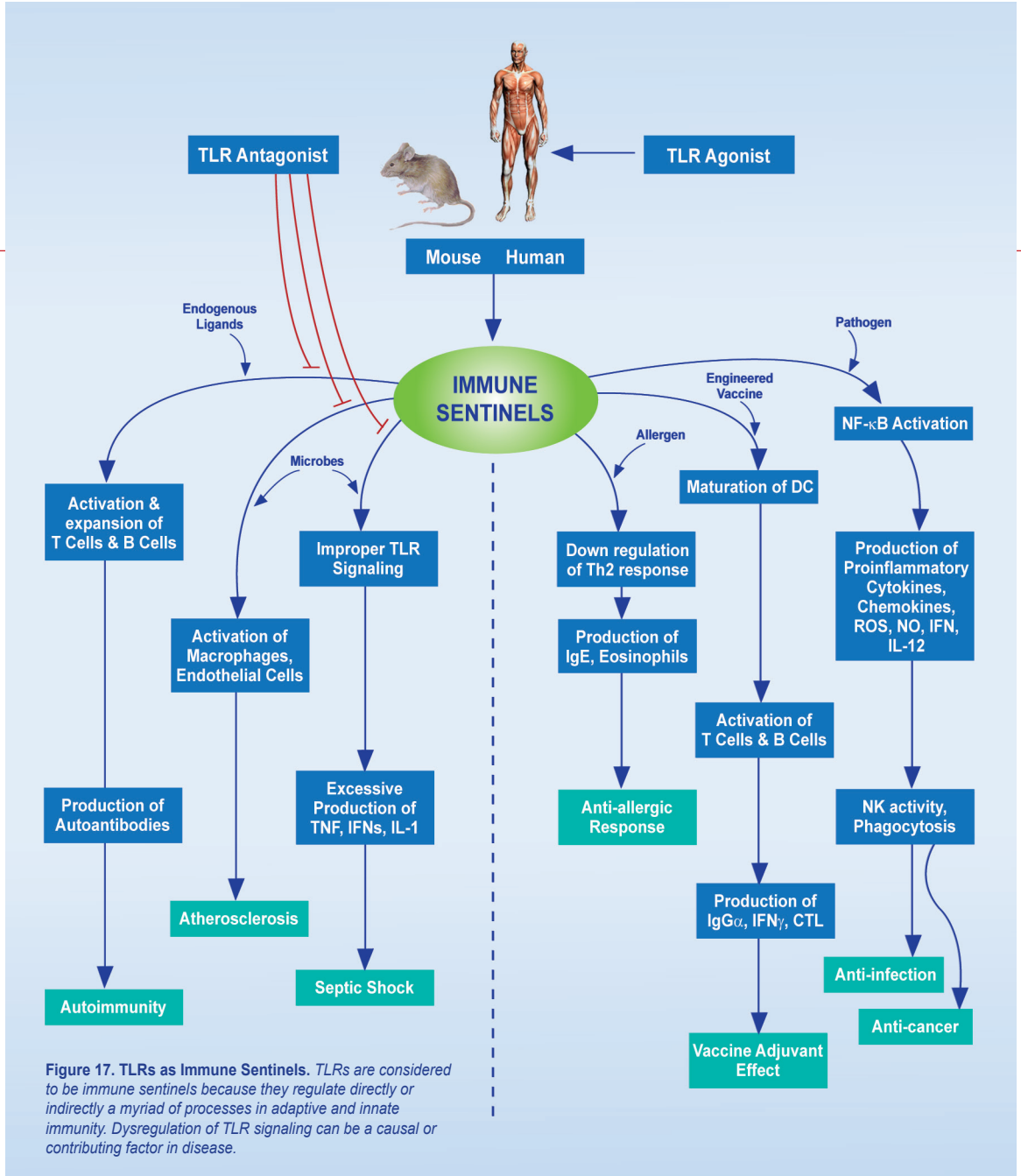


Figure 17. TLRs as Immune Sentinels. *TLRs are considered to be immune sentinels because they regulate directly or indirectly a myriad of processes in adaptive and innate immunity. Dysregulation of TLR signaling can be a causal or contributing factor in disease.*

TLRs as Immune Sentinels

present or if it should be eliminated. To respond or not to respond, that is an important question for TLRs! This is because TLRs have the dual ability to ignore commensal nonself nonpathogenic microorganisms that colonize the host, and at the same time recognize and respond to infectious pathogens.

When a TLR identifies a pathogen that it deems requires a response, watch out! The LRR region of the TLR binds to the pathogen's PAMP resulting in TLR activation and initiation of TLR effector functions. During this process, the cytoplasmic TIR domains of activated TLRs trigger downstream signaling pathways leading to the rapid production of effector cytokines, chemokines, and interferons. Figure 18 shows an overview of this process, from pathogen recognition to inflammatory response.

Activated TLRs can homodimerize or heterodimerize with other TLRs or non-TLRs, and gain their signaling specificity by interacting with different combinations of receptors and adaptor molecules. This process drives PAMP appropriate responses. For example, LPS activates DCs through TLR4 and associated adaptor molecules to produce abundant IL-12(p70), but little IL-10, and stimulates Th1 and T cytotoxic 2 (Tc2) responses. In contrast, Pam3CSK4 activates DCs through TLR2 and associated adaptor molecules to produce abundant IL-10, but little IL-12(p70), and stimulates Th2 and Tc2 responses.

In addition to cytokines, chemokines and interferons, TLR activation and downstream signaling also triggers the expression of anti-microbial host-defense peptides (HDPs) which kill a wide range of pathogens. HDPs are components of the innate immune system which have a central function in the host defense properties of skin and other epithelia, granulocytic leukocytes and mucosal surfaces. TLR activation leads to the production of the HDPs human β-defensins 2 and 3 (hBD2 and hBD3) in a variety of epithelial cell types (hBD1 expression is constitutive) (reviewed in Miller, 2008).

For example, activation of TLR5 by flagellin on human keratinocytes has been shown to result in the production of hBD2 and hBD3, as well as TNFα and IL-8. TLR activation is thought to contribute to both increased HDP levels and cutaneous inflammation found in the psoriatic lesions of psoriasis patients. However, the signaling pathways between TLR sensory functions and HDP induction remain to be fully elucidated.

TLRs as Effectors. As effectors, TLRs induce localized inflammatory responses whereby activated phagocytic cells travel to the site of infection, internalize, and then kill pathogens. The TLR effector function provides rapid protection of the host from the pathogen, and is executed through TLR signaling pathways that lead to NF-κB activation. NF-κB is a master switch for inflammation, and activated NF-κB translocates to the nucleus where it induces the expression of anti-inflammatory and

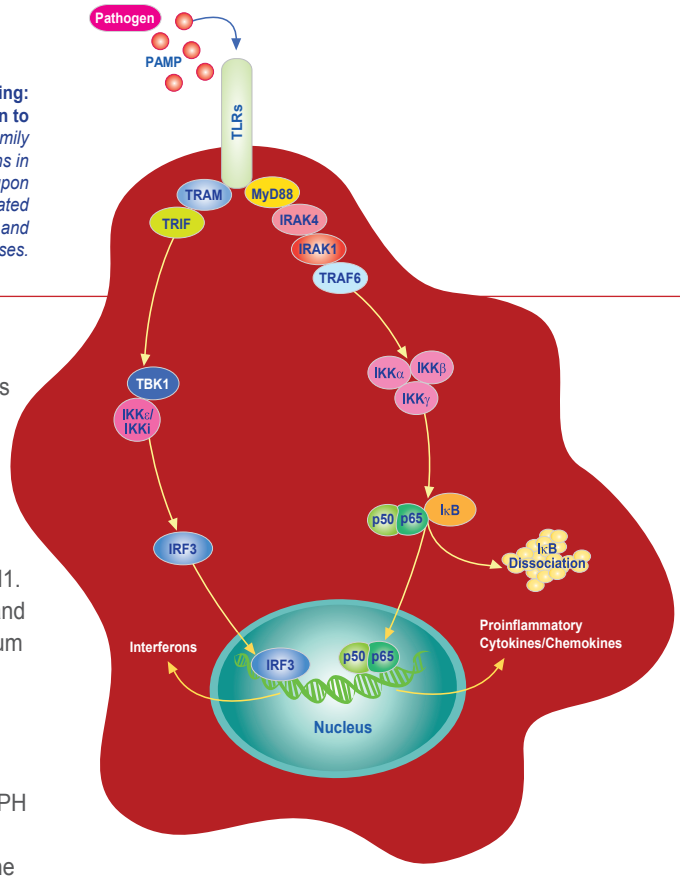


Figure 18. TLR Signaling: From pathogen recognition to inflammatory response. *TLR family members have basic functions in common. They become activated upon pathogen detection, and once activated use signaling pathways to evoke and regulate immune responses.*

antimicrobial genes (Fig 18). These genes mediate the production of HDPs, inflammatory cytokines, interferons and TNF, which in turn activate surrounding cells to produce chemokines as well as chemotactic and cell surface adhesion molecules.

For example, activation of TLRs on the endothelium leads to the surface expression of E-selectin and ICAM1. E-selectin and ICAM1 are crucial for leukocyte rolling and adhesion. The adhesion of leukocytes to the endothelium is a mechanism for recruiting leukocytes to infected tissues from the blood (reviewed in Mogensen, 2009). The leukocytes then can kill the pathogen by several processes. These processes include generation of inducible nitric oxide synthetase, activation of the NADPH oxidase complex and other nitrogen intermediates, generation of reactive oxygen species, stimulation of the neutrophil oxidative burst, or by perforin-mediated killing.

TLRs as Regulators. TLRs play a key role in regulating both the type and magnitude of adaptive immune responses (reviewed in Mogensen, 2009). They act as potent stimulators of DCs which bridge innate and adaptive immune responses. Specifically, TLR ligands activate TLR signaling to induce maturation of immature DCs into potent APCs which in turn induce co-stimulatory molecules such as CD40, CD80 and CD86. CD80 and CD86 ligands bind to CD28 on T cells and act as the second signal for T-cell activation. Understanding DC subsets through TLR "phenotyping" is an entirely new and open area of critical research. Furthermore, TLR-induced cytokines, including IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β and IL-12, direct T-cell differentiation toward either CD4+ T helper type 1 (Th1) cells or CD8+ cytotoxic T lymphocytes (CTLs).

The understanding of the roles that TLRs play as immune sentinels will continue to be reshaped as new information emerges. It is now well accepted that TLRs are inextricably involved in a myriad of biological processes in both health and disease. It is also likely that the sensor, effector, and regulator functions of TLRs are more far reaching than heretofore recognized.

TLRs as Bridges Between Innate & Adaptive Immunity

Historically, innate and adaptive immunity have been thought of as separate but complementary immune defense mechanisms against pathogens (reviewed in Akira, 2009 and Medzhitov, 2009). Both mechanisms have been extensively characterized independently of one another. The classical dogma states that innate immunity is a "non-specific" system which provides first-line defense against pathogens and participates in rapid, nonselective host protection without any resulting immunological memory. In contrast, the adaptive immune system is composed of T cells and B cells that specifically recognize foreign antigens and generate immunological memory.

The interest in innate immunity perhaps waned as advances were made in adaptive immunity which became a research focus during the 20th century. However, the 21st century is experiencing a renaissance of interest in innate immunity as new insights and advances have brought scientists from diverse research fields together. The realization that TLRs specifically recognize PAMPs and function as bridges to adaptive immunity has been key to reshaping the classical dogma of immunity (reviewed in Akira, 2009 and Medzhitov, 2009). The emerging dogma indicates that innate immunity bridges to adaptive immunity, is intertwined with adaptive immunity, and is essential for immunological memory.

Figure 19a, shows an overview of TLRs within the context of the emerging dogma of immune response which puts forth that innate and adaptive immunity is an integrated process. It is thought that TLRs may be the initiators of the entire host defense program, be it innate or adaptive immunity (O'Neil, 2004). This is because TLRs activate signaling pathways that culminate in the induction of the key immune and inflammatory genes, including MHCs, co-stimulatory molecules, antibodies, cytokines, chemokines and adhesion molecules.

The dual role concept of TLRs is illustrated in both Figures 19a and b. In their innate immune role, TLRs recognize pathogens and generate an immediate defense response by inducing the production of proinflammatory cytokines which rapidly destroy or limit the invading pathogens. This occurs through TLR signaling pathways which are activated by TLR-PAMP binding as shown in Figure 19a. In their bridging role, TLR downstream signals link innate and adaptive immunity, particularly by mediating DC maturation and activation of pathogen-specific T lymphocytes. A schematic of the TLR induced pro-inflammatory signaling cascade as a bridge connecting innate and adaptive immunity is shown in Figure 19b.

TLR bridging to adaptive immunity could be said to begin with TLR-PAMP binding which activates TLRs signaling pathways (reviewed in Mogensen, 2009). These pathways lead to the activation of professional APCs which is followed by enhanced expression of surface molecules, MHC and co-stimulatory molecules [CD40, CD80 (B7.1), CD86 (B7.2), and CD70)]. Mature DCs (highly efficient professional APCs) migrate to draining lymph nodes where they present pathogen-derived antigens to naive T cells.

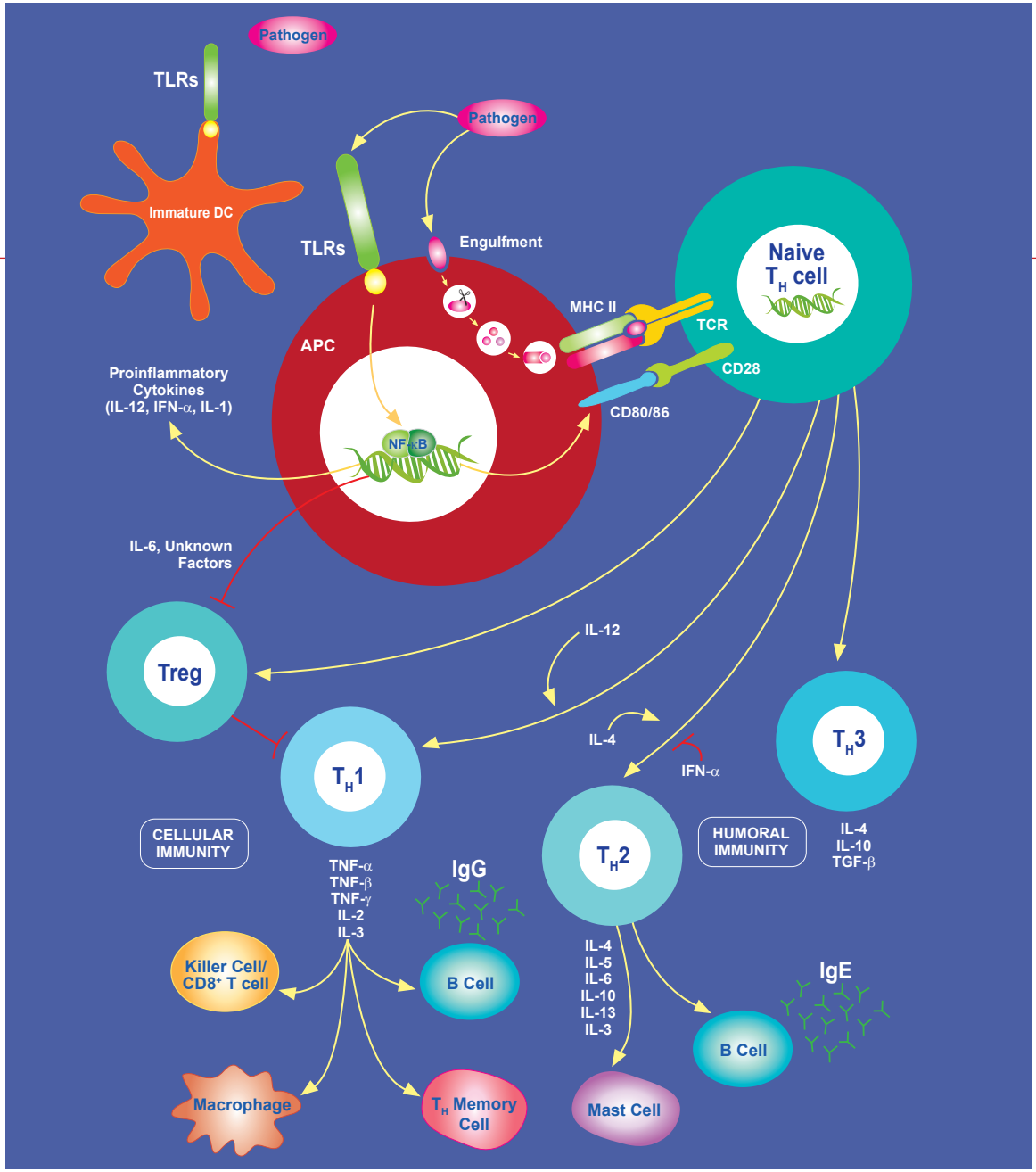


Figure 19a. TLRs in the immune response network. TLRs decode pathogen identity and activate immune signaling pathways according to the nature of the infection. This leads to the development of an immune response network tailored to eradicate a given infection. However, immune response networks are complex and remain to be fully elucidated.

TLRs as Bridges Between Innate & Adaptive Immunity

CD80 and CD86 ligands bind to CD28 on T cells and provide critical co-stimulatory signals required for the activation and differentiation of naïve T cells, particularly those of the CD4+ T lineage. CD80/CD86 co-stimulation is thereby a remote but key TLR downstream signaling mechanism for regulating activation of adaptive immune responses (Reviewed in Dorhoi et al, 2009).

Indirect APC activation and maturation has also been reported through the interaction between CD40 ligand (expressed on activated CD4+ T cells) and CD40 on APCs, resulting in the production of cytokines by APCs and increased B7 expression (reviewed in Kaiko et al, 2007). It is becoming increasingly apparant that different TLR signaling pathways trigger the production of different cytokines by APCs. Furthermore, the specific cytokine milieu regulates the differentiation of CD4+ T cells into various types of Th (Th1, Th2, Th3 and Th17) cells or Tregs, thereby faciliating cell-mediated adaptive immune responses. (Reviewed in Dorhoi and Kaufmann, 2009).

Although it was originally thought that TLR signaling favors Th1 rather than Th2 responses, later results showed that TLRs are also important for Th2 responses through both MyD88 dependent and independent mechanisms. Th1 responses are induced by inflammatory cytokines such as IL-12, IL-23, IL-27, IL-1, IL-6, IL-18, TNF-α, and chemokines. Th1 responses mediate antiviral and antibacterial immunity and are associated with the production of IFN-α and IgG2a antibodies. In contrast, Th2 responses are driven by cytokine IL-4 and other factors which remain to be fully elucidated. Th2 responses are involved in allergic reactions and immunity againsts helminths, and are associated with the production of IL-4, IL-5, IL-13, and IgG1 and IgE antibodies. Data suggests that Th2 responses are also involved in TLR2 signaling via several ligands including microbial lipoprotein, P. gingivalis, LPS, Pam-3-Cys, and Schistosomal egg antigen.

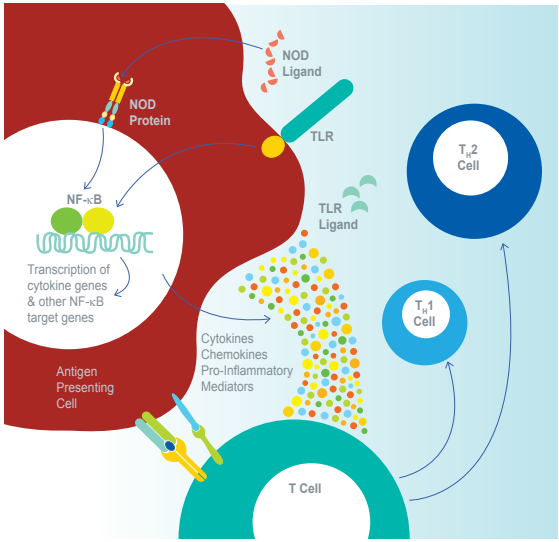


Figure 19b. TLRs as bridges between innate and adaptive immunity. The current dogma puts forth that TLR activation results not only in innate immune responses, but also in signals which serve to bridge innate and adaptive immunity. For example, cytokines, chemokines and pro-inflammatory mediators, produced through TLR activation, activate T cells and other components of adaptive immunity.

The type of Th responses triggered by TLRs is also determined by the milieu of the organism. For example,

LPS stimulation of TLR4 in mutant mice lacking the TLR adapter MyD88 uncovered a defect in the activation of antigen-specific Th1 responses compared to wild-type mice (reviewed in Horner, 2006). However, when the MyD88 null mice were challenged with a low dose of LPS, Th2 responses were triggered through the modulation of DC co-stimulatory signals. The mechanisms that control the differential activation of antigen-specific Th immune responses *in vivo* remain to be fully elucidated.

TLRs also play a critical role in the activation and maturation of B cell responses during infection and vaccination. This occurs through both T cell dependent and independent pathways which induce B cell proliferation, immunoglobulin isotype class switching and somatic hypermutation as well as MHCII expression, and the production of low affinity IgM antibodies. The observed transient increases in circulating antibodies and increased glomerular deposition of IgG2b and IgG3 (Th1 isotypes) may also be indicative of B cell-mediated effects.

TLR links to both the initial phase of immunity (innate defense) and the establishment of adaptive immunity ensure that TLR signaling will continue to be an important research area in immunology. Elucidating the myriad of TLR signaling mechanisms involved in bridging innate and adaptive immunity is a key goal. It is critical for understanding immune disease mechanisms and for developing diagnostics and therapeutics. The study by Schneeman et al (2005) is an example of how Novus' Abs are being used to help define the signaling links between innate and adpative immunity (Fig 20).

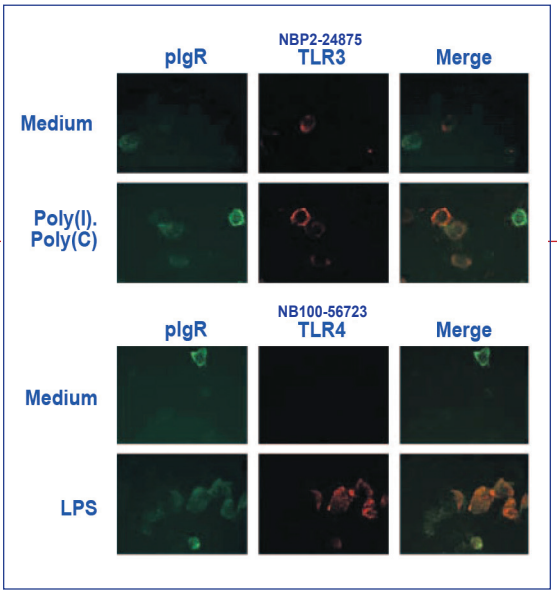


Figure 20. Colocalization analysis of pIgR and TLR3 (NBP2-24875) or TLR4 (NB100-56723). *Secretory IgA (SIgA) Abs play a key role in maintaining homeostasis at mucosal surface by providing an initial adaptive immune defense against the constant barrage of pathogens, while suppressing inflammatory responses to commensal organisms and food antigens. When IgA antibodies are bound to the polymeric IgG receptor at the apical epithelial cell surface they can neutralize pathogens intracellularly or block their transport through polarized epithelia.*

The purpose of this study was to test the hypothesis that TLR signaling upregulates the polymeric IgA receptor (pIgR) which could then enhance IgA mediated homeostasis by providing additional cell surface binding sIgA binding sites. HT29 human adenocarcinoma cells were incubated with medium alone or treated with poly I:C (TLR3 synthetic dsRNA ligand:100 ug/ml) or LPS (TLR4 ligand: 1 ug/ml)* for 24 hr. pIgR + TLR3 or pIgR+ TLR4 expression was detected in unfixed cells by immunofluorescence confocal microscopy.*

The data shows that TLR ligands upregulated both pIgR and their cognate TLR receptors. TLR4 and pIgR co-localized in LPS treated cells. In contrast, TLR3 and pIgR did not always co-localize in dsRNA treated cells. These results suggest that TLR innate immune signaling may augment adaptive immune responses through pIgR upregulation. However, the significance of the variation between pIgR and TLR4 (co-localized) and pIgR and TLR3 (did not always co-localize) staining patterns remains to be elucidated.
**The Poly(I),Poly(C) TLR3 Ligand (NBP2-25288) and the LPS TLR4 Ligand (NBP2-25295) are also available from Novus.*
Source: Schneeman et al (2005).

TLR Signaling Pathways

Advancements in structural biochemistry, cell biology, and immunology are elucidating mechanisms that enable specificity and selectivity of TLR signaling in response to diverse PAMPs and endogenous ligands. For example, Ranjith-Kumar et al (2007) defined structure-function relationships between the TLR3 ligand binding ECD and TLR3 activation using a mutational analysis system and cell based assays. The Novus TLR3 mAb (IMG-315A) helped demonstrate that a mutant with a particular ECD deletion resulted in ECD structural changes and abolished TLR3 activity, but did not prevent TLR3 protein expression (Fig 21). Hence, the antibody results contributed to defining ECD structural features that are critical for TLR3 signaling, but not required for TLR3 protein expression.

There are a number of excellent reviews in the literature focusing on TLR signaling pathways, and the rest of this chapter will focus on information distilled from some of them including Barton and Kagan (2009), Montero and de Andres (2008), O'Neil (2008) and Tapping (2009). Most of this information is common to all of these reviews unless otherwise noted.

Vertebrate TLR signaling is highly conserved and involves a sequence of stimulus-induced conformational changes. A pathway overview illustrating the current dogma of TLR signaling from ligand binding through transcriptional activation is shown in Figure 22. The high binding affinity of ligands and adaptor complexes to TLRs suggests that signaling is unidirectional and irreversible once TLRs are activated. It is thought that the termination of TLR signaling may require endocytic removal of TLRs, ligands and associated complexes from the cell membranes.

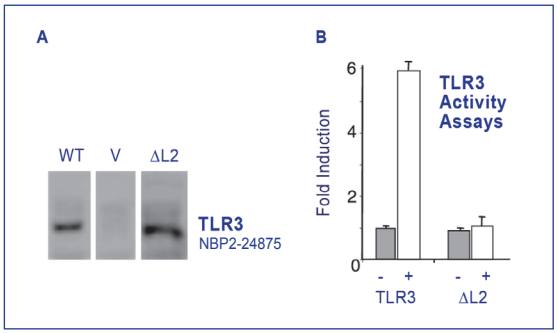


Figure 21. Analysis of WT and mutant TLR3 protein expression and activity. *Western blots: HEK293T cells were transiently transfected with WT TLR3, mutant TLR3(deltaL2) or control pcDNA (V), and cell lysates were probed with the TLR3 mAb Clone 40C1285.6 (NBP2-24875). TLR3 expression was detected in the both the WT and mutant lysates, but not in the control. Lack of expression in the V control shows that 293T cells did not express detectable levels of endogenous TLR3.*

TLR3 activity assays: HEK293T cells were transiently transfected with a mixture of three plasmids, one to express either WT or mutant TLR3, a second to express the firefly luciferase driven from a promoter-containing NF-kB binding sites and a third to express the transfection control. Cells were left-untreated or treated with Poly(I)Poly(C) (2.5 ug/ml) to activate the TLR3 signaling pathway which leads to NF-κB activation downstream. The data from independent sets of transfected cultures shows that Poly(I) Poly(C) induced a 6 fold increase in NF-κB activation in the WT TLR3 cultures, but not in the mutant TLR3 cultures where NF-κB activity was unchanged or only slightly above baseline.

Taken together the results indicate that the mutation (a deletion in the internal loop of the TLR3 ECD) abolished TLR3 function, but not protein expression.

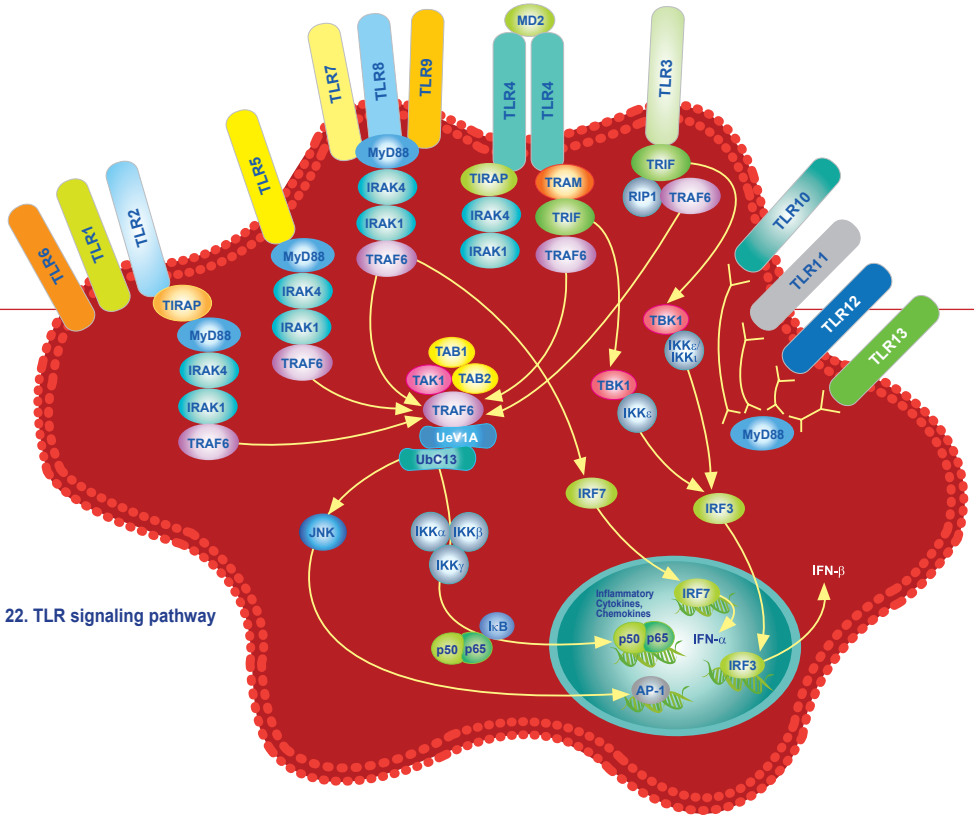


Figure 22. TLR signaling pathway

Signaling by both Toll and TLRs involves ligand-induced dimerization or oligomerization. Many TLRs function as homodimers; TLR4 homodimers are shown as an example in Figure 22. In contrast, TLR2 heterodimerizes with TLR1 or TLR6, with each dimer having different ligand specificity. Additionally, other co-receptors can also be required for full TLR ligand sensitivity. For example, TLR4 and MD2 form a symmetrical complex when activated by LPS, with CD14 and LPS binding protein facilitating the presentation of LPS to MD2.

TLR activation is a sequential process which is initiated when TLR ligand binding induces a conformational change in the C-terminal region of the ECD. This induces stability in TLR-TLR interactions, which in turn induce conformational changes in the TLR transmembrane helices of the receptor dimer. The conformational changes in the TLR transmembrane domains are critical for transmitting the signal from the cell surface to the cytoplasm, whereas the TLR-TIR domains are required for coupling receptor dimerization to adaptor proteins that transmit signals downstream. Activated TLRs recruit specific sets of adaptors through their TIR domains, resulting in the formation of TLR-adaptor complexes as can be seen in Figure 22. TLR dimerization induces a change in their TIR domains which generates the binding specificity required for adaptor recruitment. Adaptors also have TIR domains, and conserved sequences in the TLR and adaptor TIR domains enable TLR-adaptor interaction.

It is noteworthy that although many TLR-specific antibodies can crosslink two TLRs, crosslinking does not always translate into induction of TLR signaling. This is because specific TLR-TLR interactions, such as

TLR Signaling Pathways

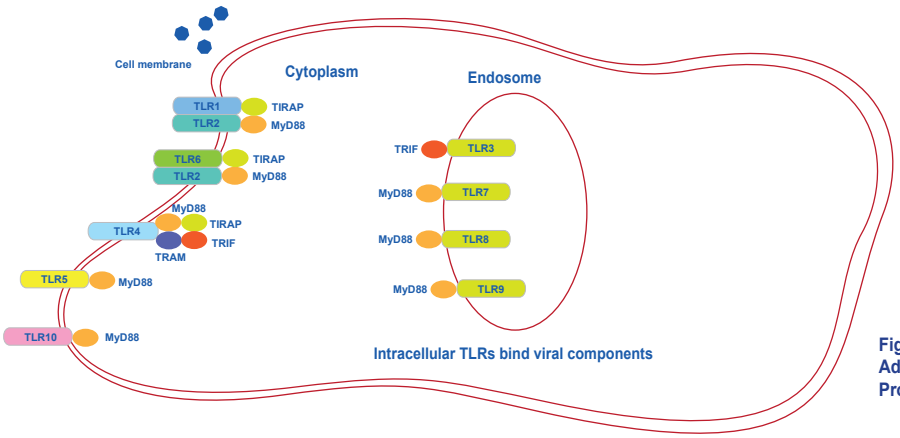


Figure 23. TLR Adaptor Signaling Proteins

C-terminal ECD alignment, are accompanied only by specific ligands.

In addition to the cell surface, TLRs are also expressed intracellularly. For example, TLR3 is primarily located intracellularly on the endosome. TLR3 has also been detected on the cell surface in some model systems. TLR3 is part of a specialized signaling mechanism that enables specific recognition of viral nucleic acids. This mechanism results in delivery of viral nucleic acids to the endosome where they are recognized by TLR3. It indirectly enables TLR3 to distinguish non-self viral DNA from self DNA because self DNA is not normally present in the endosome.

Both cell surface and intracellular TLRs utilize adaptor proteins to transmit signals downstream (reviewed in Waters et al, 2007). Downstream signaling directly or indirectly leads to activation of NF-κB, MAPKs, JNKs, p38, ERKs, and IRF (IRF3, IRF5 and IRF7) signaling pathways as illustrated in Figure 22. The adaptors help impart signaling specificity in order to obtain the appropriate biological effect.

Most of the TLRs (except for TLR3) and IL-1R family members signal through the adaptor protein MyD88 (Fig. 23). Other TLR adaptors include TRIF, TIRAP, and TRAM; TLR3 signals through TRIF. Additionally, various combinations of adaptor proteins can be utilized during TLR signaling. For example, TLR4 can signal through both the MyD88 and TRIF adaptor pathways.

TLR/MyD88 Signaling

MyD88 is a signaling adaptor protein that has a key role in numerous immune modulated processes including host defense, infection, inflammation, and disease (Fig 24) (Reviewed in O'Neil, 2008). MyD88 contains a C-terminal TIR domain and an N-terminal death domain. It was the first adaptor molecule found to be critical for TLR signaling. TLR signaling is typically caterogerized as MyD88-dependent (D) or MyD88-independent (I) as is illustrated in Figure 25 (reviewed in Montero and de Andres, 2008). The MyD88-D and MyD88-I pathways are the two dominant intracellular TLR signaling pathways and all TLRs utilize the MyD88-D pathway, with the exception of TLR3 which signals through the MyD88-I pathway. TLR4, however, signals through both the MyD88-D and MyD88-I pathways.

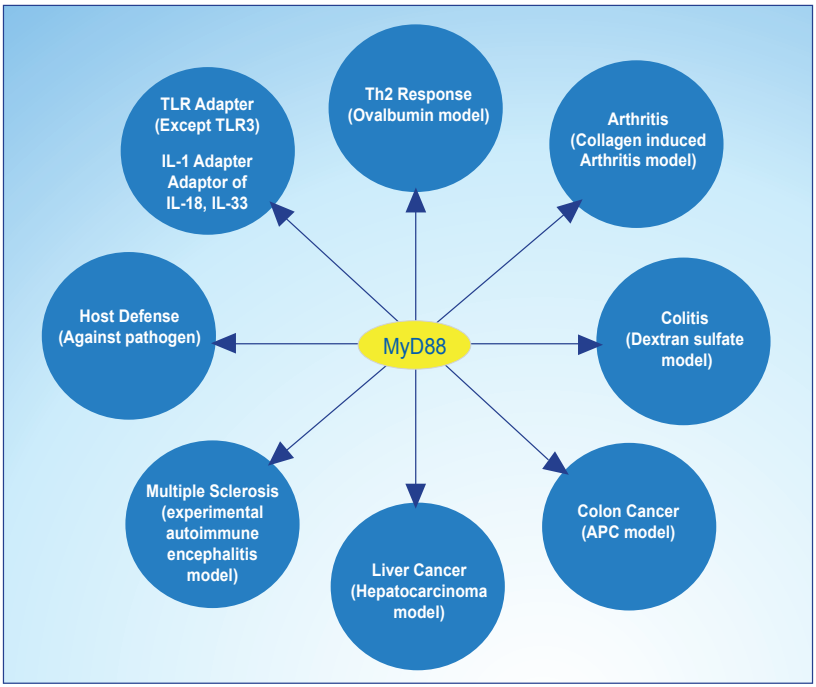


Figure 24. MyD88 Roles in immune modulation. *Adapted from O'Neil (2008)*

MyD88-D Signaling

In MyD88-D signaling, the MyD88 TIR domain interacts with TLRs, and the MyD88 death domain recruits IRAK family members, including IRAK1, IRAK2, IRAK4, and IRAKM as can be seen from Figure 22 (reviewed in Montero and de Andres, 2008). IRAK4 is the central mediator of the MyD88-D pathway and associates with IRAK1 and activates it. Activated IRAK1 then activates TRAF6 by binding to its TRAF domain. The association of IRAK4/IRAK1/TRAF6 induces conformational changes and this complex disengages from the receptor complex.

IRAK1/TRAF6 are then transferred to a preformed membrane complex consisting of TAK1, TAB1 and TAB2. Formation of the IRAK1/TRAF6/TAK/TAB1/TAB2 complex leads to its phosphorylation by a local kinase. TRAF6/TAK/TAB1/TAB2 then dissociates from IRAK1 and translocates to the cytosol where it forms a large complex with other proteins such as the E2 ligases Ubc13 and Uev1A. IRAK1 remains bound to the membrane where it degrades.

TRAF6 acts as ubiquitin ligase in concert with Ubc13 and Uev1A to catalyze its own Lys63-linked ubiquitination. This induces TAK1 activation which triggers the activation of IKKα and IKKβ, thereby activating the IKK complex. The active IKK complex (IKKα/IKKβ/IKKγ) catalyzes the phosphorylation and degradation of IκB, an inhibitory protein that sequesters NF-κB in the cytoplasm. IκB degradation enables the liberation and activation of NF-κB. Active NF-κB translocates to the nucleus where it binds to target inflammatory cytokine genes and activates their transcription. Activated TAK1 also facilitates MyD88-D activation of MAP kinases, such as p38 and JNK, which like NF-κB activation, leads to inflammatory cytokine gene expression.

TLRs obtain specificity through MyD88-D signaling by various mechanisms including adaptor combinations, pathway bifurcations and specialized cell types. For example, the TIRAP adaptor protein is recruited to the plasma membrane when the LPS ligand binds to TLR4. TIRAP mobility, in turn, recruits MyD88 to the plasma membrane leading to MyD88-TLR4 TIR-TIR interactions (reviewed in Barton and Kagan, 2009). These interactions trigger TLR4 pro-inflammatory signal transduction. This type of signaling pathway is referred to as MyD88/TIRAP dependent. In MyD88/TIRAP dependent pathways, TIRAP is required for MyD88-TLR TIR-TIR domain interactions and the initiation of downstream TLR signal transduction.

It is notable that TIRAP-deficient mice have TLR2 and TLR4 signaling impairments similar to MyD88-deficient mice. Additionally, MyD88/TIRAP double deficient mice have no additional impairments beyond MyD88 or TIRAP single deficient mice. These findings are consistent with an interdependent relationship between TIRAP and MyD88 in TLR2 and TLR4 signaling.

Pathway bifurcation and cell type plays an important role in TLR9 subfamily (TLR7, TLR8 and TLR9) MyD88-D

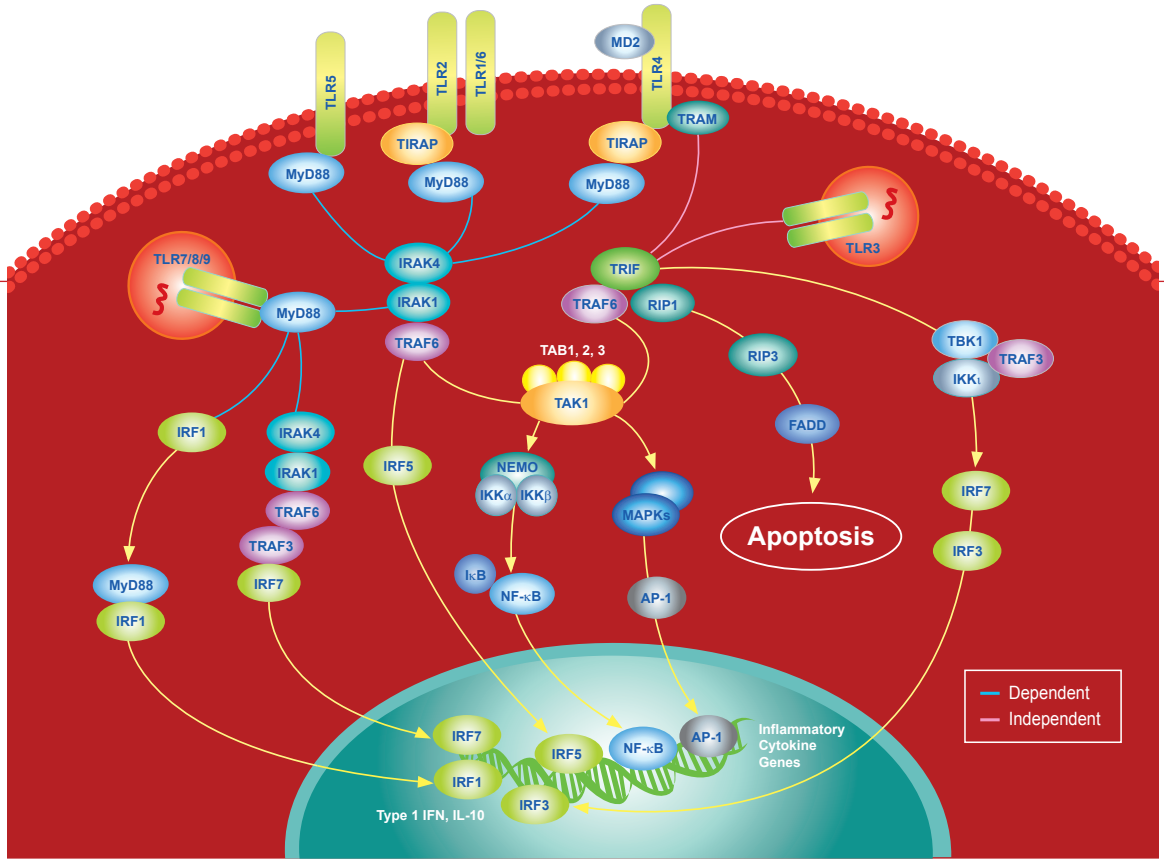


Figure 25. MyD88 dependent (D) and independent (I) pathways. *Adapted from Montero and de Andres (2008).*

signaling. For example, signaling through TLR9 subfamily members bifurcates into the NF-κB and IRF7 pathways in plasmacytoid dendritic cells (pDC). In the NF-κB branch, the NF-κB transcription factor is activated leading to the production of inflammatory cytokines as described above. In the IRF7 branch, TRAF3 is recruited to the MyD88-IRAK4-IRAK1-TRAF6 complex leading to activation of the IRF7 transcription factor. IRF7 is the main inducer of type 1 IFNs (IFN-α/β) which are critical to immunity against viruses. pDCs are a specialized cell population capable of producing large amounts of IFN-α/β and are often referred to as IFN-producing cells. The mechanisms which enable pDCs, but not other cell types, to activate the MyD88-IRF7 pathway remains to be fully elucidated.

MyD88-I Signaling. This signaling occurs in both the TLR3 and TLR4 pathways: TLR3 utilizes the adaptor protein TRIF to activate the MyD88-I TRIF-dependent pathway as is shown in Figure 25 (reviewed in Montero and de Andres, 2008). Ligand binding to TLR3 results in TRIF recruitment through TIR-TIR domain interactions. TRIF has a domain in its N-terminus which then recruits IKKs, IKKi and TBK1. TRAF3 is also involved in this

recruitment and is thought to facilitate TRIF's association with TBK1.

IKKi and TBK1 then phosphorylate and activate IRF3 and IRF7. Activated IRF3 and IRF7 form homodimers in the cytoplasm and translocate to the nucleus where they induce high levels of IFN-β and also regulate the expression of other host defense genes. For example, TRIF-dependent activated IRF3 is thought to promote the expression of TNFα which then binds to the TNFαR and initiates late phase NF-κB activation through autocrine signaling mechanisms (reviewed in Montero et al, 2008).

The N-terminus of TRIF also contains a domain that mediates interaction with TRAF6, and a C-terminal domain that interacts with RIP. RIP, TRIF and TRAF6 form a complex with TRAF6. Complex formation facilitates TAK1 activation which results in the activation of NF-κB and MAP kinase. Additionally, when RIP3 becomes part of the complex, RIP1 blocks RIP1-induced NF-κB activation and the death domain of RIP1 recruits FADD to induce apoptosis.

TLR4 I Signaling. TLR4 signals through both the MyD88-D and MyD88-I pathways as can be seen in Figure 25 (reviewed in Montero and de Andres, 2008). Historically speaking, the observation that MyD88-deficient mice could still respond to LPS gave the first indication that TLR4, in addition to MyD88-D signaling, also ultilizes an MyD88-I pathway. TLR4 signaling activated NF-κB and MAPKs in a delayed manner in MyD88-deficient compared to wild type mice. The delayed responses fail to induce inflammatory cytokine gene expression, but were sufficient for DC maturation and the expression of IFN-α and other host defense genes. Gene expression was found to be induced through IRF3/IRF7 activation.

Functional analysis of TRIF and with TRIF-deficient mice showed that MyD88-I pathway activation in both TLR3 and TLR4 occurs through the TRIF-dependent pathway which leads to IRF3/IRF7 and NF-κB activation, and can also lead to apoptosis. Analysis also showed that TLR3 directly associates with TRIF, whereas TLR4 requires another adaptor, TRAM in order to associate with TRIF.

Pathogens hijacking TLR signaling pathways

TLR pathways can be highjacked by pathogens that have developed mechanisms to interfere with signaling in order to produce results in favor of their own benefit (reviewed in Shames et al, 2009). For example, the *Yersinia entercolitica* secreted LcrV protein activates the TLR2 pathway in mice to promote macrophage production of the immunosuppressive cytokine IL-10. Hence, the host immune system is suppressed giving *Yersinia entercolitica* more of a chance to survive.

In line with this, TLR2-deficient mice are found to be less susceptible to Yersinia entercolitica infection than wild-type mice. This supports the concept that the LcrV PAMP utilizes the TLR2 pathway to diminish the host response against the virus. This enables the virus to proliferate more freely in WT mice and cause more damage in WT than mutant hosts.

Another example of pathogen interference with TLR activation pathways involves the immunosuppressive effect of the vaccinia virus protein A52R. A52R contains a TIR domain and physically associates with IRAK-2 and TRAF6, two cytoplasmic proteins required for signal transduction by TLRs. The association of A52R with IRAK2 and TRAF6 has an immunosuppressive effect on the host. Hijacking mechanisms are interesting and potentially important as targets, but their effect on human disease remains to be elucidated.

Signaling pathways are at the core of TLR research. A rapidly emerging area focuses on defining the cellular machinery underlying TLR function and regulation (Reviewed in Barton and Kagan, 2009). As discussed in the chapter 'TLRs: Structure, Ligands & Expression (section Subcellular Localization),' the mechanisms that recruit TLRs to specific cellular compartments are just now being described. New knowledge about these and other cell machinery mechanisms will likely result in retooling the current concepts we have about TLR signaling pathways and immune signal transduction networks overall.

Another signaling area with exciting developments is that of the role of post translationally modified, cleaved, or mutated TLR proteins. For example, Ranjith-Kumar et al (2007) showed that certain mutations resulted in changes in the TLR3 ECD structure and abolished TLR3 signaling, but not TLR3 protein expression (Fig 21).

Additionally, the importance of proteolytic cleavage has been demonstrated for TLR9 where cleavage in an endolysosomal compartment appears to be required for TLR9 activation (reviewed in Barton and Kagan, 2009). It is thought that full-length TLR9 protein is sorted in the ER, trafficked through the Golgi and then routed to the endolysosome, where it is cleaved by resident proteases. As a result, receptor activation is limited to the endolysosome. Full-length TLR9 present on the cell surface appears to be non functional.

Putative TLR cleavage fragments are often observed in western blot analysis for TLR9 as well as other TLRs. Heretofore their significance has been elusive. However, the role of cleavage-activation is well known in other fields, particularly apoptosis. It is exciting to think that evidence demonstrating TLR9 cleavage may be an indication that cleavage-activation also has essential roles in TLR signaling overall.

TLR Expression in Tregs

A Treg is a CD4+ T cell that reduces or suppresses the immune responses of B cells or of other T cells to an antigen. Tregs were discovered in 1970 and coined "Suppressor T cells," later the name was changed to "Regulatory T cell (Treg)." However, the understanding of Treg function remained nebulous following their initial discovery, and their suppressive ability was recognized as more of a phenomenon than a regulated process. About thirty years after their discovery, the concept of Tregs as integral components of the immune system began to take hold and a research area focused on Tregs emerged. Tregs are now key players in the global research effort to understand immune regulation (reviewed in van Maren et al, 2008 and Walker, 2009).

The current dogma puts forth that Tregs are vital for maintaining tolerance and homeostasis, and for preventing the immune system from becoming overactive through a highly tuned process. Additionally, Treg dysregulation can cause aberrant immune responses, and the absence of Tregs results in immune chaos.

Tregs can be broadly divided into two groups:

1. Tregs that originate in the thymus: referred to as 'naturally occurring Tregs'
2. Tregs that develop in the periphery: referred to as 'adaptive Tregs'

The most well characterized Tregs are the naturally occurring CD4+CD25+ Tregs, which constitute 5-15% of the total CD4+ T cell population. Naturally occurring Tregs express the transcription factor FOXP3 which is induced by TGF-β. FOXP3 appears to be the most specific marker for naturally occurring Tregs, although several other markers have been identified, including CTLA-4, GITR, CCR8 and the absence of CD127. Figure 26 is an example from Bell et al (2007) of using FOXP3 as a Treg marker to help characterize TLR expression in Tregs versus non-Tregs.

At least two types of adaptive CD4+ Tregs have been characterized, Type I regulatory (Tr1) and Type 3 regulatory (Th3) T cells. Tr1 cells arise after repeated TCR stimulation in the presence of IL-10. Th3 cells differentiate from naïve CD4+ T cell precursors through repeated TCR stimulation in combination with exposure to high levels of TGF-β. The field of Tregs is rapidly expanding, and it is likely additional Treg subsets remain to be be discovered.

Both naturally occurring and adaptive Tregs monitor the activity of effector T cells (Teffs), where they regulate the initiation, expansion, and retraction of Teff responses. Teff responses must be closely controlled to prevent extensive immune-mediated tissue damage or autoimmune disease. Once activated, Tregs can suppress Teff proliferation and cytokine production as well as APC function, thereby controlling Teff responses. Tregs have been best characterized for regulating the activity of CD4+ Teffs; however they also influence the activity of CD8+ Teffs, B cells, and cells of the innate immune system.

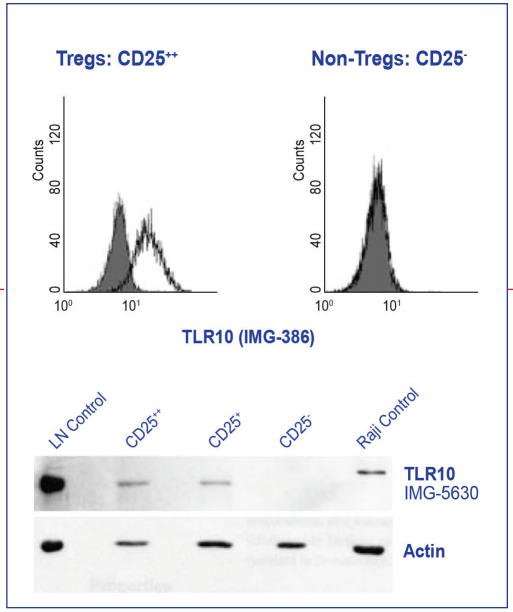


Figure 26. Comparison of TLR10 expression in human Treg and non-Treg cells by flow cytometry [(cell surface), IMG-386 (Clone 158C114)] and western blot analysis (IMG-5630). *Human Treg* cells were isolated from healthy donors by Ficoll separation and magnetic bead sorting using a CD4+CD25+ Treg cell isolation kit. Cells of the highest CD25 expression (CD25++) were selected through incubation with a limiting quantity of anti-CD25 Ab beads. Non-Treg CD25- cells were selected by collecting flow through from saturating amount of anti-CD25 Ab beads. Cells of intermediate CD25 expression (CD25+) were collected using a non-limiting (moderate) amount of CD25 Ab beads.*

The data shows that TLR10 was detected in the Treg (CD4+CD25++ and CD4+CD25+) cell, but not the non-Treg (CD4+CD25-) cells. Normal lymph node (LN) and Raji cells were used as a positive western blot control for TLR10 expression. The Western Blot Loading Control Kit (NBP2-25090) containing a Beta Actin polyclonal antibody (IMG-5142A-050) is recommended for detecting actin or other housekeeping proteins.

** Treg CD4+CD25++ cells were >60% FOXP3 positive and non-Treg CD4+CD25- cells were <3% positive for FOXP3 by intracellular staining. The suppressive phenotype of the Treg CD4+CD25++ cells was confirmed through in vitro suppression assays. Source: Bell et al, 2007.*

The discovery that TLR signaling directly or indirectly regulates the suppressive capability of Tregs is among the farthest-reaching developments in the Treg field (reviewed in van Maren, 2009). The expression of TLRs in Tregs was first shown in 2003 by Caramaldo and her colleagues who demonstrated that a subset of CD4+ T cells known to exert regulatory functions expressed TLRs 4, 5, 7 and 8. Since then, TLR expression in Tregs has been reported in a number of studies. For example, Bell et al (2007) found that TLR10 was constitutively expressed in primary human Treg cells isolated from the peripheral blood of healthy donors as is shown in Figure 26.

Overall, TLR expression profiling studies have indicated that multiple TLRs are expressed in naturally occurring CD4+CD25+ Tregs. Additionally, TLR expression has been shown to be functionally relevant in various Treg model systems. Collectively, results suggest that as a family TLRs have both positive and negative effects on the suppressive function of CD4+CD25+ Tregs. That is, TLR signaling has been shown to both enhance and reduce the ability of these Tregs to suppress Teffs. Additionally, TLR signaling can also enhance Treg proliferation and may also act to inhibit it. These results are summarized below and reviewed in van Maren et al (2008):

1. **Enhance Treg suppression**
   a. Hsp60 (TLR2)
   b. LPS (TLR4)
   c. Flagellin (TLR5)

TLR Expression in Tregs

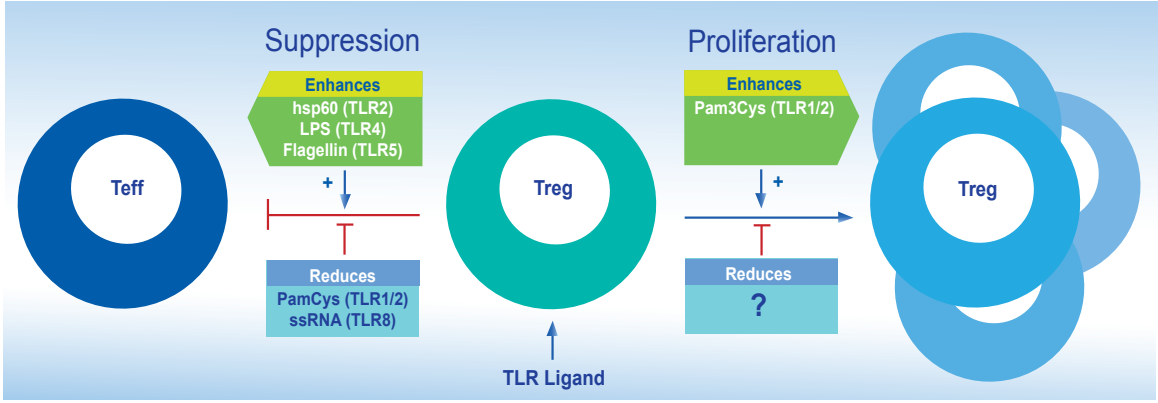


Figure 27. TLRs and modulation of Tregs. *Ligand activated TLR signaling is thought to directly modulate Treg function. This includes enhancing or reducing Treg suppressive and/or proliferative ability. Adapted from Van Maren et al (2008).*

2. **Reduce Treg suppression**
   a. Pam3Cys (TLR1/2)
   b. ssRNA (TLR8)
3. **Enhance Treg proliferation**
   - Pam3Cys (TLR1/2)
4. **Reduce Treg proliferation**
   - Remains to be defined

A conceptual overview of ligands and TLRs with respect to modulation of Treg activity is shown in Figure 27. However, the role of TLR activation itself on Tregs as well as the mechanisms underlying modulation of Treg function, e.g. to suppress or not to suppress, remain to be fully elucidated. Critical questions regarding the dynamics of TLR expression on Treg subsets during homeostasis, infection and inflammation will need to be answered in order to understand the role of TLR expression on Tregs in both health and disease.

Elucidating relationships between TLRs, Tregs and cancer is of particular interest in the TLR/Treg field. Tregs can infiltrate tumors and suppress the anti-tumor response in mice, and increased levels of Tregs have been found in the peripheral blood of cancer patients. Both naturally occurring and adaptive Tregs have been found in tumor microenvironments. Since TLRs can reduce the suppressive activity of Tregs as shown in Fig 27, manipulating TLR signaling could potentially lift Treg suppression of the anti-tumor immune response. Hence, leveraging TLR signaling on Tregs may offer new opportunities for shifting the balance between tolerance and immunity, and lead to new treatments for cancer and other diseases.

TLRs in Cell Survival & Death Pathways

TLRs as a family have the capacity to promote both cell survival and cell death signaling pathways (reviewed in Salaun et al, 2007). The pathway(s) traveled depends on various factors, including the specific TLR, the trigger leading to TLR activation and the experimental model system utilized. For example, global gene profiling of PBLs treated with different ligands showed an inflammatory and pro-survival gene signature reflecting transcriptional activation of NF-κB dependent genes. Likewise, a number of genes primarily involved in apoptosis were also upregulated. The rest of this chapter will focus on mechanisms and examples of TLRs in cell survival and death pathways.

Cell survival. TLR activation in B cell chronic lymphocytic leukemia (B-CLL) cells with resiquimod (R-848) led to NF-κB dependent transcriptional activation of iNOS, increased NO production and apoptosis resistance (Hammadi et al, 2008). An MTT assay, which measures cell proliferation/viability, showed that R-848 treated cells had increased viability compared to untreated cells. R-848 is a TLR7/8 agonist. However, primarily B-CLL cells do not express TLR8 (Shi et al, 2007) and hence the results suggest that TLR7 may play a role in B-CLL cell survival pathways by increasing cell viability in a survival pathway involving NF-κB activation.

It is noted that the IMGENEX' IκBα ActivELISA™ Kit (IMK-501) was used as a readout of NF-κB activation in this study (Fig 28). This kit measures IκBα phosphorylation, a marker of NF-κB activation occuring through the classical NF-κB activation pathway as shown in Figure 29.

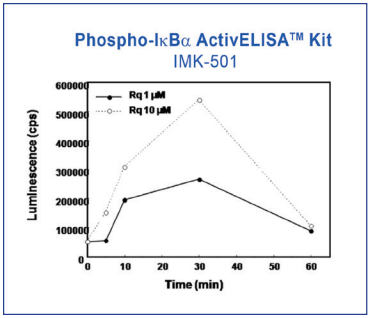


Figure 28. Analysis of NF-κB activation in resiquimod treated B-CLL cells with an IκBα phosphorylation assay. *Cells were treated with 1 or 10 uM of resiquimod and the phosphorylation of IκBα was measured with the IκBα ActivELISA™ Kit (IMK-501) over a 1 hr time period. IκBα is a cytoplasmic marker of NF-κB activation as illustrated in Fig 29. The kit utilizes a pair of antibodies in a sandwich ELISA to specifically detect phosphorylated IκBα. The data shows that NF-κB activation as measured by IκBα phosphorylation peaked at 30 min and then diminished over the next 30 min to near baseline. Source: Hammadi et al, 2008.*

TLR activation of the PI3K-Akt pathway has also been shown to promote cell survival. For, example TLR activation of PI3K–Akt signaling promoted cell survival in monocytes exposed to LPS by inhibiting proinflammatory and apoptotic events (reviewed in Cinei and Opal, 2009). Additionally, TLR signaling has also been associated with the increased expression of anti-apoptotic or survival proteins including cIAP1, cIAP2, XIAP and Bcl2 family members such as A1 (reviewed in Rakoff-Nahoum and Medzhitov, 2009).

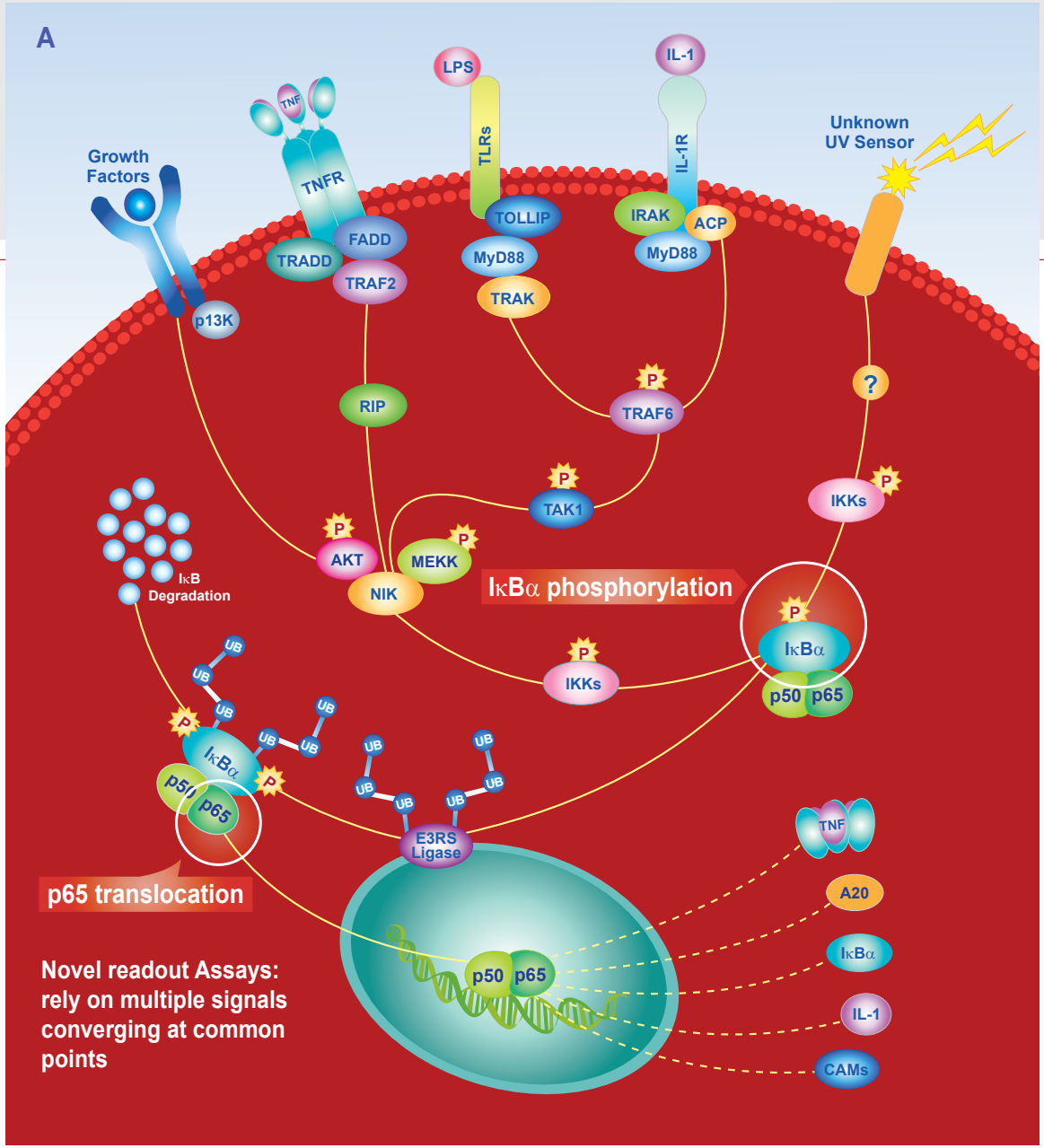


Figure 29. IκBα-P: Upstream NF-κB Activation Marker. *A. Multiple signals in the NF-κB signaling pathway converge at IκBα phosphorylation rendering it a useful marker of NF-κB activation. B. The IκBα ActivELISA™ Kit (IMK-501) utlizes a pair of antibodies to specifically detect IκBα-P levels in a readout assays. IκBα-P (Ser32/36) and IκBα–biotinylated mAbs are used as capture and detection antibodies, respectively.*

B

Indicators of NF-κB Activation: IκBα Phosphorylation Assays

Pair of IκBα monoclonal antibodies

- **IκBα**
  - **Detects the total protein; unphosphorylated + phosphorylated fractions**
- **IκBα-phos (Ser32/36)**
  - **Detects the phosphorylated form of the protein; unphosphorylated form not recognized**

Cell death. The understanding of TLR signaling cascades from ligand activation down to cell activation has rapidly progressed over the last few years. TLR activation of cell death pathways is increasingly being recognized as among the repertoire of defense mechanisms utilized by the innate immune system. Figures 30-32 show overviews of TLRs within the context of the major cell death pathways: necroptosis, (Fig 30), apoptosis (Fig 31), and autophagy (Fig 32).

Although many of the molecular players and mechanisms in TLR death pathways remain to be fully elucidated, it is apparent that TLRs as a family utilize both overlapping and separate mechanisms. As an example, Figure 33 illustrates signaling pathways linking TLRs 2,3 and 4 to apoptosis (reviewed in Salaun et al, 2007).

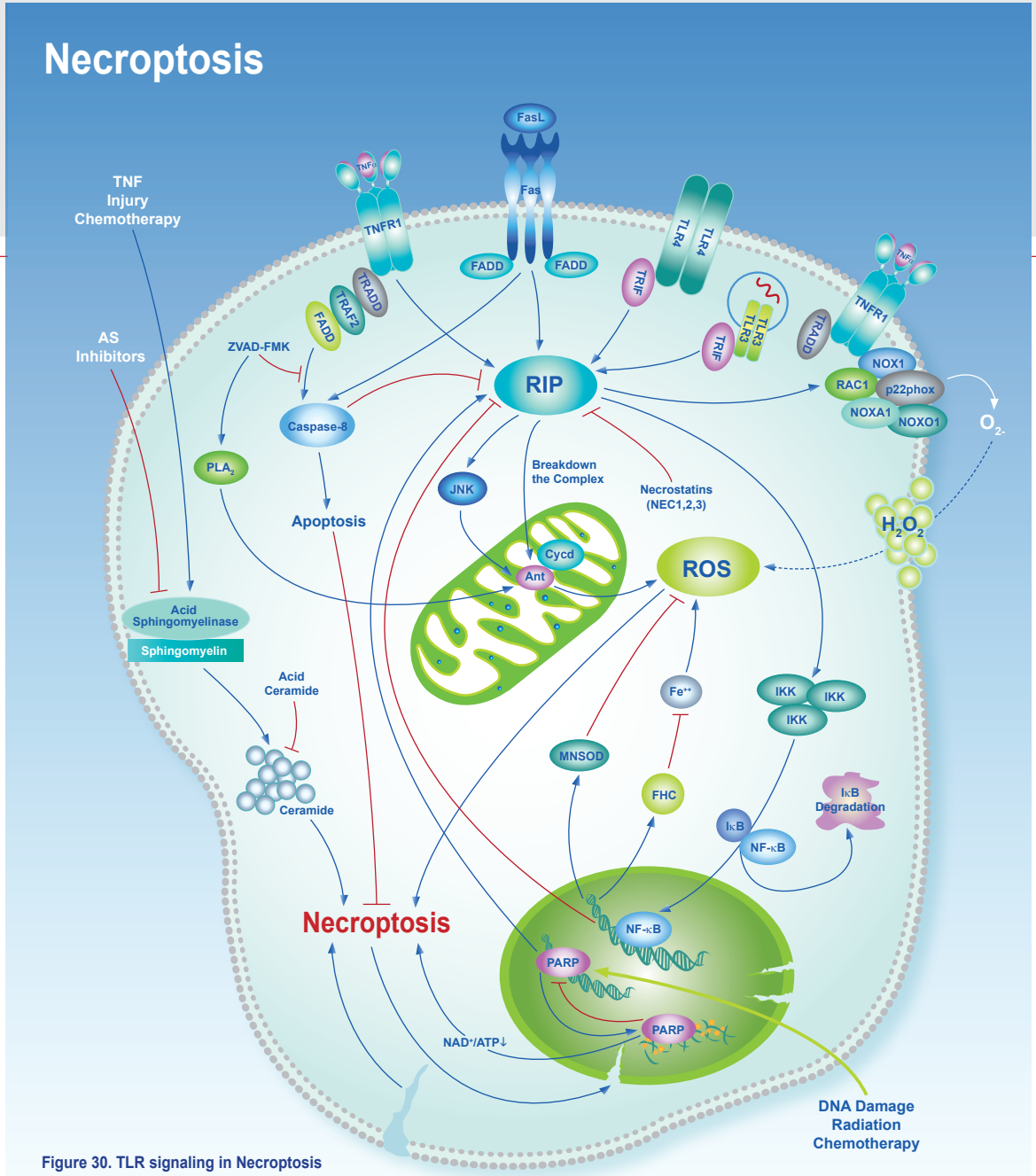


Figure 30. TLR signaling in Necroptosis

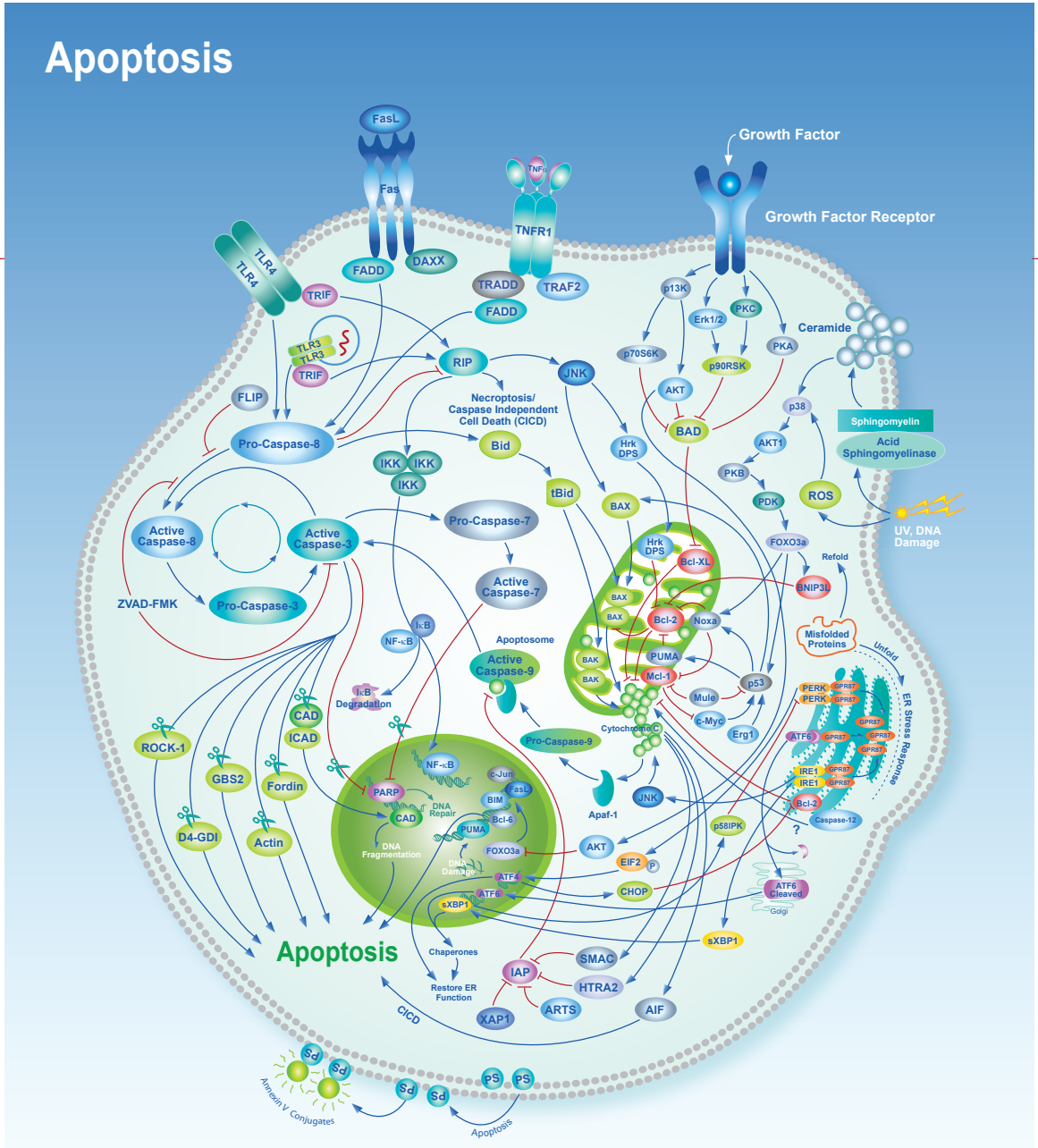


Figure 30. TLR signaling in Apoptosis

Autophagy

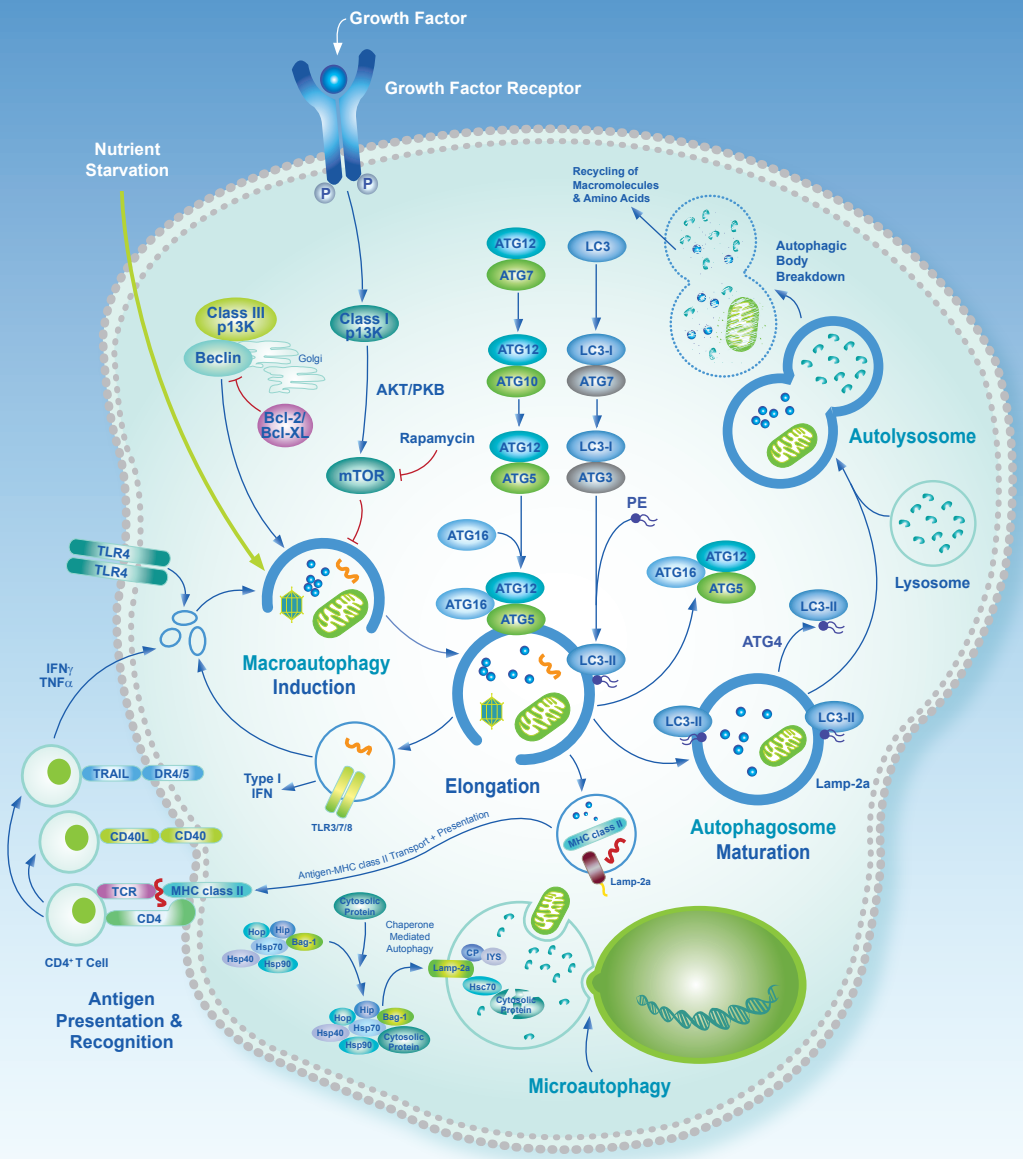


Figure 32. TLR signaling in Autophagy

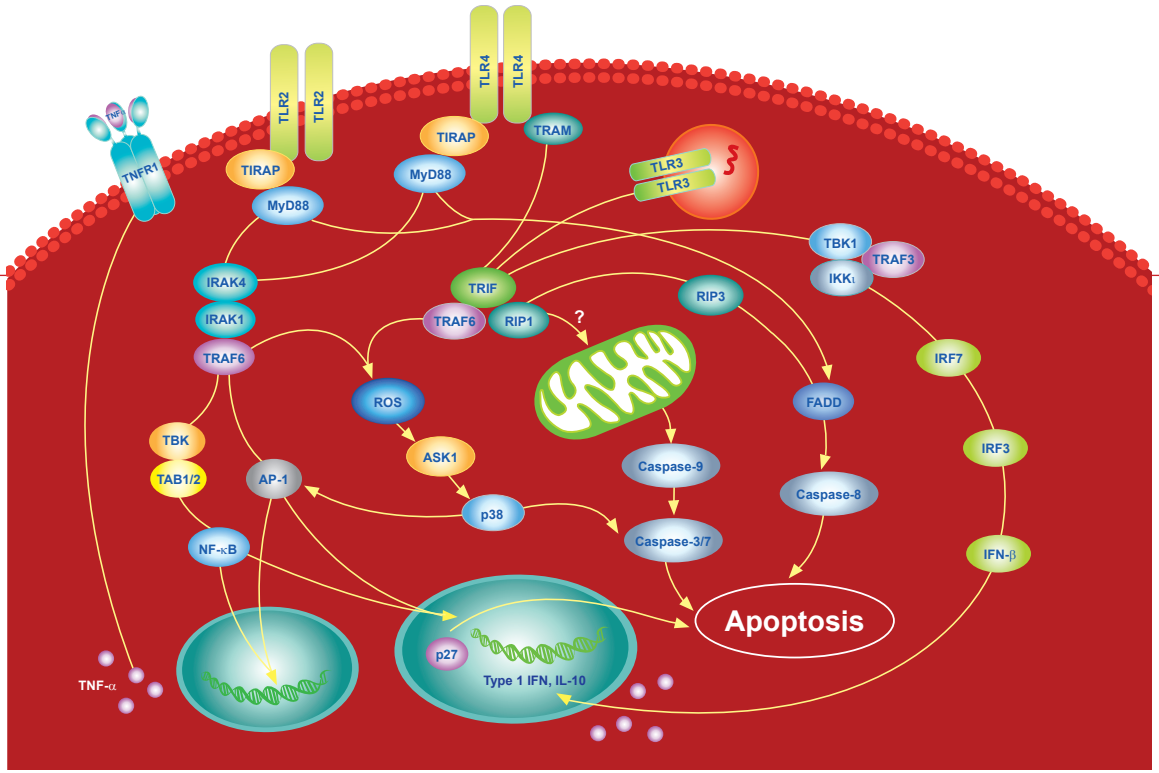


Figure 33. Signaling pathways linking TLRs 2,3 and 4 to apoptosis. *Adapted from Salaun et al, 2007.*

As shown in Figure 33, TLR2 and TLR4 recruit FADD through MyD88 and activate caspase-8, leading to caspase-dependent apoptosis. TLR2 and TLR4 also upregulate NF-κB/AP-1 dependent production of TNF-α which contributes to cell death through activation of its cognate death receptor, TNFR.

However, TLR4 can also activate caspase-dependent apoptosis through the TRIF I pathway. The TLR4 TRIF pathway can also induce the IRF3 dependent secretion of IFN-β which prevents cell cycle entry through p27 stabilization, leading to apoptosis.

TLR3 recruits FADD through TRIF/RIP1 to activate caspase-dependent apoptosis. TLR3, like TLR4, induces IRF3 dependent secretion of IFN-α thereby also inducing apoptosis through p27 stabilization and cell cycle arrest. As denoted in Figure 33, the role of the mitochondrial apoptosis pathway in TLR induced apoptosis remains to be defined.

From a physiological perspective, it is thought that PAMP triggered apoptosis is a conserved innate immune defense strategy. Whereas activation of innate immunity always involves some degree of inflammation, apoptosis is thought to be used as a mechanism to kill infected cells. For example, killing infected cells by apoptosis can be an effective means to prevent microbes from spreading thoughout the entire organism.

TLRs in Cell Survival & Death Pathways

In 1999, TLR2 became the first TLR family member to be described as a death inducing receptor when bacterial lipoproteins (TLR2 ligands) were shown to induce apoptosis in TLR2 transfected cells. Follow-up studies showed that activation of native TLR2 induced apoptosis in a number of cell types, including macrophages, neutophils, trophoblasts, Schwann cells, and microglial cells. Since then, there have been a number of reports showing the involvement of various TLRs in cell death pathways.

Among the most exciting emerging developments in the cell death field is the recognition that TLR signaling is important in the autophagic mechanisms that eliminate pathogens intracellularly (reviewed in Delgado and Deretic, 2009). For example, LPS activation of TLR4 has now been shown to induce autophagy in mouse macrophage RAW 264.7 cells by several different research groups. Likewise, ligand activation of TLRs 3, 7 and 8 have also been shown to be important in activating autophagy. As shown in Figure 32, the initial autophagic signaling mechanisms activated by TLR signaling vary between cell surface TLR4 and intracellular TLRs 3, 7, and 8.

Both TLRs and autophagy are evolutionarily ancient innate immune mechanisms that are regulatory and effector in nature. The recent developments linking these two heretofore seemingly separate cell defense mechanisms suggest that TLR-autophagy connections represent a fully integrated immunity process. Autophagy interacts with both cell death (apoptosis and necrosis) and cell survival pathways. It is thought that autophagy may have been an early innate immune defense mechanism used to eliminate pathogens that managed to gain entry into the cytoplasm of eukaryotic cells. It could have been that when pathogens outgrew autophagic defenses, evolution led to a second stage process resulting in cell death in order to limit pathogen spread. However, these and other proposed mechanisms linking innate immunity, TLRs, autophagy and cell death/cell survival pathways remain to be fully elucidated.

Manipulating TLR pathways: A strategy for analyzing cell survival and death

TLR ligand agonists are among the tools researchers utilize for assessing the role of TLRs in survival and death signaling pathways as shown in Figure 28 where CLL cells were treated with resiquimod (TLR7/8) which induced cell survival and inhibited apoptosis. Another example is the study by Francois et al (2008) where TLR agonists delayed spontaneous apoptosis of polymorphonuclear neutrophils (PMN) and extended their functional lifespan in whole blood. These agonists included LPS (TLR4), PGN (TLR2), R-848 (TLR7/8), CpG-DNA (TLR9), Pam3CSK4 (TLR1/2), and MALP-2 (TLR2/6) (Fig 34). This is an important study because PMN are usually short-lived immune cells and prolonging their life span would be expected to increase their effectiveness against pathogens.

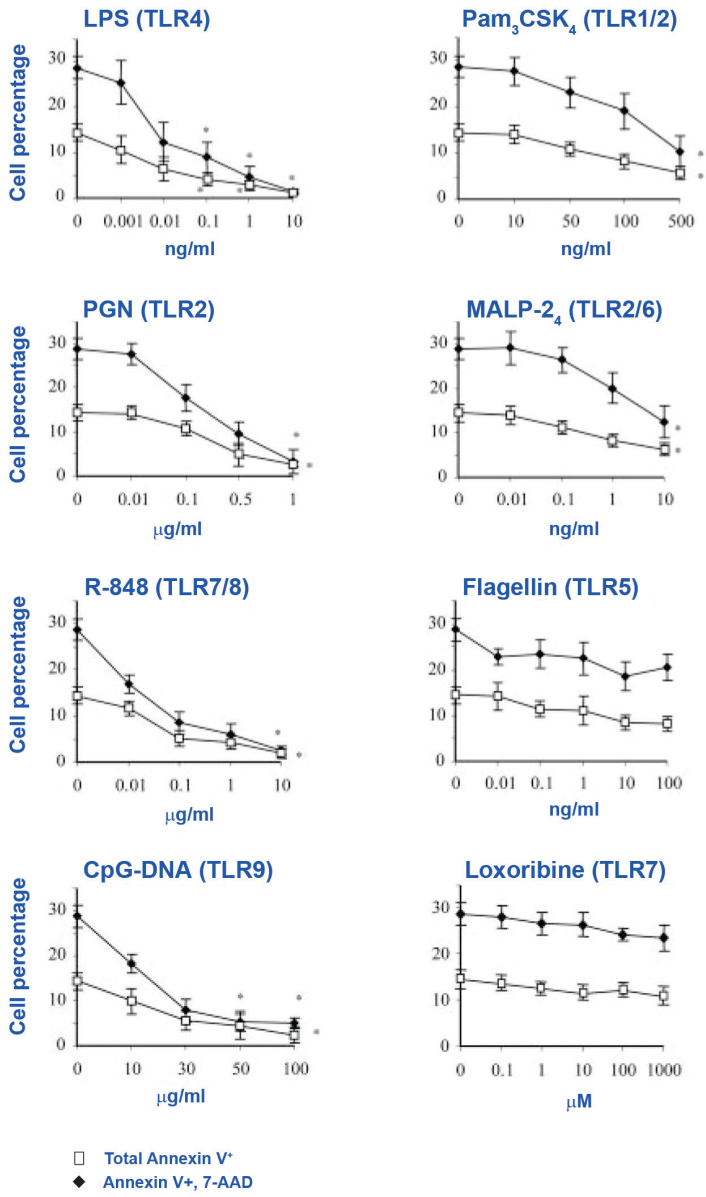


Figure 34. Flow cytometry analysis of concentration-dependent TLR ligand inhibition of spontaneous PMN apoptosis. **Whole blood samples from healthy human donors were incubated with PBS or TLR ligands at various concentrations for 8h.*

Apoptosis was analyzed using Annexin V reagents. Results are expressed as the percentage of Total Annexin V+ (Annexin V+ 7AAD+ and Annexin V+7ADD-) which are early through late stage apoptotic cells and Annexin V+ 7AAD- which are early stage apoptotic cells.

The results show ~26% percent of the untreated cells were Annexin V+ after 8 hrs. This is compared to 0.64% at 0 hr (data not shown) and results from spontaneous apoptosis, a known feature of PMN cells. All of the TLR ligands except flagellin (TLR5) and loxoribine (TLR7) inhibited apoptosis in a concentration-dependent manner as evidenced by a reduction in the percentage of Annexin V positive cells. Source: Francois et al, 2005.

**PMN apoptosis was analyzed by flow cytometry in whole blood in order to avoid artifacts, like spurious cell surface expression of molecules, which can be induced during isolation procedures and accelerate spontaneous apoptosis. A CD15 antibody was used to identify PMN as CD15+ cells and gate out other cells, erythrocytes, and debris.*

Note: TLR3 agonists were not used in the Francois study because PMN did not express TLR3 as is shown in Fig 35.

Novus' CytoGlo Annexin V-FITC Apoptosis Kit (NBP2-29373) includes Annexin V-FITC, vital dye, buffers and control cells for 100 Flow Cytometry tests.

Novus offers the following TLR ligands: CpG-ODN (human: NBP2-26232; mouse: NBP2-26235), Flagellin (NBP2-25289), R-848 (NBP2-26231), Imiquimod (NBP2-26228), LPS (NBP2-25295), MALP-2 (NBP2-26219), Pam3CSK4 (NBP2-25297), and Poly(I). Poly(C) (TLR3 ligand: NBP2-25288).

TLRs in Cell Survival & Death Pathways

The data in Figure 34 also shows that the agonists flagellin (TLR5) and loxoribine (TLR7) did not inhibit apoptosis. However western blot analysis showed that PMN expressed both TLR5 and TLR7 (Fig 35). Taken together, the agonist and western blot results suggest that although TLR5 and TLR7 were expressed, they were apparently not linked to cell survival pathways in PMN. TLR3 agonists were not used because PMN did not express TLR3 (Fig 35).

Further analysis showed that the apoptosis delaying TLR agonists activated the PI3K-Akt cell survival signaling pathway. This activation was associated with a PI3K-dependent increase in Hsp27 and a PI3K-dependent phosphorylation of the Bcl-2 family member Bad. Hsp27 is thought to be important to PMN survival, and phosphorylation of Bad suppresses apoptosis and promotes cell survival.

The TLR-induced delay in apoptosis was also associated with increased levels of Mcl-1 and A1 which are anti-apoptotic Bcl-2 family members. The anti-apoptotic action of the TLR agonists required activation of both the PI3K-Akt and NF-κB pathways as evidenced by reversal of agonist anti-apoptotic effects of PI3K-Akt and NF-κB inhibitors.

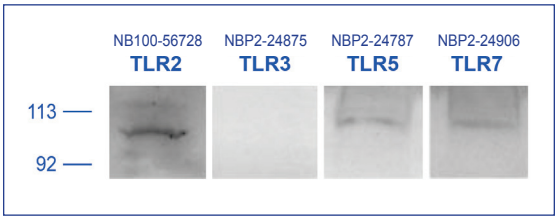


Figure 35. Western blot analysis of TLRs 2, 3, 5 and 7 in human PMN.
This western blot was performed because TLR5 (Flagellin) and TLR7 (Loxoribine) ligands were unable to inhibit spontaneous apoptosis in PMN whereas a number of other TLR ligands such as PGN (TLR2) were (Fig. 34). Therefore, the authors wanted to study the status of TLRs 5 and 7. The data shows that both TLRs 5 and 7 were expressed in PMN. Taken together with the data in Fig 34, these results suggest that the TLR5 and and TLR7 expressed in PMN were not linked to apoptosis inhibition. Source: Francois et al, 2005.

Note: TLR2 and TLR3 were used as positive and negative western blot controls, respectively. Note: Hyashi et al (2003) found that human PMNs express TLRs 1-2 and 4-10, but not TLR3 mRNA by QPCR analysis. However, other studies including Wong et al (2007) detected.

TLRs & Immune Tolerance

The immune system has evolved at least three mechanisms to ensure tolerance to self-antigens: central, peripheral, and Treg-linked (reviewed in Pasare and Medzhitov, 2003; Sakaguchi et al, 2008, and Westerberg et al, 2008). Tolerance prevents the activation of lymphocytes specific to host-derived antigens, and downstream autoimmunity whereby the immune system attacks its own antigens (self).

Tolerance Mechanisms

Immune tolerance is not simply a failure to recognize an antigen, but rather is an active response to a particular epitope. Tolerance responses are considered just as specific as immune responses.

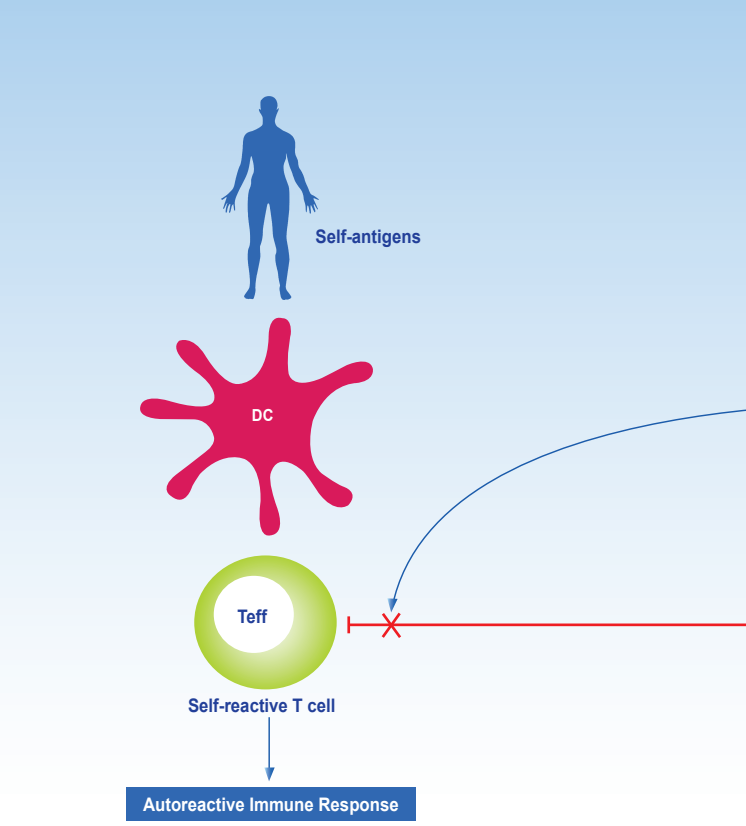
1. Central tolerance: Central tolerance occurs in the thymus (T cells) and bone marrow (B cells) as maturing lymphocytes are exposed to self antigens. During central tolerance, cells recognizing self antigens are clonally deleted before they develop into fully immunocompetent mature lymphocytes, preventing autoimmunity.

Although clonal deletion ensures that most self-reactive T cells are deleted in the thymus, the mechanism isn't perfect and some self-reactive cells mature and escape into the periphery where they are potentially vulnerable to T cell priming. T cell priming occurs following the first encounter of mature naïve T cells with antigen peptides on APCs, and results in the induction of a primary immune response. However, priming of self-reactive T cells that have inadvertently escaped in the periphery can result in hyperimmune responses or lead to autoimmune disease. Fortunately, the mechanisms of peripheral tolerance are designed to prevent priming of self-reactive mature T cells.

2. Peripheral tolerance: Mature T cells in the periphery ignore or "tolerate" immature APCs presenting self antigens, but lacking expression of co-stimulatory molecules like B7 which are induced in APCs during inflammation. Peripheral tolerance can be maintained because APCs remain in a relatively immature state during immune homeostasis.

T cell priming is induced only when both peptide-MHC complexes and B7 molecules are expressed on the same APC, and engage TCR and CD28 on mature T cells. DCs, prior to their activation into APCs, act as sentinels in the peripheral tissues where they scan for the presence of pathogens. DCs express low levels of MHC and co-stimulatory molecules. DCs also express TLRs and when TLRs are activated by PAMP or DAMP ligand binding (indication of infection or injury), TLR signaling triggers a DC maturation process. DCs differentiate into APCs by upregulating MHC molecules bearing pathogen-derived peptides and B7 co-stimulatory molecules. APCs then migrate to the nearest draining lymph node to prime pathogen-specific T cells. Thus, this second mechanism of tolerance (central tolerance being the first) is based on tight control of co-stimulation of DCs in the periphery, which ensures that self-tolerance is maintained in the absence of infection.

TLRs & Immune Tolerance



As part of their life cycle, DCs also migrate to the lymph nodes in the absence of infection. Self-reactive T cells that come into contact with these DCs either ignore them or become anergized. Anergy is a reduction or lack of an immune response to a specific antigen and consists of a direct induction of peripheral tolerance. There is evidence to suggest that self-reactive T cells that ignore DCs or become anergized can become Tregs.

3. Treg linked tolerance: This mechanism of Treg linked tolerance is based on the ability of different subsets of Tregs to suppress self-reactive cells. This concept is illustrated in Figure 36. Tregs express TLRs and the activation of TLRs on Tregs can increase or decrease Treg suppressive activity as discussed in the chapter 'TLR Expression in Tregs' and is illustrated in Figure 27. In the absence of infection, when there are no TLR signals, CD4+CD25+ Tregs directly suppress self-reactive T-cell responses. CD4+CD25+ Tregs (natural Tregs) develop in the thymus under the control of FOXP3 and are found in all the secondary lymphoid organs. It is thought that CD4+CD25+ Tregs evolved to suppress self-reactive T cells that escaped the thymus. The importance of Tregs in tolerance is underscored by the association of self-reactive (automimmune) diseases such as colitis, diabetes, gastritis and thyroiditis with a lack of CD4+CD25+ Treg cells.

The downside of their suppressive function is that CD4+CD25+ Tregs can potentially also suppress pathogen-specific T cell responses, because they recognize a broad repertoire of antigens. That is, these Tregs can not necessarily distinquish between self antigens and PAMPs. Hence, Treg suppression of legitimate T cell responses to PAMPs must be overcome in order for immune resonses to be launched during infection. TLRs play a role in this by indirectly suppressing the activity of Tregs through DC activation: TLR activated DCs present pathogen-derived peptides, and these DCs overcome CD4+CD25+ Treg mediated suppression by secreting IL-6 and other cytokines which leads to T-cell priming.

On the flipside, continual TLR signaling and cytokine productiion during chronic infections can inadvertenly lead to priming of bystander self-reactive T cells causing subsequent destruction of host-tissues and autoimmune disease. This destruction can be managed by Tr1 and

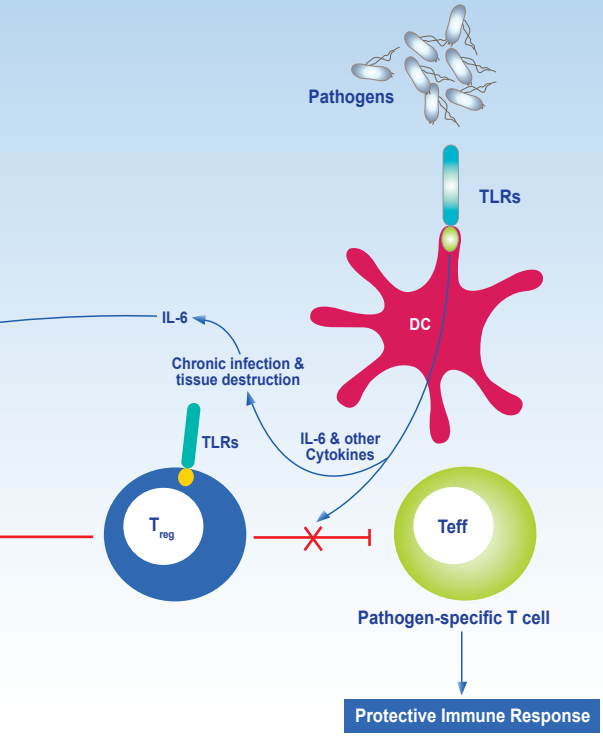


Figure 36. Tregs and TLRs: maintaining and breaking tolerance to self antigens. *TLRs are thought to play an important role in T cell priming and autoimmunity. DCs migrate to lymph nodes during steady-state conditions and present self-antigens (self-peptides) on their MHC. However, the priming of Teffs reactive to self-peptides is blocked by Tregs.*

During infection the PAMPs of pathogens activate TLR signaling on DCs, leading to DC activation. TLR activated DCs that present pathogen derived peptides overcome Treg suppression by secreting IL-6 and other cytokines. This leads to Teff priming and protective immune responses. However, pesistent TLR signaling and cytokine production during chronic infections can lead to inadvertent priming of bystander autoreactive Teffs. This can lead to destruction of host tissue and autoimmune disease. Adapted from Pasare and Medzhitov, 2003.

Th3 cells which are involved in suppressing chronic or excessive inflammatory processes. Tr1 and Th3 cells express TLRs, and TLR activation enhances their activity which can in turn help suppress bystander T cell activity during chronic T-cell priming. TLR-induced tolerance through upregulation of Tr1 and Th3 activity may be important in inhibiting excessive immune TLR responses resulting from persistent microbial infections. This could be considered to be a negative TLR feedback mechanism to prevent the host from continuing to respond to excessive TLR signaling.

TLR-induced tolerance and therapeutics?

It has been discussed throughout this handbook that TLR stimulation results in the activation of innate immune cells, leading to the production of proinflammatory cytokines. However, repeated signaling through TLRs can result in a reduction of subsequent proinflammatory cytokine responses. This phenomenon, known as TLR tolerance, was actually identified prior to the discovery of TLRs and called endotoxin tolerance. TLR/endotoxin tolerance is considered to be a homeostatic phenomenon. It is thought to help maintain homeostasis in the gut and liver which are continually exposed to a range of microbial components from commensal organisms in the gut (reviewed in Abreu et al, 2006).

The induction of TLR tolerance is emerging as a potentially viable therapeutic approach for subduing inflammation in allergy and autoimmune diseases, as well as other diseases including cancer. For example, the imidazoquinoline S28690 (a synthetic TLR7 agonist) induced TLR7 tolerance in malignant B-CLL cells and sensitized them to cytotoxic chemotherapeutic agents (Shi et al, 2007). The chemotherapeutic drug vincristine induced more cell death in tolerized than non-tolerized cultures.

S28690 is actually a TLR7/8 agonist, but primary CLL cells do not express TLR8. Hence S28690 is considered to activate TLR7 but not TLR8 signaling in CLL cells. In the study by Shi et al, TLR7-induced tolerance was demonstrated by showing that primary stimulation with S28690 resulted in TNFα production, but lack of TNFα production upon restimulation. These results suggested that induction of tolerization in TLR7+ cancer cells may be a viable stategy for increasing their susceptibility to cytotoxic chemotherapeutic drugs.

Concepts of TLRs in Disease

TLR activation is thought of as a double-edged sword because it is linked to both health and disease. TLR activation is best known for its role in host defense against pathogens where it activates multiple inflammatory pathways and coordinates systemic responses that eliminate infectious agents. This role of TLR activation in host defense is integral for the health of the host.

However, the ability of TLRs to recognize DAMPs released during disease progression or following tissue injury can set up cycles of destructive inflammatory responses. Excessive secretion of pro-inflammatory cytokines upon TLR activation can exacerbate microbial infections and lead to septic shock, tissue damage and also undermine protective immunity through increased function of regulatory T cells. Hence, TLR signaling must be tightly regulated in order to protect the host from immune and inflammation-linked disorders. Otherwise, inappropriate TLR activation followed by overproduction of cytokines and other downstream signaling events can result in the induction, progression or exacerbation of infectious diseases, autoimmune and immunodeficient diseases, inflammatory disorders, dementia, cancer and other diseases. Table IX provides an overview of TLRs linked to various diseases.

There is a growing body of literature focusing on elucidating the underlying mechanisms involved in TLR-linked pathology. For example, TLR activation appears to contribute to Alzheimer's disease (AD) pathogenesis by promoting the production of inflammatory mediators and the uptake of AD associated amyloid β peptides (Aβ) (reviewed in Salminen et al, 2009). TLR4 expression is increased in the brain tissue of AD patients and AD transgenic mice. Additionally, microglial cells are involved in the inflammatory pathogenesis of AD, and Aβ have been shown to enhance both TLR2 and TLR4-induced inflammatory responses in mouse microglial cells.

Although the precise interaction and activation of TLRs with Aβ is undefined, it is now recognized that inflammation is a significant pathological factor and not merely a bystander in AD and other neurodegenerative diseases. Pathogenesis may result from chronic activation of the innate immune system, which is perpetuated by the release of DAMPs, including Aβ, from dying tissue. In this scenario, DAMPs then activate TLR signaling leading to more inflammation, cell death, more DAMP release and more TLR activation *ad nauseum*.

Kim et al (2008) developed an *in vitro* TLR3 model system to explore the concept that DAMP-linked inflammation plays a causal role in neurodegenerative disease pathology. Human cellular RNA is a potential TLR3 DAMP ligand, and it is known that RNA is a

Table IX. TLRs in Disease

TLR	PATHOLOGY	REFERENCE
TLR1	Polymorphisms Bacterial and fungal infections leading to sepsis	Am J Respir Crit Care Med 178:710-720 (2008)
	Renal disease	J Am Soc Nephrol 15:854–867 (2004)
	Skin diseases due to bacterial infection like: Leprosy Psoriasis Lyme disease	J Invest Dermatol 125:1-8 (2005)
TLR2	Gastrointestinal tract disorders due to bacterial infection by: Helicobacter pylori Shigella flexneri Salmonella Entamoeba histolytica	Infect Immun 72:6446–6454 (2004) Infect Immun 69:6248–6255 (2001) Infect Immun 71:6058–6062 (2003) Parasite Immunol 27:127–137 (2005)
	Polymorphism Arg677Trp and bacterial infection leading to skin diseases including: Lepromatous leprosy in a Korean population Lyme disease Psoriasis Acne vulgaris	Hum Genet 116:413–415 (2005) Immunol Med Microbiol 31:53–58 (2001) J Invest Dermatol 125:1-8 (2005)
	Polymorphism Arg677Trp, Arg753Gln and bacterial infection leading to: Respiratory disorders including tuberculosis Reduced responses to Gram-positive bacteria	Clin Diagn Lab Immunol 11:625–626 (2004) Immunol Cell Biol 84:333–341 (2006) Eur Respir J 23:219–223 (2004)
	Polymorphism Arg753Gln and bacterial infection leading to: Sepsis Staphylococcal disease	Infect Immun 68:6398–6401 (2000)
	Renal Diseases including: Sepsis-induced ATN Renal infection CAPD peritonitis Toxic or immune injury Ischemia-reperfusion ATN Immune complex GN or renal vasculitis	J Am Soc Nephrol 15:854–867 (2004)
	Respiratory tract disorders due to bacterial and fungal infection by: Chlamydia pneumoniae Mycobacterium tuberculosis Legionella pneumophila Cryptococcus neoformans	Eur JI 36:1145–1155 (2006) Am J Pathol 164:49–57 (2004) Infect Immun 74:3325–3333 (2006) Infect Immun 72:5373–5382 (2004)
	Viral infection leading to: Genital herpes	J Infect Dis 196:505–509 (2007)
TLR3	Autoimmune disorder: Rheumatoid arthritis	Arthritis Rheum 52:2656–2665 (2005)
	Renal diseases: Hepatitis-associated glomerulonephritis HIV nephropathy glomerulonephritis	J Am Soc Nephrol 15:854–867 (2004)
	Respiratory tract disorders due to viral infection by: HSV encephalitis Influenza A virus	Science 317:1522–1527 (2007) PLoS Pathog 2006;2:e53

Concepts of TLRs in Disease

Table IX. TLRs in Disease

TLR	PATHOLOGY	REFERENCE
TLR4	Autoimmune disorders including: Rheumatoid arthritis Atherogenesis SLE Systemic inflammatory response syndrome Experimental autoimmune encephalomyelitis	JI 176, 7021–7027 (2006) New Engl J Med 347:185–192 (2002) Circulation 104:3103-3108 (2001)
	Gastrointestinal tract disorders due to bacterial infection by: H. pylori Entamoeba histolytica Salmonella	JI 173:1406–1416 (2004) Parasite Immunol 27:127–137 (2005) Infect Immun 71:6058–6062 (2003)
	TLR4s and viral infection leading to: RSV bronchiolitis	J Infect Dis 189:2057–2063 (2004)
	Polymorphism in Asp299Gly/Thr399Ile leading to disorders: Hyporesponsiveness to LPS Predisposition to severe malaria among African children	Nat Genet 25: 187–191 (2000) J Commun Dis 38:230–245 (2006)
	Polymorphism and bacterial and viral infection leading to: Susceptibility to infection with gram-negative bacteria Malaria Meningococcal sepsis Legionnaires' disease	PNAS 103:177–182 (2006) PNAS 100:6075–6080 (2003) J Exp Med 198:1563–1572 (2003)
	Renal diseases including: Immune complex GN or renal vasculitis Interstitial fibrosis Ischemic, toxic, or obstruction-related tubular injury Renal infection CAPD peritonitis Sepsis-induced tubular necrosis	J Am Soc Nephrol 15:854–867 (2004)
	Respiratory tract disorders due to bacterial and fungal infection by: Mycobacterium tuberculosis Cryptococcus neoformans	Microbes Infect 8:1790–1800 (2006) Infect Immun 72:5373–5382 (2004)
TLR5	Autoimmune disorders including: SLE Psoriasis (Skin Disease)	PNAS 102:10593–10597 (2005) J Invest Dermatol 125:1-8 (2005)
	Gastrointestinal tract disorders due to bacterial infection by: H. pylori Enteroaggregative E. coli	Int J Med Microbiol 295:179–185 (2005) Immunol 112:651–660 (2004)

Table IX. TLRs in Disease

TLR	PATHOLOGY	REFERENCE
TLR5	Polymorphism leading to respiratory disorder including: P392STOP leading to susceptibility to Legionnaires' disease	J Exp Med 198:1563–1572 (2003)
	Renal infection	J Am Soc Nephrol 15:854–867 (2004)
TLR6	Renal infection	J Am Soc Nephrol 15:854–867 (2004)
	Skin Infection leading to: Lyme disease	J Invest Dermatol 125:1-8 (2005)
TLR7	Renal infection	J Am Soc Nephrol 15:854–867 (2004)
	Viral infection leading to: Hepatic fibrosis in chronic HCV infection	J Hepatol 47:203–211 (2007)
	Viral infection leading to: Asthma	Clin Exp Allergy 34:1314-1320 (2004)
TLR8	Polymorphism leading to coronary artery disease (CAD)	Mol Biol Rep 36(7):1897-1901 (2009)
	Renal infection	J Am Soc Nephrol 15:854–867 (2004)
TLR9	Autoimmune disorders: Susceptibility to SLE Rheumatoid arthritis Experimental autoimmune encephalomyelitis	Ann Rheum Dis 66:905–909 (2007) Eur JI 38: 565–575 (2008)
	Gastrointestinal tract disorders due to bacterial infection by: H. pylori	Int J Med Microbiol 295:179–185 (2005)
	Increased risk of low birth weight in malaria during pregnancy	J Infect Dis 194:184–188 (2006)
	Pulmonary disorders including: Asthma Allergy	J Exp Med 202:1715–1724 (2005)
	Renal diseases including: Immune complex GN or renal vasculitis CMV-induced allograft dysfunction BK virus-induced allograft dysfunction HBV-associated vasculitis GN or lymphoma Drug-induced SLE Lupus nephritis	J Am Soc Nephrol 15:854–867 (2004)
	Respiratory tract disorders due to bacterial infection: Mycobacterium tuberculosis	J Exp Med 202:1715–1724 (2005)
	Viral infection leading to: Rapid progression of HIV infection	AIDS 21:441–446 (2007)

component of AD plaques. Therefore, RNA released from dying necrotic cells could potentially activate TLR3 signaling in brain cells expressing TLR3 receptors. Kim et al (2008) used an Novus TLR3 mAb (IMG-315) to show that TLR3 was expressed in primary human astrocytes (Fig 37). TLR3 expression was upregulated by inflammatory stimulation including IFN-γ (Fig 37), poly(I).poly(C) or LPS treatment. Their results supported the concept that TLR3 signaling could potentially play an active role in neurodegeneration by participating in and perpetuating chronic inflammation and cell death cycles.

Concepts of TLRs in Disease

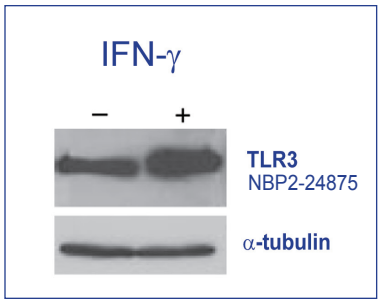


Fig 37. Western blot analysis of TLR3 in normal human primary astrocytes using NBP2-24875 (Clone 40C1285.6). *Cultures were left untreated or treated with INF–γ for 5 hr (1000 U/ml). The blots show that the human astrocytes express TLR3 which can be upregulated with INF--γ treatment. Source: Kim et al, 2008.*

It should be kept in mind that much of what is known about the mechanisms of TLR signaling in disease comes from mouse and other animal models. It can, however, be difficult to extrapolate data from animal studies and apply it to human disease (reviewed in Sabroe et al, 2008). Humans and animals are susceptible to different pathogens. Hence, signaling pathways and disease patterns induced by a particular pathogen may vary between species.

Additionally, heterogeneous genetic backgrounds and complex environmental factors render the studies of aberrant TLR signaling in human diseases more complicated than in TLR disease models using inbred mice. Mapping the strengths and weaknesses of animal models as they pertain to human health and disease is a key goal for the TLR field.

The identification of polymorphisms and mutations in TLRs (Table IX) is an important avenue for elucidating the role of TLRs and underlying mechanisms in human pathology. An increasing body of literature is supporting associations betweeen single nuclear polymorphisms (SNPs) in TLRs and the increased risk of bacteria or viral infections (reviewed in Garanziotis et al, 2008, and Misch and Hawn, 2008). For example:

1. **TLR4 Asp299Gly:** This single SNP results in the replacement of a conserved aspartic acid residue with glycine at amino acid 299. The substitution alters the extracellular domain of the receptor. The extracellular domain SNP Thr399Ile often cosegregates with Asp299Gly. Together, these polymorphisms have been linked to decreased airway sensitivity to inhaled LPS. Additionally, *in vitro* studies showed that cells with the Asp299Gly polymorphism have defective responses to LPS.

   It is thought that the Asp299Gly and Thr399Ile polymorphisms increase susceptibility for diseases linked to endotoxin exposure, including asthma, allergy, and sepsis. They also increase risk for severe malaria which are examples of TLR polymorphisms affecting disease progression rather than overall disease susceptibility.

2. **TLR2 Arg753Gln:** This is the most studied TLR2 SNP and is located within the intracellular TIR domain. It results in decreased TLR2 immune stimulation with bacterial lipopeptide, and has been associated with increased risk of cytomegalovirus infection, leprosy and tuberculosis.

3. **TLR5 392STOP:** This mutation changes the arginine at amino acid 392 to a stop. It is predicted to truncate TLR5 in the extracellular domain, causing the loss of the transmembrane domain and the entire signaling cytoplasmic tail. It is associated with susceptibility to infection by flagellated organisms, including increased risk for Legionnaires' disease.

4. **TLR9 1635A/G and 1174G/A:** These SNPs in linkage disequilibrium were associated with rapid progression of HIV-1 infection. This is another example of TLR polymorphisms, like TLR4 polymorphisms in malaria, affecting disease progression rather than disease acquisition.

TLR SNPs can also hinder the development and progression of disease. For example the TLR4 Asp299Gly SNP that renders the receptor less responsive to ligands has been associated with decreased atherosclerosis, decreased risk for heart attack, and improved response to statin treatment. Although the exact mechanism resulting in the benefical effect of this SNP is unknown, it may be linked to a reduction of the TLR signaling induced inflammatory processes that are associated with the development of atherosclerosis.

SNPs in TLR signaling pathway molecules have also been linked to disease. For example, IRAK4 (stop codon at 287) and IκBα (missense Serine 32) SNPs have been associated with increased risk for pneumonia and immunodeficiency, respectively.

The expanding body of data related to TLRs is uncovering important relationships between human disease and TLR signaling pathways. The knowledge of how TLR genetics relates to TLR signaling and disease should be helpful for developing strategies for individualized medicine, including prophylactic and disease treatment.

Concepts of TLRs in Disease

TLRs IN SEPSIS

In 1992, sepsis was defined by a Critical Care Medicine consensus panel as the systemic inflammatory response syndrome (SIRS) due to infection (reviewed in Kessel et al, 2009). The infection may be bacterial, viral, fungal, or parasitic. SIRS disrupts immune homeostasis by inducing an initial intense systemic inflammatory response, amplified through signaling cascades (reviewed in Kessel et al, 2009). The initial phase rapidly leads to negative feedback of anti-inflammatory processes, resulting in a secondary SIRS phase where immune responses are inhibited.

The secondary inhibition phase of SIRS has been referred to as an 'immunoparalysis state', a.k.a systemic endotoxin tolerance. This state is characterized by a decrease of phagocytes and B, T and NK cells, as well as a reduction in proinflammatory cytokine release and antigen presenting capacity. Persistence of immunoparalysis decreases host resistance to secondary infections and greatly increases mortality risk. However, sepsis exists on a clinical continuum from low grade with self limited infection to severe infection with multiple organ failure and increased risk of death. Treatments are often suboptimal and sepsis continues to be a leading cause of mortality in intensive care units worldwide. Elucidating the underlying mechanisms of SIRS is key for developing effective treatment strategies and improving the outcome of sepsis patients. The immunoparalysis state is of particular interest because inhibition of the immune response is a key contributor to increased risk of sepsis associated morbidity and mortality.

In this regard, it is known that CD4+CD25+ Tregs play an important role in maintaining immunologic tolerance to self and foreign antigens by suppressing aggressive Teff responses. Emerging evidence suggests CD4+CD25+ Tregs can actively suppress adaptive immune responses, and may be a causal factor in septic immunoparalysis. In this regard, studies have found a relative increase in the number of CD4+CD25+ Tregs during sepsis, as well as an increase in their suppressive function. Other studies showed a higher level of FOXP3 expression in Tregs from sepsis patients compared to controls. FOXP3 is important for CD4+CD25+ Treg function and increased FOXP3 levels have been correlated with increased suppressive activity.

Since TLR signaling can both enhance and reduce the ability of CD4+CD25+ Tregs to suppress Teffs as shown in Figure 27, leveraging TLR pathways might be a viable strategy for fine tuning Treg function to manage both the hyperimmune and immunoparalysis states associated with sepsis. Figure 38 shows TLRs within the context of a model showing the interactions between Tregs and effector cells during sepsis. In this model, Tregs decrease Th1 proliferation function and increase monocyte and neutrophil apoptosis. Increased expression of FasL and Fas was found on the PBMCs of sepsis patients, providing a putative mechanism for apoptosis induction through activation of the Fas death pathway. Tregs are thought to be

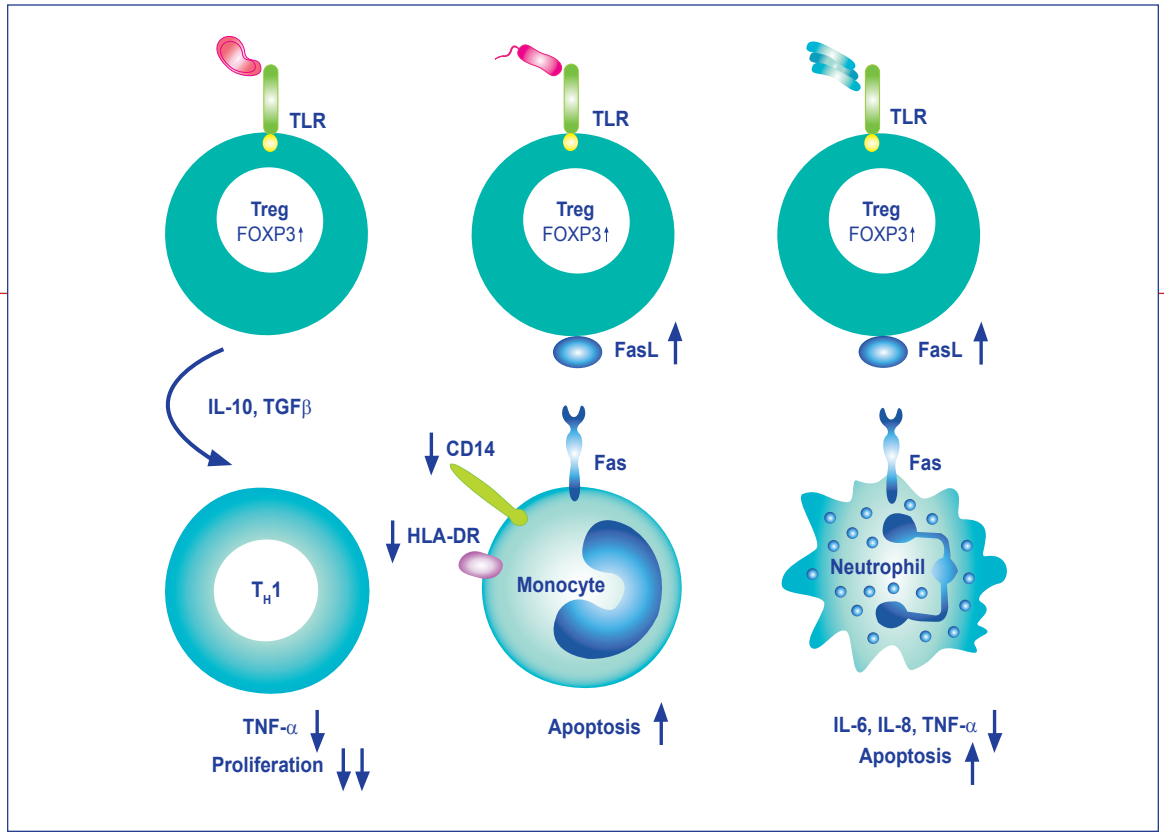


Figure 38. Treg and effector cell interactions during sepsis. *The suppressive function and numbers of CD4+CD25+ Tregs increases during sepsis. TLR signaling and FOXP3 expression is correlated with the extent of Treg suppressive function. An increase in Treg suppressive function decreases Th1 proliferation and secretion of the inflammatory cytokine TNFα. It also increases monocyte (Mφ) and neutrophil (N) apoptosis as well as decreases TNFα, IL-6 and IL-8 secretion by neutrophils. Adapted from Kessel et al, 2009.*

involved in the downregulation of CD14 and HLA-DR receptor expression on monocytes observed in sepsis patients. Since down regulation of the CD14 receptor also triggers monocyte apoptosis, this could be a cell death mechanism that occurs during sepsis. Down regulation of HLA-DR on monocytes results in reduced proinflammatory cytokine release and antigen-presenting capacity and could be a contributing factor to decreased monocyte function during sepsis.

It is important to note that there are discordant results within the literature regarding the roles of Tregs in sepsis, as well as some concerns about interepretation of results. Hence, additional work is needed to determine if Treg manipulation would be an effective therapeutic approach.

In a related body of sepsis literature, it is thought that TLRs may also play a role in coordinating responses of the adrenal and immune system essential for survival during sepsis (reviewed in Tran et al, 2007). Steroids released from the adrenal glands help prevent excessive proinflammatory immune responses in sepsis patients. However, failure of the hypothalamic-pituitary-adrenal (HPA) axis and adrenal insufficiency occurs in many

sepsis patients leading to increased risk of mortality. The molecular mechanisms underlying HPA axis failure and insufficient adrenal function during inflammation and their potential relationship to TLRs is being investigated.

For example, it is known that bacterial DNA containing CpG motifs can initiate an innate immune response through TLR9, potentially leading to septic shock. However, historically little has been known about the relationship between TLR9 signaling and the adrenal glands. Tran et al (2007) found that TLR9 was expressed in both murine (Fig 39) and human adrenal glands. In adrenal function studies, the CpG-ODN TLR9 agonist led to a corticosterone and inflammatory response in WT but not in TLR9-/- mice (Table X). Taken together with the IHC results, these results show that TLR9 is expressed in the adrenal cortex and suggest that TLR9 plays a role in regulating the HPS axis when bacteria are present. The authors also established the Y-1 adrenal cortex tumor cell line as an *in vitro* adrenal model system (Fig 40 and Table X). The Y-1 cells, like the WT mice, respond to CpG-ODN challenge by increasing cytokine production.

TLR research findings are contributing to major advances in understanding the pathogenesis of septic shock. Coupled with the high failure rate of conventional sepsis treatments, this has led to the concept of TLR pathway based therapeutics. Many therapeutic strategies are based on leveraging TLR antagonists to counteract

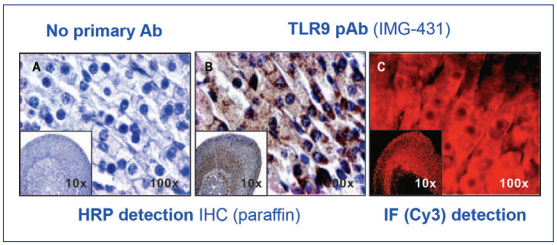


Figure 39. Immunohistochemical staining of formalin-fixed, paraffin embedded adrenal tissue from WT mice using TLR9 pAb (IMG-431). *TLR9 staining was detected in the adrenal cortex, particularly in the zona fasciculate, but not in the adrenal medulla. A. Adrenal gland sections without the TLR9 antibody were used as a negative control. B. Overview (10X), and C. Zona fasciculata (100X).These authors validated the specificity of the TLR9 antibody by western blot (see Figure 9). Source: Tran et al, 2007.*

the excessive pro-inflammatory responses associated with sepsis and other systemic microbial infections. This includes using soluble TLRs to bind and neutralize microbial ligands, small molecules or antibodies to block ligand-receptor binding or binding of receptor-protein complexes. Peptide and peptidomimetic inhibitors are also being used to block the common signaling pathways activated by ligand–TLR interactions.

For example, studies on MyD88-deficient mice have shown LPS activation of TLR4 plays a major role in the pathology of sepsis. Thus, interruption of TLR4 signaling may inhibit the toxic effects of LPS. Additionally, CD14 monoclonal antibodies are being tested in humans

Table X. Cytokine levels in plasma from WT and TLR9-/- mice and in Y-1 cell supernatants in response to the TLR9 agonist ligand CpG-ODN.

MICE						
	WT		TLR9$^{-/-}$		Mouse Adrenal Y-1 Cell Line	
Cytokine (pg/ml)	Plasma				Cell Supernatant	
	Control	120 min	Control	120 min	Control	24 h
GM-CSF	10 ± 2	46 ± 12	23 ± 12	29 ± 6	31 ± 4	202 ± 11
IL-1β	6 ± 2	7 ± 2	nd	0.5 ± 0.5	56 ± 30	534 ± 6
IL-2	45 ± 12	94 ± 31	13 ± 5	22 ± 4	4 ± 4	nd
IL-4	1 ± 0.3	18 ± 11	nd	nd	2 ± 1	0.9 ± 0.9
IL-5	9 ± 2	90 ± 24	11 ± 3	61 ± 19	nd	nd
IL-6	11 ± 2	2349 ± 12	5 ± 2	75 ± 19	48 ± 21	821 ± 36
IL-10	5 ± 1	186 ± 65	nd	nd	nd	nd
IL-12	180 ± 15	5302 ± 168	174 ± 60	190 ± 71	nd	183 ± 16
IFN-γ	0.1 ± 0.1	10 ± 3	0.1 ± 0.1	0.3 ± 0.2	nd	nd
TNFα	8 ± 2	243 ± 78$^{\alpha}$	2 ± 1	4 ± 2$^{\beta}$	288 ± 140	5118 ± 158

The data shows that WT and TLR9-/- control mice had similar basal levels of cytokines. CpG –ODN challenge in WT mice caused a significant increase in all cytokines (except IL-1β and IL-2) ranging from 5 to 213 fold as compared to control mice. In contrast, only IL-5 and IL-6 increased in TLR9-/- mice and the increase in IL-6 was much less dramatic than that in TLR WT mice.
Note: Mice were IP injection sensitized with D-Galn for 30 min prior to IP injection of saline or CpG-ODN and then challenged with either saline (control) or CpG-ODN or for 2 hr. The levels of a number of cytokines in the Y-1 supernatant of the mouse adrenal cortex Y-1 cell line also increased following CpG-ODN treatment. Taken together, these results suggest that activation of the TLR9 receptor is associated with an adrenal response both in vivo and in vitro. Novus offers both human (NBP2-26232) and mouse (NBP2-26235) CpG-ODN. Source: Tran et al, 2007.

as a potential therapy for sepsis. Moreover two lipid A analogs that act as potent antagonists of TLR4 have advanced into clinical trials for sepsis. The pertubation of TLR activation and downstream signaling pathways for preventing or treating sepsis is a major scientific advance and should help to reduce the high rate of morbidity and mortality associated with sepsis.

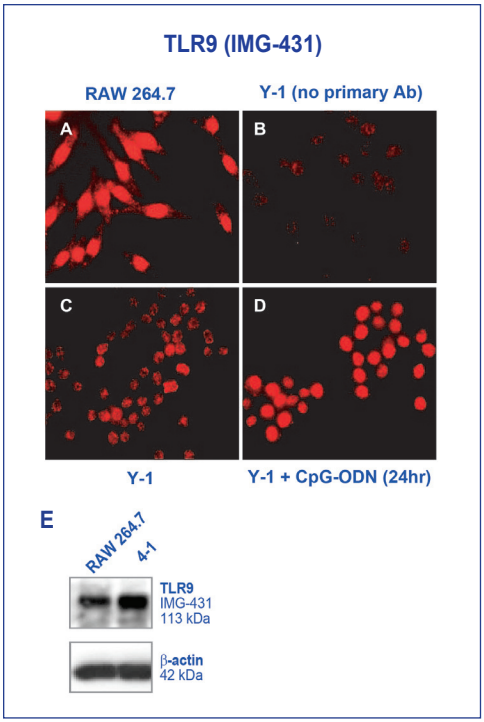


Figure 40. Immunofluorescence and western blot analysis in Y-1 and RAW 264.7 cells using TLR9 pAb (IMG-431). *RAW cells were used as a positive control for TLR9 expression for both imunofluorescence microscopy (A) and western blot analysis (E). Y-1 cells were left untreated (B, C, E) or treated for 24 h (D) with CpG-ODN. B. Y-1 cells without primary antibody were used as a negative control. The data shows the Y-1 cells constitutively expressed TLR9 (C, E) and that immunofluorescence staining intensity increased (D) following CPG-ODN stimulation.*

Beta-actin was used as a protein loading control antibody. These results, taken together with Table X showing cytokine upregulation in Y-1 following CPG-ODN treatment suggest that Y-1 may be useful as an in vitro sepsis model system. Source: Tran et al, 2007.

Novus offers both human (NBP2-26232) and mouse (NBP2-26235) CpG-ODN. The Western Blot Loading Control Kit (NBP2-25090) containing a Beta Actin polyclonal antibody (NBP2-24780) is recommended for detecting actin or other housekeeping proteins.

Concepts of TLRs in Disease

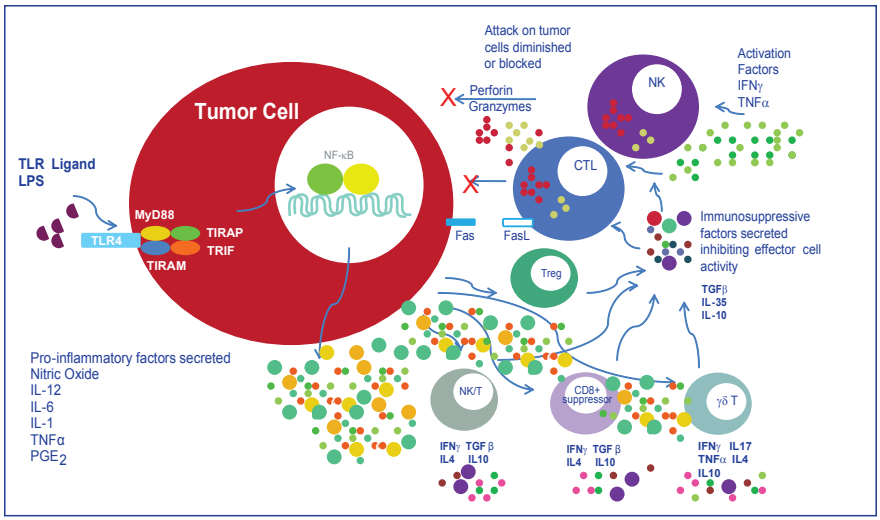


Figure 41. TLRs, inflammatory processes and cancer. *Cancer cells can express functional TLRs. PAMP and DAMP activated TLR signals on cancer cells may promote cancer progression, inhibition of apoptosis or other cell death pathways, or resistance to host immune responses.*

TLRs IN TUMORIGENESIS

It is increasingly recognized that inflammatory processes play a key role in tumorigenesis. TLRs, as in other human diseases, appear to act as double edged swords in tumorigenesis (reviewed in Conroy et al, 2008 and Huang et al, 2008). For example, infection and TLR activation have been linked to both tumor inhibition and tumor promotion.

Tumors are infiltrated with various types of immune cells, and immune cells can be the major cell population in the tumor microenvironment. Although tumor cells are dependent on the microenvironment for growth and survival signals, tumor cells are also under immune surveillance. Immune cells in the microenvironment can recognize tumor cells as foreign, and mechanisms such as TLR signaling can be activated to inhibit or destroy them.

However, tumors have multiple mechanisms that evade or overcome immune surveillance and avoid attack by the host's immune system. Emerging evidence suggests that TLR signaling is among these mechanisms. Figure 41 shows an overview of how TLR signaling may participate in helping tumor cells evade attack by the host immune

Table XI. TLR Expression and Polymorphisms in Tumors

TLR	TYPE OF CANCER	NOTES	APPLICATION	REFERENCES
TLR1	Colon cancer (RNA)	Low Expression	RT-PCR	Immunol 115:565–574 (2005)
	Lymphoma (DNA)	Single-nucleotide polymorphism	Functional Assay	Genes Immun 7:615–624 (2006)
	Prostate cancer (DNA)	Single-nucleotide polymorphism	Functional Assay	J Natl Cancer Inst 97:525–532 (2005)
TLR2	Colon cancer (RNA, protein)	Slightly Increased Expression	RT-PCR, Functional Assay, IHC	J Int Med Res 29:409–420 (2001) Immunol 115:565–574 (2005)
	Gastric cancer (protein)	Moderate Expression	IHC	Mol Pharmacol 66:1465–1477 (2004)
	Hepatocellular carcinoma (RNA)	Increased Expression	RT-PCR	S Biol Pharm Bull 28:886–892 (2005)
	Laryngeal carcinoma (protein)	Increased Expression	IHC	Eur Arch Otorhinolaryngol 264:525–530 (2007)
	Lung cancer (RNA)	Weak Expression	RT-PCR	Mol Immunol 44:2850–2859 (2007)
	Lymphoma (DNA)	Single-nucleotide polymorphism	Functional Assay	Genes Immun 7:615–624 (2006)
	Ovarian tumor (protein)	Increased Expression	IHC	Cancer Immunol Immunother 58(9):1375-1385 (2009)
	Pancreatic carcinoma (Incompletely resectable)	Moderate Expression	Functional Assay	Br J Cancer 97:598-604 (2007)
TLR3	Breast cancer (RNA)	Moderate Expression	RT-PCR	JI 176:4894–4901 (2006)
	Colon cancer (RNA)	Increased Expression	RT-PCR	Immunol 115:565–574 (2005)
	Hepatocellular carcinoma (RNA)	Increased Expression	RT-PCR	S Biol Pharm Bull 28:886–892 (2005)
	Laryngeal carcinoma (protein)	Moderate Expression	IHC, IF	Eur Arch Otorhinolaryngol 264:525–530 (2007)
	Lung cancer (RNA)	Moderate Expression	RT-PCR	Mol Immunol 44:2850–2859 (2007)
	Melanoma (protein)	Increased Expression	IP, WB, Functional Assay	Clin Cancer Res 13:4565–4574 (2007)
	Ovarian tumor (protein)	Increased Expression	IHC	Cancer Immunol Immunother 58(9):1375-1385 (2009)
TLR4	Breast cancer (RNA)	Increased Expression	RT-PCR	Mol Immunol 44:2850–2859 (2007)
	Colon cancer (RNA)	Increased Expression	RT-PCR	Immunol 115:565–574 (2005)

system. Collectively, research studies suggest that TLRs as a family are involved in both inhibiting and promoting cancer (Rakoff-Nahoum and Medzhitov, 2009). Examples from the literature will be provided in this chapter.

Research focusing on TLRs in tumor biology is growing rapidly and TLRs as well as various polymorphisms have now been identified in a number of tumor types as summarized in Table XI. Tumor cell lines are also widely used to study TLRs, examples are shown in Tables V (protein) and VI (RNA).

Novus antibodies are widely used to analyze TLRs expression in both tumor tissue and tumor cell lines. For example, Droemann et al (2005) analyzed the expression of TLR9 in both human lung carcinoma and in the A549 human lung carcinoma cell line (Fig 42). The results showed

Table XI. TLR Expression and Polymorphisms in Tumors

TLR	TYPE OF CANCER	NOTES	APPLICATION	REFERENCES
TLR4	Gastric carcinoma (protein)	Increased Expression	IHC, Functional Assay	Int J Med Microbiol 295:179–185 (2005)
	Hepatocellular carcinoma (RNA)	Low Expression	RT-PCR	S Biol Pharm Bull 28:886–892 (2005)
	Laryngeal Carcinoma (protein)	Low Expression	IHC	Eur Arch Otorhinolaryngol 264:525–530 (2007)
	Lung cancer (RNA, protein)	Increased Expression	RT-PCR, FACS	Mol Immunol 44:2850–2859 (2007)
	Lymphoma (DNA)	Single-nucleotide polymorphism	Functional Assay	Genes Immun 7:615–624 (2006)
	Melanoma (RNA, protein)	Increased Expression	RT-PCR, IHC, Functional Assay	Cancer Lett 235:75–83 (2006)
	Nasopharyngeal carcinoma (RNA)	Increased Expression	RT-PCR	Cancer Biol Ther 5:1285–1291 (2006)
	Neuroblastoma (protein)	Moderate Expression	RT-PCR, FACS, Functional Assay	BMC Cancer 6:281 (2006)
	Ovarian tumor (protein)	Increased Expression	IHC, WB, RT-PCR	Cancer Immunol Immunother 58(9):1375-1385 (2009)
	Prostate cancer (DNA)	Single-nucleotide polymorphism	Functional Assay	Cancer Epidemiol Biomarkers Prev 16:352–355 (2007)
TLR5	Cervical squamous cell carcinoma (protein)	Increased Expression	IHC, Functional Assay	Int J Gynecol Cancer 18:300-305 (2008)
	Gastric carcinoma (protein)	Increased Expression	IHC, Functional Assay	Int J Med Microbiol 295:179–185 (2005)
	Lymphoma (DNA)	Single-nucleotide polymorphism	Functional Assay	Genes Immun 7:615–624 (2006)
	Ovarian tumor (protein)	Increased Expression in benign tumors, epithelial tumors, and ovarian cancer cell lines	RT-PCR, IHC	Cancer Immunol Immunother 58(9):1375-1385 (2009)
TLR6	Hepatocellular carcinoma (RNA)	Increased Expression	RT-PCR	S Biol Pharm Bull 28:886–892 (2005)
	Ovarian tumor (protein)	Variable Expression in benign tumors, epithelial tumors, and ovarian cancer cell lines	IHC	Cancer Immunol Immunother 58(9):1375-1385 (2009)

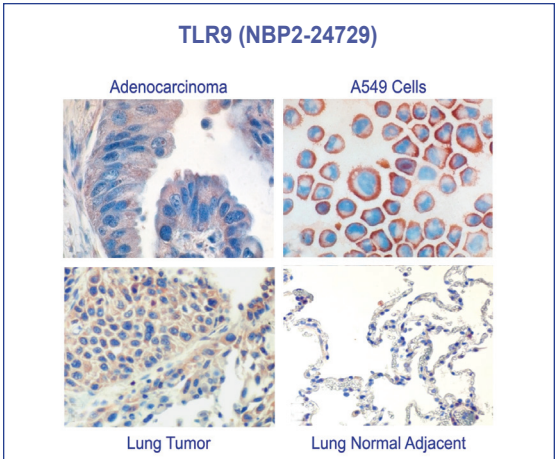
Table XI. TLR Expression and Polymorphisms in Tumors

TLR	TYPE OF CANCER	NOTES	APPLICATION	REFERENCES
TLR6	Pancreatic carcinoma (Incompletely resectable)	Moderate Expression	Functional Assay	Br J Cancer 97:598-604 (2007)
	Prostate cancer (DNA)	Single-nucleotide polymorphism	Functional Assay	J Natl Cancer Inst 97:525–532 (2005)
TLR7	Chronic lymphocytic leukemia (RNA, protein)	Moderate Expression	RT-PCR, WB	Leuk Lymphoma 46:935-939 (2005)
	Melanoma (IV stage) (protein)	Moderate Expression	Functional Assay	Clin Cancer Res 14:856-864 (2008)
	Ovarian tumor (protein)	Increased Expression	RT-PCR, IHC	Cancer Immunol Immunother 58(9):1375-1385 (2009)
TLR8	Chronic lymphocytic leukemia (RNA, protein)	Moderate Expression	RT-PCR, WB	Leuk Lymphoma 46:935-939 (2005)
	Ovarian tumor (protein)	Variable Expression in benign tumors, epithelial tumors, and ovarian cancer cell lines	RT-PCR, IHC	Cancer Immunol Immunother 58(9):1375-1385 (2009)
TLR9	Breast cancer (RNA, protein)	Increased Expression	RT-PCR, WB, IHC, FACS, Functional Assay	Mol Cancer Res 4:437–447 (2006)
	Cervical squamous cell carcinoma (protein)	Increased Expression	IHC, Functional Assay	Mol Carcinog 46:941–947 (2007)
	Gastric carcinoma (protein)	Increased Expression	IHC	Int J Med Microbiol 295:179–185 (2005)
	Glioma (protein)	Increased Expression	Functional Assay	Glia 54:526–535 (2006)
	Hepatocellular carcinoma (RNA)	Increased Expression	RT-PCR	S Biol Pharm Bull 28:886–892 (2005)
	Lung cancer (RNA, protein)	Increased Expression	RT-PCR, IHC, Functional Assay	Respir Res 6:1 (2005)
	Lymphoma (DNA)	Single-nucleotide polymorphism	Functional Assay	Genes Immun 7:615–624 (2006)
	Melanoma (IIIb/c or IV stage) (RNA)	Moderate Expression	RT-PCR	J Clin Oncol 24:5716-5724 (2006)
	Ovarian tumor (protein)	Increased Expression	IHC	Cancer Immunol Immunother 58(9):1375-1385 (2009)
	Prostate cancer (protein)	Increased Expression	WB, IHC, Functional Assay	Prostate 67:774–781 (2007)
	Recurrent glioblastoma (RNA)	Moderate Expression	RT-PCR	Neuro- Oncol 8:60-66 (2006)
	Recurrent non-Hodgkin lymphoma (RNA)	Moderate Expression	RT-PCR	J Immunother 29: 558-568 (2006)
TLR10	Nasopharyngeal carcinoma (RNA)	Increased Expression	RT-PCR	Cancer Epidemiol Biomarkers Prev 15:862–866 (2006)
	Prostate cancer (DNA)	Single-nucleotide polymorphism	Functional Assay	J Natl Cancer Inst 97:525–532 (2005)

Concepts of TLRs in Disease

that there was high TLR9 expression in the cytoplasm of tumors in most lung cancer specimens. TLR9 was also expressed in the cytoplasm of A549 and other tested tumor cell lines. In contrast, there was only sporadically weak TLR9 expression in non-malignant (normal adjacent) lung tissue. The specificity of the antibody was verified in GFP-TLR9 A549 cells where TLR9 mAb and GFP fluorescence colocalized (Fig 43).

The significance of TLR expression patterns in cancer and their variation from normal/normal adjacent tissue as well as tumor-tumor variation remains to be fully elucidated. However, there is evidence that TLRs expressed in tumor cell lines are functional. For example, Droemann et al (2005) showed that A549 expressed TLR9 (Fig 42), and that CpG-ODN induced secretion of the chemokine MCP-1 which inhibited apoptosis. These results are indicative of functional TLR9 receptors in A549 tumor cells.



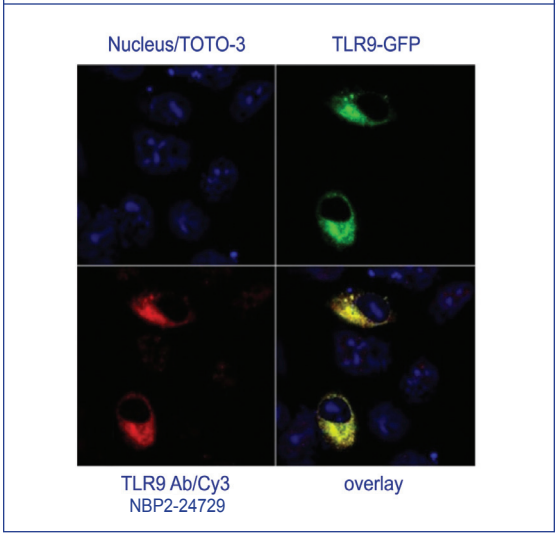


Figure 42. Immunohistochemisty (formalin-fixed, paraffin embedded) analysis of TLR9 in lung tumor/normal adjacent tissue and in A549 lung carcinoma cells using TLR9 mAb, Clone 26C593.2 (NBP2-24729). *Lung tumor and normal adjacent tissues were obtained from the same lung. TLR9 expression is observed in the cytoplasm as shown by the brown stain. Much less TLR9 staining is seen in the normal adjacent than the tumor tissue. Source: Droemann et al, 2005.*

Figure 43. Specificity validation of TLR9 mAb, Clone 26C593.2 (NBP2-24729) by confocal microscopy using GFP-TLR9 transfected cells. *Researchers sometimes perform specificity verification of antibodies in their own hands to determine the suitability of antibodies for their model systems. A549 cells were transiently transfected with a human GFP-TLR9 vector. TOTO-3 is a nuclear dye. GFP and TLR9 signals were cytoplasmic, and the GFP signals coincided with TLR9 mAb signals (overlay) indicating the specificity of the antibody for TLR9. Source: Droemann et al, 2005.*

Elucidating the expression patterns of TLRs and their function in tumors is key for developing TLR-based cancer therapeutic strategies. Particular focus is being directed towards understanding which TLRs inhibit or augment tumor growth pathways as well as their underlying mechanisms. The rest of this chapter will focus on TLR-based mechanisms in cancer inhibition/promotion, including examples of specific TLRs and their links to cancer and therapeutics [reviewed in Rakoof-Nahoum and Medzhitov, 2009 (unless otherwise denoted)]. It is interesting to note that some therapeutics, like BCG for bladder cancer, were found to act through TLR pathways many years after their establishment as effective treatments. For BCG, it took about 30 years.

TLR ligand activation and tumor inhibition

TLR ligand activation has been shown to exert anticancer effects against various tumors including:

1. Bacillus Calmette–Guérin (BCG), a vaccine derived from attenuated *Mycobacterium bovis*, has been a standard and effective therapy for superficial bladder cancer since the 1970s. However, it wasn't until 2003 that BCG was found to activate both TLR2 and TLR4 signaling.
2. LPS (TLR4), delivered intravenously, has been used in Phase II clinical trials for treating colorectal and non-small cell lung cancer.
3. OK-432, a drug consisting of lyophilized group A *Streptococcus pyogenes* is used for treating cervical, gastric and oral squamous cell carcinoma. OK-432 was used as a therapy before it was found to activate TLR4 signaling.
4. Flagellin (TLR5) has been shown to lead to tumor regression in mice transplanted with murine tumors.
5. Imiquimod (TLR7 and TLR8) are under investigation for treatment of skin cancer and chronic lymphocytic leukemia.
6. CpG ODN (TLR9) is being developed for treatment of brain, skin and renal cancer, and lymphoma.

TLRs and anti-tumor mechanisms

1. **Apoptosis:** Poly(I:C) activation of TLR3 induces apoptosis in both vascular endothelial tumor cells and cells in the tumor microenvironment. This involves IFN-β and type I IFN autocrine signaling, and the activation of IRAK4, NF-κB and caspases.

2. **Apoptosis (iNOS-dependent):** The TLR2/4 agonist OM-174, a lipid A derivative, induces iNOS which increases NO production. NO derived from TLR stimulated macrophages induces apoptosis in many types of tumors.

   NO can induce apoptosis of chemotherapy-resistant tumor cells (reviewed in Garay et al, 2007). This is because tumors frequently have mutations in p53 and are resistant to apoptosis by conventional chemotherapeutic drugs. However, NO can induce apoptosis irrespective of the p53 phenotype.

3. **Breaking tolerance:** Many models have shown that TLR activation can break tolerance to tumor self antigens through up regulation of the co-stimulatory signals which augment the adaptive immune response. The property is known as adjuvanticity and has led to the use of TLR agonists as adjuvants in cancer vaccines. Adjuvanticity has also been leveraged for generating anti-tumor antigen-specific T cells *in vitro* for adoptive transfer.

   Studies in mice have supported the importance of TLR activation in augmenting the host response to cancer treatment. For example, the ability of chemotherapeutic agents to kill established, adoptively transferred tumors was decreased in TLR4- and MyD88- deficient mice. The HMBG1 alarmin protein is a TLR4 DAMP released by necrotic cells during chemotherapy-induced cell death. Released HMBG1 activates the TLR4 MyD88 pathway in DCs, where it is processed and presented. This process breaks tumor-induced immune tolerance, thereby allowing the development of anti-tumor T-cell immunity. However, the absence of TLR4 in the mutant mice presumably results in the inablity to induce TLR4 mediated activation, thereby blocking the establishment of anti-tumor T-cell immunity.

   Likewise, breast cancer patients who carry a TLR4 loss-of-function allele relapsed more quickly after chemotherapy and radiotherapy than those carrying the WT allele. It is thought the patients with the WT allele mounted immune responses through TLR4 signaling that aided in tumor erradication. These results suggest that TLR4 is a clinically relevant immunoadjuvant pathway that can be triggered by tumor cell death, presumably through DAMP induced activation.

4. **Vascular permeability:** The TLR2/4 agonist OM-174 also induces TNFα which permeabilizes neoangiogenic tumor vessels, thereby allowing increased passage of chemotherapeutic drugs in solid tumors. This enhances the anticancer efficacy of cytotoxic drugs, resulting in tumor regression (reviewed in Garay et al, 2007).

TLRs and tumor promotion

We've discussed how TLR activation can inhibit tumor growth in adoptively transferred tumor murine models through breaking tolerance. Likewise, TLR ligands can augment the growth of adoptively transferred tumors. For example, systemic LPS treatment increased migration, invasion and angiogenesis at secondary sites of a metastatic mammary adenocarcinoma cell line that had been intravenously injected into mice. In another LPS study, LPS injection increased proliferation and decreased apoptosis in metastatic tumors that had already formed in mice after adoptive transfer of a colon adenocarcinoma cell line. Results suggested that the tumor microenvironment played an essential

role in this outcome, as TLR4 signaling in the host was required for LPS-induced tumor growth. It is thought that a mechanism involving host-dependent increase of circulating TNFα led to upregulation of NF-κB regulated antiapoptotic factors, including Bcl-X, cIAP1 and cIAP2, in the tumor cells.

TLR stimulation of a variety of tumor cells and cell lines has also been shown to increase survival and proliferation. For example, plasma cells isolated from patients with multiple myeloma expressed an increased repertoire of TLRs compared with plasma cells from healthy donors. TLR stimulation with various TLR ligands led to increased myeloma cell proliferation, in part due to autocrine secretion of IL-6, indicating that the TLRs expressed in the myeloma cells were functional.

TLR activation is also thought to play a role in promoting tumors that develop from chronic bacterial infection-induced inflammation. For example, injection of *Listeria monocytogenes* into mouse tumors, generated by injection with mouse H22 hepatocarcinoma cells, accelerated tumor growth (Huang et al, 2007). This effect could be abrograted *in vitro* by silencing TLR2, but not TLR4, with siRNA suggesting that TLR2 activation was the underlying mechanism responsible for tumor promotion.

In an *in vitro* human bacterial infection tumor model, gastric tumor cells from *H. pylori* negative patients were cultured in the presence of live *H. pylori* isolated from *H. pylori* positive gastric cancer patients (Huang et al, 2007). Intact *H. pylori* activates TLR2 signaling, and *H.* pylori increased proliferation of the gastric tumor cells from the *H. pylori* negative patients. *H. pylori* is a known risk factor for gastric carcinoma, and the results supports the concept that *H. pylori* plays at least a contributing role in the pathogenesis of gastric tumors. It also supports a putative link between TLR2 signaling, inflammation and tumor promotion, and strengthens the argument for antibiotic therapy in the treatment of bacterial-infection associated cancer.

Though the exact role of TLRs in tumorigenesis is not yet clear, it is evident that TLR pathways are an important player in both tumor inhibition and promotion. For example, both apoptosis and broken immune tolerance can be induced through the pathway, thereby inhibiting tumors. Yet tumors also utililize TLRs to promote tumor invasion and metastasis. Harnessing the TLR signaling pathway for cancer immunotherapy and vaccines may prove to be among the most promising cancer prevention and management strategies of the twenty-first century.

Concepts of TLRs in Disease

TLRs IN ALLERGY

Allergic diseases are widespread in developed countries and are increasing worldwide. There is accumulating evidence that environmental and pharmalogical exposure to TLR ligands can influence the genesis and duration of allergic reactions. This chapter will focus on the roles of TLRs in allergies, and strategies for leveraging TLR signaling to modulate allergic reactions (reviewed in Broide, 2009; Garantziotis et al, 2008; Goldman, 2007; and Horner, 2006):

The process of allergic reaction occurs sequentially and includes:

1. Immune system recognition of allergen.
2. IgE production.
3. Interaction of the allergen with effector cells bearing Fcε receptors such as eosinophils, resulting in effector cell activation.
4. Release of a variety of pro-inflammatory and vasoactive mediators from activated eosinophils and other effector cells, including histamines, prostaglandins, leukotrienes, and cytokines.
5. Allergic inflammatory response: Eosinophils express multiple TLRs as demonstrated by Wong et al (2007) (Figs 44-46). Wong et al propose that PAMP activation of eosinophils through TLRs may be a key link between PAMP induced immune responses and allergic inflammation. Figures 44-46 also show the expression of multiple TLRs in neutrophils, another effector cell involved in allergic response that may link PAMP activation and allergic inflammation through TLRs.

Many of the characteristics of allergic inflammation result from the actions of Th2 cell-derived cytokines, and hence Th2 cells have an integral role in the pathophysiology of allergic disease. There has been a prevailing dogma that TLR activation induces APCs to produce cytokines that favor Th1 immune responses, thereby avoiding the deleterious Th2 responses associated with allergic reactions. However, this dogma is being reshaped as studies show that TLR ligands also elicit Th2 responses. As a result, researchers are focusing on understanding how TLR signaling influences allergic phenotypes and Th1/Th2 profiles. A key goal is to translate this knowledge into developing TLR ligand-based therapeutics that can shift allergic immune reactions away from deleterious Th2 responses.

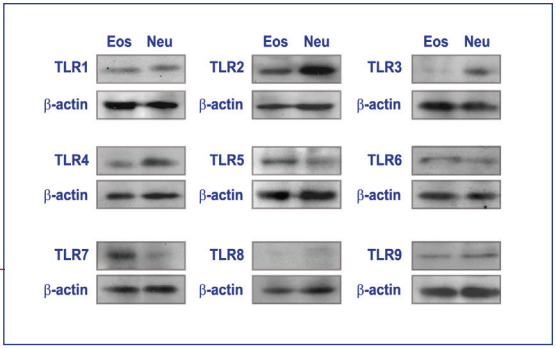


Figure 44. Western blot analysis of TLRs in human eosinophils (Eos) and neutrophils (Neu). *Cells were isolated from fresh human buffy coat obtained from normal donors. TLR molecular weights and catalog numbers are shown in (Fig. 45). Actin was used as loading control. Eosinophils expressed TLRs 1,2, 4-7, and 9. Neutrophils expressed TLRs 1-7, and 9**. The TLR WB profiles correlate with those obtained by flow cytometry (Fig 46). Source: Wong et al, 2007.*

TLR	WB	kDa	Flow
1	NB100-56563	87	NBP2-24984
2	NB100-56722	90	NB100-56058
3	NBP2-24875	120	NBP2-24899
4	NB100-56566	100	NB100-56059
5	NBP2-24787	97	NB200-571
6	NB100-56536	92	NBP2-24971
7	NB100-56682	120	NB100-56588
8	NBP2-24917	120	NBP2-24972
9	NBP2-24729	120	NBP2-24908

Figure 45. TLR antibodies utilized in neutrophil and eosinophil study. *The table shows the Novus antibodies used for WB (Fig 44) and flow cytometry (Fig 46 in the study by Wong et al, 2007). Observed molecular weights (kDa) in western blots are delineated. *Recommended WB antibodies for TLRs 3-5. Source: Wong et al, 2007.*

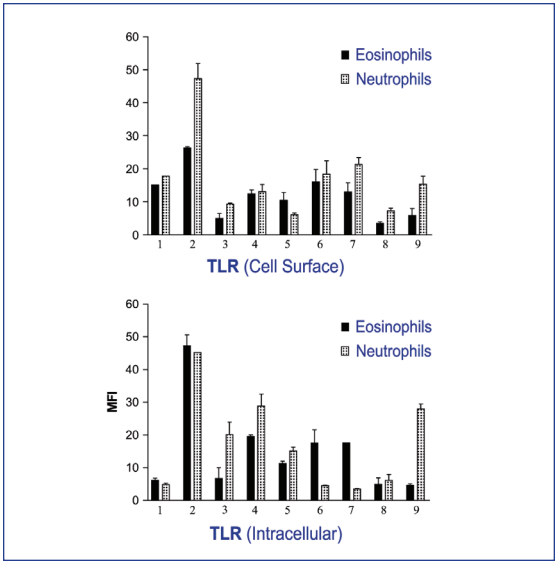


Figure 46. Flow cytometric analysis of TLRs in human eosinophils (Eos) and neutrophils (Neu). *Cells were isolated from fresh human buffy coat obtained from normal donors. Cat nos of TLR antibodies used are shown in (Fig. 45). Eosinophils expressed TLRs 1,2, 4-7, and 9. Neutrophils expressed TLRs 1-7, and 9**. TLR flow expression is shown as MFI subtracting corresponding isotype control and expressed as the mean plus SD of three independent experiments. The TLR flow cytometry files correlate with those obtained by WB (Fig 44). Source: Wong et al, 2007.*

***It is noted that the TLR3 results are in contrast with those of Francois et al (2005) and Hyashi et al (2003) where TLR3 expression was not detected by WB (Fig 35) or QPCR respectively. This is an example of discordant TLR results in the literature. See the 'TLRs: Structure, Ligands & Expression' chapter for a discussion on inconsistencies in the TLR literature.*

Concepts of TLRs in Disease

TLR4 and Allergy

Low levels of inhaled LPS (TLR4 ligand) trigger Th2 responses including IgE production and eosinophil maturation in *in vivo* mouse models of allergic asthma. However, high LPS levels trigger Th1 responses. *In vitro* studies provided insight into the seemingly disparate LPS signaling mechanisms by showing that LPS-activated wildtype and MyD88-deficient DCs induced Th1 and Th2 responses, respectively. The wildtype/MyD88-deficient mouse model results suggested that:

1. Th2 responses were induced through TLR4 MyD88-I signaling. This is because these responses were induced in the MyD88-deficient mice, and hence not dependent on the MyD88-D pathway.
2. Th1 responses were induced through TLR4 D signaling. This is because they were induced in the WT but not the MyD88-deficient mice, and hence dependent on the MyD88-D pathway.

Taken in context with the *in vivo* asthma model and other results, it was hypothesized that:

1. Low doses of LPS signal through the TLR4 MyD88-I pathway resulting in Th2 responses.
2. High doses of LPS signal through the TLR4 MyD88-D pathway resulting in Th1 responses.
3. LPS at low doses, comparable to endotoxin levels found in the environments of asthmatic children, lead to Th2 responses.
4. LPS at doses typical of an infection lead to inflammatory Th1 responses.

However, TLR4-based ligands are not considered to be promising allergic disease therapies due to their Th2 adjuvant potential. Nevertheless, immunotherapy strategies that utilize the TLR4 D, but not the I pathway could potentially be employed to shift the immune system in the direction of Th1 responses and away from Th2 deleterious allergic responses.

TLR9 and Allergy

The synthetic immunostimulatory sequence oligodeoxynucleotides (ISS-ODNs) are TLR9 ligands that induce innate immune responses characterized by cytokines that inhibit the allergic phenotype. Induced cytokines include IL-10, IL-12, type I IFNs, and IFN-γ; mice injected with ISS-ODN maintain elevated serum levels of these cytokines for at least a week. ISS-ODN is effective in preventing and reversing Th2 hypersensitivity in mouse allergic models. For example, mice treated with ISS-ODN within hours of allergen challenge had attenuated hypersensitivity responses associated with various allergic conditions including asthma, conjunctivitis and rhinitis. Although the induced cytokine responses are one component, studies suggest that ISS-ODN actually acts through a variety of redundant and complementary immune mechanisms to promote anti-allergic responses.

Allergen ISS-ODN conjugates (AIC) were the first TLR ligand-based allergen disease interventions to proceed to clinical trials. Conjugation renders allergens resistant to binding by allergen-specific IgE, and patients treated with ragweed-specific AIC developed resistance to ragweed. Analysis showed that *ex vivo* ragweed-specific cytokine responses by PBMCs obtained from treated patients shifted away from Th2 and toward Th1 cytokine production. ISS-ODN-based therapies hold promise for reversing allergic hypersensitivities, although there is still much to be learned about AIC immune modulation with respect to safety, optimal dosing and efficacy.

Other TLRs and Allergy

The Pam3Cys TLR2 ligand induced a Th2 response and promoted asthma in mouse allergy models and human Th2 development in *in vitro* cultures. However, in another mouse study, Pam3Cys inhibited Th2 responses and established allergen airway hypersensitivity while inducing IFN-γ responses. The mechanisms of these seemingly discordant Pam3Cys remain to be elucidated. However, mechanistic studies suggest that Pam3Cys modulation of Erk1/2 pathways contribute to induction of the Th2 response.

TLR2 also appears to be involved in the pathology of allergic atopic dermatitis (a type of eczema) because patients are typically colonized by *S. aureas* (a TLR2 ligand) and show evidence of Th2-biased immune dysregulation. Additionally *S. aureas* colonization and infection sets off atopic dermatitis flare-ups which are treatable by antibiotics. Furthermore, application of *S. aureas* to mice skin induces an eczema like inflammatory response.

The roles of TLR3, TLR5 and TLR7/8 in the allergic phenotype remain to be determined. However, ligands for TLR7 and TLR8 such as R848 have been described as Th1 adjuvants. Additionally, R848 can inhibit Th2 effector cell cytokine production while shifting human allergen-specific CD4+ Th2 lymphocytes into IFN-gamma producing cells.

Although immunotherapy was shown to reverse allergic hypersensitivities in the early 1900's, efficacy has limited its clinical utility. Nearly 100 years later, the findings that select TLR ligands and ISS-ODN conjugated ligands have anti-allergic activities brings promise to the development of a new generation of immunomodulatory therapeutics. Hopefully, the growing understanding of TLR ligands and their respective signaling pathways will result in new therapeutics for preventing and treating allergic hypersensitivities and their associated diseases.

Concepts of TLRs in Disease

TLRs IN AUTOIMMUNITY

Autoimmune diseases are disorders that result from attack of the host immune system against itself. There are at least 80 different autoimmune diseases and they can be restricted to certain organs such as thyroiditis, or be systemic such as systemic lupus erythematosus (SLE). These diseases are a major health problem worldwide, and both environmental and genetic factors can increase the risk of developing autoimmune disease. Individuals have unique innate immune response signatures, and their particular responses to TLR-mediated activation is thought to play a critical role in their susceptibility to developing autoimmune disease (reviewed in Waldner, 2009). The rest of this chapter will focus on known and emerging autoimmune disease mechanisms and examples from the literature.

Autoimmune disease mechanisms

The pathological processes that lead the body to attack its own tissues are multifactorial, and involve dysregulation of both the innate and adaptive immune system. A loss of immune tolerance occurring during central and peripheral differentiation of adaptive immunity can result in uncontrolled proliferation of self-reactive B and T cells. It is well recognized that autoimmune diseases arise from overactive responses of the host's adaptive immune system against self, eg cells, tissues, and other constituents normally present in the body.

However, mechanisms of autoimmune disease development can also be directly traced and attributed to innate immune effectors. Specific cell populations in the innate immune system such as the antigen-presenting DCs are critical in promoting primary T and B responses and the induction of primary adaptive immunity. DCs are a heterogenous population of APCs. DCs are present in small quantities in tissues that are in contact with the external environment. This includes the skin where there is a specialized dendritic cell type called Langerhans cells, and the inner lining of the nose, lungs, stomach and intestines. DCs are also found in an immature state in the blood.

DC, TLR signaling and autoimmunity concepts

DCs express a variety of PPRs including TLRs, and undergo significant functional changes following TLR mediated activation. Activated DCs migrate to the lymphoid tissues where they interact with T and B cells to initiate and shape the adaptive immune response. Activated DCs induce different effector functions in T cells depending on the TLR ligand, DC subset and other yet uncharacterized or elusive variables.

DCs, among all innate cells, are known as the most potent activator and modulator of the adaptive immune system. In this regard, DCs are considered as entities that bridge the gap between innate and adaptive immunity. That is, the type of adaptive immune response that is induced in the host depends to a large extent on the PAMP or DAMP ligand type and DC subset activated. Excessive or chronic activation of innate immunity can break peripheral tolerance to self-antigens, and result in the induction of autoimmune disease, even in resistant hosts.

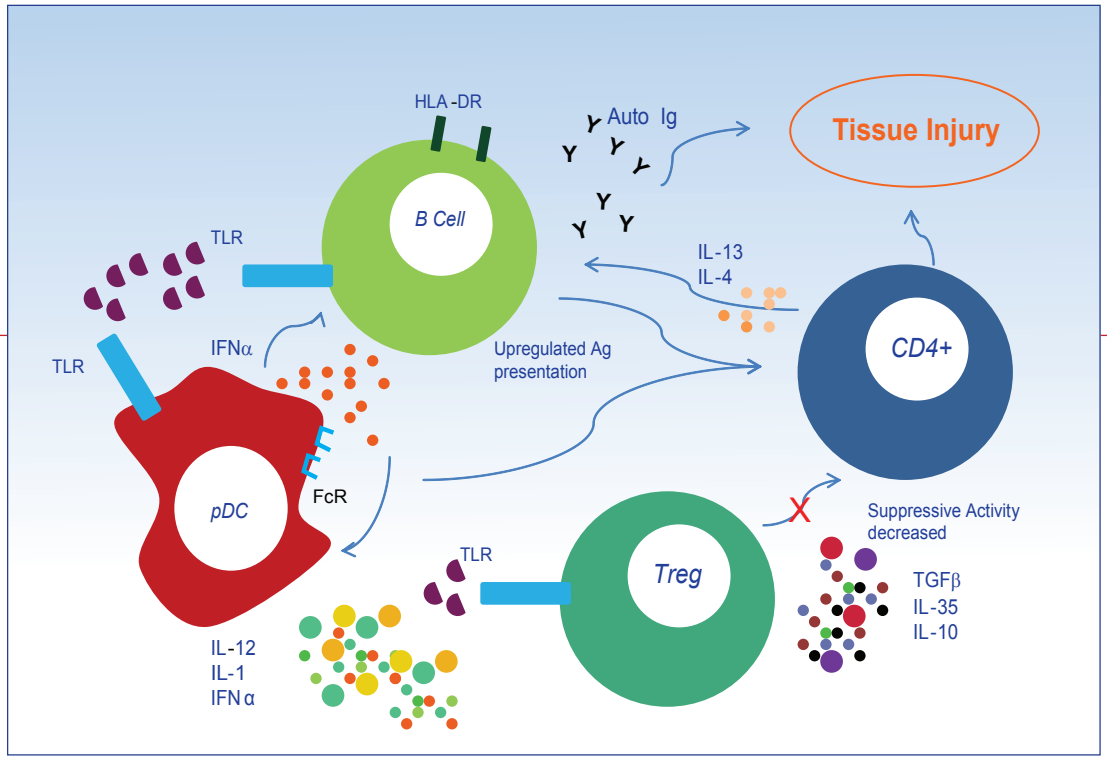


Figure 47. TLRs, Inflammatory Processes and Autoimmunity

Hence, DC bridging can also be thought of a mechanism whereby dysregulation in innate immune processes can be transmitted to adaptive immunty. For example, the actions of DC subsets have been shown to play a key role in the induction and regulations of various autoimmune diseases in mice.

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Autoimmune disease inhibitory mechanisms

Newly developing self-reactive T cells are largely deleted in the thymus during central tolerance induction. This clonal elimination process is not 100% complete though, and some self-reactive T cells escape to the periphery. The development of autoimmune disease is typically prevented by mechanisms in the periphery that render the host tolerant to these self-reactive T or B cells. However, infections or excessive stimulation of APCs can break peripheral tolerance and induce priming of self-reactive T cells in draining lymph nodes. This can lead to pathological attacks against self, resulting in promotion of autoimmune disease, particularly in predisposed hosts.

Emerging concepts in autoimmune disease mechanisms

The contribution of innate immune response in the development and regulation of autoimmune diseases is increasingly being defined. An expanding body of literature is accumulating suggesting that aberrant TLR functioning on susceptible genetic backgrounds is integrally linked to chronic inflammation and autoimmune disease. Many TLRs, including TLRs 2, 3, 4, 7, 8, and 9, can be activated by endogenous ligands and have been found to induce autoimmune reactions. An illustration of the working dogma of TLRs within the context of inflammatory processes and autoimmunity

Concepts of TLRs in Disease

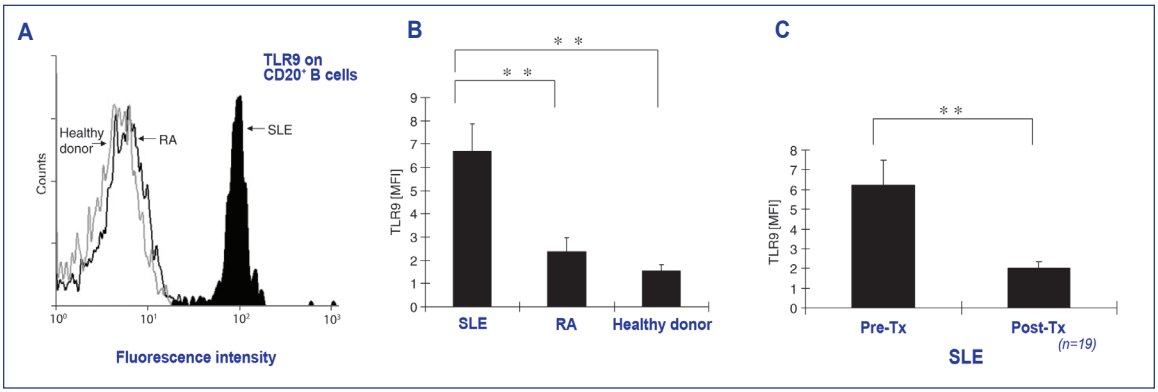

Figure 48. Flow cytometric analysis of TLR9 in human CD20+ B cells using TLR9 mAb, clone 26C593 (NBP2-24908). *B cells were isolated from peripheral blood by magnetic bead separation using a CD20 Ab.*
A. Representative histogram of TLR9 from a healthy donor, disease (RA) control and patient with active SLE.
*B. The MFI of TLR9 was significantly higher in patients with active SLE than in normal or disease controls. (**P < 0.01)*
*C. The MFI of TLR9 was significantly reduced in active SLE patients (Pre-Tx) following treatment (Post-Tx). (**P < 0.01). Patients were treated with steroids.*

The data shows that TLR9 expression on B cells is upregulated in SLE patients and suggests that upregulated TLR9 is related to SLE disease activity since treatment reduced the level of TLR9 expression. Source: Nakano et al, 2008.

is shown in Figure 47. Aberrant TLR functioning can include inappropriate TLR activation by endogenous or exogenous ligands, molecular mimicry and enhancement of autoantigen presentation through APC activation and defective Treg regulation.

Multisystem disease: SLE

SLE is a chronic multisystem autoimmune disease characterized by the production of autoantibodies. The autoantibodies are primarily dsDNA and nuclear proteins from dead cells. dsDNA is a ligand for TLR9 receptor and under normal circumstances endogenous dsDNA does not easily gain access to intracellular compartments where TLR9 is located. However, self-derived dsDNA or other DAMPs including those with autoantigenic potential can gain access to and activate TLRs when released during tissue destruction. Factors resulting in tissue

destruction can include tissue injury, increased apoptosis, necrosis, necroptosis, defective clearance of cell debris, or inflammation.

In a murine SLE model, TLR9 activated by endogenous ligands led to the activation of TLR9 MyD88-D pathway which was required for switching to pathogenic autoantibody production. Additionally, human patients with SLE had increased expression of TLR9 compared to normal and disease controls (Fig 48) (Nakano et al, 2008). Treatment that reduced SLE symptoms also downregulated TLR9, suggesting that TLR9 expression was linked to disease activity.

Organ specific disease: Type 1 diabetes (T1D)

T1D is an organ specific autoimmune disease resulting from T cell mediated destruction of insulin producing pancreatic beta cells. The development of T1D favors genetically predisposed individuals. Studies have linked TLR2 mediated signaling as a contributing factor to the induction of diabetes in mice. For example, secondary necrosis in apoptotic beta-cells activated APCs through TLR2 signaling. Activated APCs in turn primed islet-specific CD4+ T cells which contributed to the initation of T1D in the mice.

However, this finding contrasts with other studies, and the role of TLR/innate immune activation in T1D is somewhat controversial. For example, MyD88-deficient mice housed under germ free conditions were susceptible to T1D development, whereas their counterparts housed in a specific-pathogen-free (SPF) facility were not. As TLR2 signaling proceeds through MyD88, the observation that MyD88 deficient mice developed T1D in germ free conditions suggests that TLR2 or PPR linked activation of the innate immune system was not a factor in T1D induction. Hence, additional studies are needed to elucidate the role of TLR activation in T1D.

Defining the relationships between TLR activation, the host environmental milieu, and individual host immune response signatures is key for understanding autoimmune disease risk factors and pathogenesis. This knowledge will facilitate the development of overall and individualized therapeutic strategies to better prevent and manage autoimmune disease.

TLRs in Stem Cells

A stem cell is an undifferentiated cell whose daughter cells may differentiate into other cell types. In fact, stem cells are able to both renew themselves through mitotic cell division, and differentiate into a diverse range of specialized cell types. Stem cells are found in most, if not all multicellular organisms, and are an increasing research focus of scientists worldwide from diverse fields.

Scientists have long suspected that stem cells have therapeutic potential in a wide variety of diseases. On the flipside, it is increasingly being recognized that a number of diseases, particularly certain cancers, have their origins in stem cells that have gone awry. While widespread stem cell therapy lies somewhere in the future, the interest in stem cells is being fueled by treatment success stories. For example, headlines like "Stem cell cultured on contact lens restore sight in patients with blinding corneal disease" (ScienceDaily, 2009) are generating intense media and scientific interest. The treatment reported as a world-first breakthrough in stem cell research was developed by Di Girolama et al (2009) from the University of New South Wales.

Identifying stem cells and their markers as well as defining the underlying mechanisms that control their function and differentiation/de-differentiation are not easy tasks. However, widespread development of successful stem cell diagnostics and therapies will rely on elucidating the markers and mechanisms of undifferentiated and differentiated stem cells. Undoubtedly some of these markers will be new, yet others will likely come from already existing unexpected sources.

For example, initially research on TLRs focused on their expression and signaling pathways in immune cells. However, an emerging body of literature is showing that other bone marrow-derived cells, including mesenchymal stem/progenitor cells (MSCs) express TLRs (reviewed in Tomchuck et al, 2008). For example, Tomchuck et al showed that MSCs expressed multiple TLRs including TLRs 2, 3, 4 (Fig 49), 7 and 9 as well as the MyD88 TLR signaling adaptor protein. Additionally, these TLRs were functional as they responded to ligand stimulation.

The response of TLR4 expression following LPS treatment is shown in Figure 49. Flow cytometric analysis showed a decrease in cell surface expression, likely due to LPS induced receptor activation, internalization and degradation. Fluorescence microscopy supported receptor internalization, including a relocalization of TLR4 staining from the surface to endosomal-like compartments following LPS treatment. Additionally, LPS and other ligands led to the activation of downstream signaling pathways including NF-κB, AKT and MAPK, and the induction of cytokines, chemokines and other established TLR-regulated genes. hMCS migration was also promoted by ligand exposure whereas neutralizing TLR antibodies inhibited migration. These results

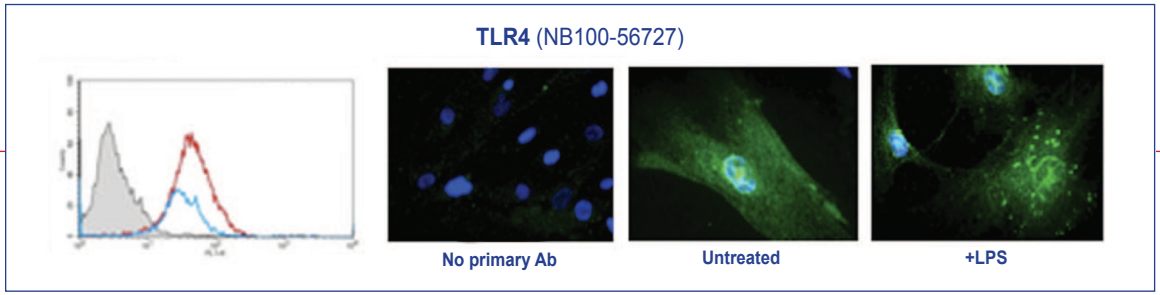

Figure 49. Analysis of TLR4 expression in adult human bone marrow-derived mesenchymal stem cells (hMSCs) before and after LPS ligand stimulation using TLR4 mAb, clone HTA125 (NB100-56727). *Primary MSCs were obtained from normal human donor pools. Prior to experimentation, they were verified to be MSCs by testing for homogeneity, and established positive and negative surface markers, as well as their potential to differentiate into chondro, osteo, and adipogenic lineages.*

Flow cytometry: Cells were left untreated or treated for 30 min with 10 ng/ml LPS and stained for cell surface expression of TLR4. An Alexa 488-conjugated secondary Ab was used for detection. Treatment resulted in diminished TLR4 levels, most likely because of receptor activation, internalization, and degradation, as evident from the leftward shift of the plot measured for the LPS treated sample (blue line).

Fluoresence microscopy: Cells were grown to near confluence on chamber slides and left untreated or treated for 1 hr with 10 ng/ml LPS. TLR4 antibody staining was performed following fixation and membrane permeabilization (primary antibody omitted as a control). An Alexa 488-conjugated secondary Ab was used for detection. Treatment led to relocalization of TLR4 from the surface to intracellular endosomal-like structures. Source: Tomchuck et al 2008.

Other Novus antibodies used in this study include MyD88 (NB100-56698), TLR2 (NB100-56722), TLR5 (NBP1-97728), TLR4 (NB100-56727), TLR7 (NB100-56682) and TLR8 (NBP2-24917). LPS is available from Novus as Cat no. NBP2-25295.

suggested TLRs play a role in regulating MSCs in immune responses and may be one mechanism that drives their migration to injured tissue sites.

MSCs are widespread in adult organisms and are thought to be involved in tissue maintenance and repair as well as the regulation of hematopoisesis and immunological response. *In vitro*, they can be induced to differentiate into cells of the mesodermal lineage including osteocytes, adipocytes, chondrocytes, myocytes, tenocytes and hematopoietic supportive stroma. In their repair function, human MSCs are recruited to sites of stress and inflammation. There are studies, based on their repair function, to develop human MSCs as therapeutic delivery agents to assist in the repair of damaged tissues. Since the function of MSCs can be manipulated through TLRs, the TLR pathway may be a novel target that can be exploited to develop therapeutic delivery and other stem cell-based therapies. Additional studies will need to be done to determine the scope of TLR expression on stem cells in general, and how the TLR pathway can potentially be manipulated to modulate stem cell function or differentiation.

TLRs in Therapeutics & Vaccines

TLR signaling is rapidly being recognized is an important phenomena in both health and disease. Although TLR signaling was first described in innate immunity, we know that it is also linked to most known fundamental biological processes and many diseases. Throughout this handbook we have discussed TLR linkage to induction and regulation of adaptive immunity, maintenance of homeostasis, cell death and survival pathways. We have also considered the role of TLR signaling in a number of diseases including cancer, neurodegeneration, autoimmunity, sepsis, and allergy. The quest for new therapeutics targeting TLR pathways is rapidly expanding and Table XII shows a summary of TLR drugs in clinical trials (reviewed in Makkouk A, AM Abdelnoor, 2009 and Parkinson, 2008). This clinical focus has revealed that some TLR agonists, such as BCG for bladder cancer, were already being used as effective therapeutics without knowing their mechanism of action.

Likewise, imiquimod was developed as a topical treatment of genital warts, basal cell carcinoma and actinic keratosis before it was identified as a TLR7 agonist. Imiquimod is marketed by 3M Pharmaceuticals as Aldara. Each discovery that identifies effective existing therapeutics as a TLR agonist or otherwise acting through TLR signaling pathways opens up a door of opportunities for leveraging TLR signaling in therapeutic milieus.

Targeting TLR pathways is an emerging avenue for immunotherapy of a number of diseases including infectious diseases, such as sepsis, autoimmune disorders, allergic diseases such as asthma and cancer. It is thought that TLR-based therapies may provide new hope for certain diseases. For example, the incidence and mortality rates for renal cell carcinoma (RCC) have been steadily increasing throughout the world, and it is now one of the 10 most frequent malignanices in developed countries, accounting for ~3% of all malignancies. Unfortunately, it is also one of the most drug-refractory, hence there is an urgent need for new therapies.

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TLR3 has now been identified as a potential therapeutic target in clear cell RCC (CCRCC) (Morikawa et al, 2007). TLR3 overexpression was found in over 70% of CCRCCs, yet entirely absent in chromophobe RCC (Fig 50). These results suggested that the TLR3 pathway may be a novel target for CCRCC treatment. The authors then went on to show that the TLR3 agonist poly(I)poly(C) had a growth inhibitory effect in RCC cell lines, supporting the concept of the TLR3 pathway as a novel therapeutic for CCRCC. As shown in Table XII, a TLR3 agonist is also being considered for breast cancer treatment and is already in preclinical trials.

Table XII. TLR Based Agonists and Antagonists in Clinical Trials

TARGET	PRIMARY INDICATION	NAME	MANUFACTURER	CLINICAL STATUS
TLR3 agonist	Breast cancer	IPH-31XX (dsRNA)	Innate Pharma	Preclinical
	Chronic fatigue syndrome (CFS)	Ampligen (PolyI:Poly C12U)	Hemispherx	Filed for NDA
	HIV	Ampligen (PolyI:Poly C12U)	Hemispherx	Phase IIb
TLR4 agonist	HPV prophylactic vaccine	Fendrix [HBV antigen + Monophosphoryl Lipid A (MPLA)]	GSK	Approved
	HPV prophylactic vaccine	Cervarix (HPV antigens 16 & 18 + alum + MPLA)	GSK	Approved
	HPV prophylactic vaccine	Supervax [HPV antigen + RC-529 (synthetic MPLA)]	Dynavax	Approved
	Lung cancer (non-small cell)	Stimuvax/BLP25 (MUC1 + MPLA in a liposomal vehicle)	Biomera/Merck	Phase III
	Rhinitis	Pollinex Quattro (modified multiple antigens +MPLA)	Allergy Therapeutics	Marketed in Europe
	Rhinitis	RagWeed Pollinex (ragweed pollen + MPLA)	Allergy Therapeutics	Phase III
TLR4 antagonist	Septic shock	Eritoran [E5564 (lipid A derivative)]	Eisai	Phase III
	Septic shock	Resatorvid [TAK-242 (lipid A derivative)	Takeda Pharmaceuticals	Phase III
TLR5 agonist	HPV prophylactic vaccine	Hepsilav [Recombinant HPV antigen +1018 ISS (CpG ODN)]	Dynavax	Phase III (on hold)
	Influenza	Matrix Protein-2 + Flagellin	Vaxxinate	Phase I
TLR7 agonist	Actinic Keratosis	Aldara (Imidazoquinoline)	3M Pharma	Approved
	Basal cell carcinoma	Aldara (Imiquimod, topical 5% cream)	3M Pharma	Approved
	Hepatitis C virus (HCV)	Aldara (Imidazoquinoline)	3M Pharma	Phase II
	HPV warts	Aldara (Imidazoquinoline)	3M Pharma	Approved
	HPV	852A (small molecular structurally related to imidazoquinoline)	3M Pharma/Takeda	Phase II
	Melanoma	852A (small molecular structurally related to imidazoquinoline)	3M Pharma	Approved

Table XII. TLR Based Agonists and Antagonists in Clinical Trials

TARGET	PRIMARY INDICATION	NAME	MANUFACTURER	CLINICAL STATUS
TLR9 agonist	Anthrax prophylactic vaccine	AV7909 (Biothrax + VaxImmune)	Coley/DARPA	Phase I
	Asthma	1018 ISS (CpG-ODN) + Amb a 1 (a ragweed allergen)	Dynavax	Phase II
	Asthma	AVE0675 (CpG-ODN)	Coley/Aventis	Phase I
	Asthma/COPD	AZD1419 (CpG-ODN)	Dynavax/Astra Zeneca	Phase I
	Colorectal cancer	1018 ISS (CpG-ODN) + chemo-therapy	Dynavax	Phase II
	Colorectal cancer (meta-static)	DSLIM [(double stem loop immuno-stimulator (GpG ODN)]	Mologen	Phase Ib
	HCV	IMO-2125	Idera Pharm	Phase I
	HIV	Remune (inactivated HIV-1 + HYB2055)	Idera/Immune Re-sponse Corp	Phase II
	Influenza	Matrix Protein-2 + nucleoprotein + Tri valent flu vaccine + Iss (ODN)	Dynavax/Novartis	Preclinical
	Lung cancer (non-small cell)	MAGE-A3 (tumor specific antigen) + VaxImmune	Coley/GSK	Phase III
	Melanoma	CYT004-MelQbG10 [MART1 (melanoma antigen recognized by T cells) + MelQbG10 (DNA sequence from Mycobacterium tuberculosis)]	Cytos	Phase II
	Melanoma	Melan-A + IFA + CpG ODN 7909	Coley/GSK	Phase I
	Renal cell carcinoma	IMOxine [HYB2055 (CpG ODN) + IMO2055 (CpG ODN)]	Idera Pharmaceuticals	Phase II
TLR7 + TLR9 antago-nists	Autoimmune disorders	DV1079 (CpG ODN)	Dynavax/GSK	Preclinical
TLR7 + TLR8 + TLR9 antagonists	Autoimmune disorders	CpG 52364	Coley Pharmaceuticals	Phase I

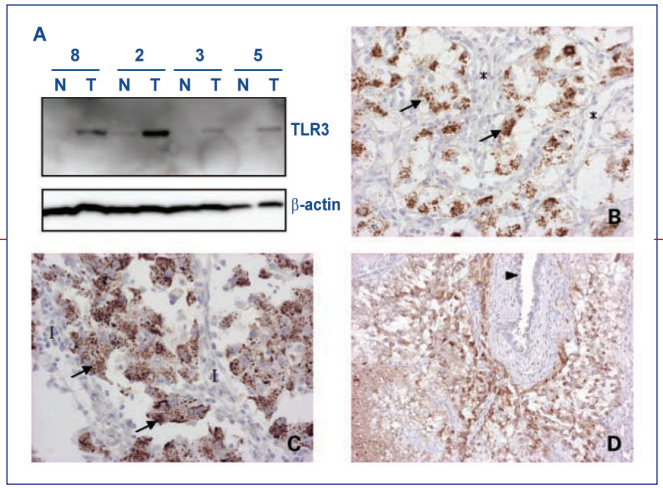

Figure 50. Analysis of TLR3 expression in human RCC using TLR3 mAb, clone 40C1285.6 (NBP2-24875). *A. Western blot analysis of four normal adjacent/tumor matched sample pairs of CCRCC. β-actin was used as an internal control. TLR3 expression was identified in the tumor, but not normal adjacent kidney. B-D. Immunohistochemistry of formalin-fixed, paraffin embeded RCC tumors, strong staining was observed in all 3 CCRCC tumors as follows: B: Grade 1 tumor, staining was observed only in cancer cells (arrows) not in blood vessels (*). Magnification, X400; C: Grade 3 tumor, staining was observed only in cancer cells (arrows) not in tumor-infiltrating inflammatory cells (I). Magnification, x400; D: CCRCC lung metastasis, staining was observed in metastatic CCRCC but whereas normal bronchial epithelium (arrowhead) had minimal staining. Table. TLR3 over expression was identified in over 70% cases of CCRCC and paipillary RCC, but in none of the chromophobe cases. Source: Morikawa et al (2007).*

Immunohistochemical analysis of TLR3 in RCCs

	No. cases	TLR3 staining			
		0	1+	2+	3+
CCRCC	189	5	45	85	54
Papillary RCC	11	2	3	4	2
Chromophobe RCC	8	8	0	0	0

TLR pathways can also be leveraged as adjuvants in prophylactic vaccines. Vaccine adjuvants act as shields against infectious diseases such as HPV and HCV, and as therapeutic immunization against noninfectious diseases, such as cancer and Alzheimer's disease. There are a number of prophylactic vaccines based on TLR agonists already approved or in clinical trails such as TLR4 (HPV), TLR5 (HPV) and TLR9 (anthrax) as shown in Table XII.

Specific TLR agonists must be designed to have reduced toxicity but increased potency, as compared to traditional adjuvant candidates in order to fulfill the stringent safety criteria required for prophylactic vaccines. The principal cellular targets of these vaccines include APCs, primarily the DCs which link initial innate responses with subsequent adaptive immune responses. Two improved adult HPV vaccines that use TLR4 agonists as adjuvants have been approved and are more effective than alum-based vaccines. An analogous HBV vaccine using CpG-ODN is in Phase III studies and appears to be superior to conventional HBV vaccines because it can overcome age-related declines in responsiveness. A papilloma virus vaccine nearing approval also uses a monophosphoryl lipid adjuvant. There are additional studies directed towards exploring the adjuvant activity of other TLR ligands.

TLRs in Therapeutics & Vaccines

TLR agonists designed for vaccines against cancer and chronic viral diseases function by enhancing CD8+ T cell responses to protein antigens, by cross-presention of peptides generated from exogenous antigens or by overcoming self-tolerance. Overcoming self-tolerance is necessary for generating responses to tumor-associated antigens that are nearly similar to normal self-antigens. Stimulation of TLR9 with CpG ODNs has been shown to increase the immunogenicity of peptide-based, DNA- based, tumor cell-based and DC-based vaccines. Preclinical studies suggest that TLR3, TLR4, TLR7 and TLR7/8 agonists also have potential to enhance therapeutic vaccination for cancer and chronic viral infections, including HIV and HBV.

Prevention of infectious diseases through immunization is clearly one of the most profound achievements of modern medicine. Vaccines containing live attenuated or inactivated microorganisms have long been used to stimulate immune responses. We now know that vaccines generate potent immunogenicity by stimulating innate immune responses via TLR signaling and subsequent activation of adaptive immune responses. Linkage of a TLR agonist (adjuvant) to antigen increases antigen uptake by DCs thus reducing the required antigen dose. It may also facilitate antigen processing and MHC class I and II antigen presentation. Additionally, adjuvant activity of most TLR agonists can be enhanced by formulation in lipid emulsions, microparticles, or virus-like particles containing antigens.

Potential clinical applications of TLR antagonists require either the development of structural analogs of agonists that bind to the receptor but fail to signal or produce anti-TLR antibodies or small molecule antagonists selected from compound libraries. The use of TLR antagonists to TLR7 or TLR9 appears quite promising for a number of inflammatory and autoimmune diseases.

In summary, though significant progress has been made in the area of TLR therapeutics, determining the efficacy, feasibility and safety behind this use of TLR signaling pathways is still, for the most part, in its infancy.

Epilogue

As we go to press with our Updated Edition of the TLR Handbook, new developments in TLR research and innate immunity continue to emerge. Hence the "Story Toll'd" will undoubtly be reshaped as time goes on to reflect emerging advancements, concepts and dogmas. For example, autoinflammatory diseases are a relatively new classification of diseases that are different from autoimmune diseases or allergies as reviewed by Masters et al (2009) in "Horror Autoinflammaticus: The molecular pathophysiology of autoinflammatory disease." This group of diseases involve overactivation of the innate immune system, and present as unprovoked episodes of inflammation without accompanying high titer autoantibodies or antigen-specific T cells.

The term autoinflammatory was coined to distinguish these diseases from the classically recognized autoimmune diseases such as SLE and rheumatoid arthritis where the hallmarks of adaptive immunity are more evident. Autoimmflamatory diseases appear to be driven by the innate system and are thought to be mediated by inflammasomes. Whereas NLRs have been associated with some of these diseases, including Crohn's, Blau, and Guadaloupe periodic fever, the overall role of PRRs including TLRs remains to be defined. It is thought these diseases may represent examples from a heretofore nebulously defined inflammasome-mediated disease group where cross-talk between the innate and adaptive immune systems is absent or dysfunctional. In the overall picture, this suggests that innate and adaptive immune systems don't necessarily always communicate or link with one another. Hence, there are compelling reasons why we may need to rethink and or reshape the dogma regarding the bridges or interplay that link adaptive and innate immunity.

We encourage researchers to keep us abreast with their new findings and publications. New products and information are continually being added to our TLRSystem™ portfolio, and one of the most valuable components is documentation of your publications.

Notes

Appendix

Apoptosis/Cell Death Assays

Cell death is a fundamental aspect of the life cycle of the eukaryotic cell. During the 1990's, research in the field of cell death grew exponentially, and fundamental aspects regarding the molecular nature and physiological regulation of the dying process were elucidated. By the end of the decade, it was recognized that cell death occurs through a network of mechanisms.

These mechanisms act in series or in parallel, each of which can be variably expressed in different eukaryotic species, within the same species, and between different cell types. By the early 2000's assays to analyze cell death had become essential research and diagnostics tools in laboratories worldwide. By 2005, many different models of cell death had emerged including necrosis, apoptosis, anoikis, caspase-independent apoptosis, autophagy, endoplasmic reticulum stress, Wallerian degeneration, excitotoxicity, erythropoiesis, platelets, cornification and lens (reviewed in Kroemer et al, 2009).

However, apoptosis continues to be a popular, although loosely defined, term that is used differently by different investigators to measure and describe cell death. In general, anything that looks like apoptosis has been called apoptosis, which has contributed to the complexity of the cell death literature (Kroemer et al. 2009). Researchers should keep in mind that cell death assays detect a biological aspect or mechanism of cell death versus being exclusive to a particular type of cell death such as apoptosis or necrosis. Novus offers a wide range of products and assays for detecting and analyzing cell death. Collectively, these products encompass a variety of techniques including flow cytometry, western blot analysis, immunohistochemistry, fluorescence microscopy, and ELISA. As cell death occurs through different mechanisms, which may be present in various combinations, the Nomenclature Committee on Cell Death (NCCD) encourages investigators studying cell death to use more than one assay whenever possible (Kroemer et al. 2009). We will highlight the most common two approaches described for detecting apoptosis, the Annexin V Assay and caspase analysis. Please visit the Novus website (www.novusbio.com) for a comprehensive listing of our products for studying cell death.

Reference

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Annexin V Apoptosis Kit with Controls

CytoGlo™ Annexin V-FITC Kit : NBP2-29373, 100 Assays

The Annexin V Assay is a classical technique for detecting apoptosis. The assay is based on Annexin V, which is a calcium-dependent phospholipid binding protein that has a high affinity for phophatidylserine (PS), a plasma membrane phospholipid (Fig 1

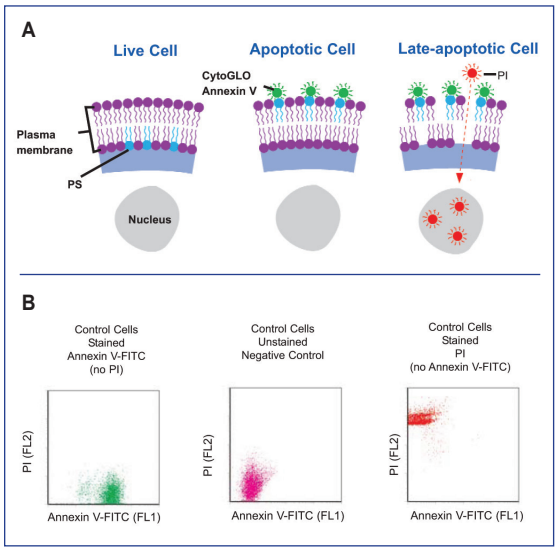

Figure 1. Analysis of apoptosis with Annexin V and PI. *A. Annexin V detection of phosphatidylserine on the Cell Surface. The plasma membrane loses asymmetry during early apoptosis. During asymmetry loss, PS translocates from the cytoplasmic to the external face of the plasma membrane where it is detectable by Annexin V conjugates. As apoptosis progresses, the plasma membrane becomes compromised and permeable to vital dyes like PI. PI is often used in conjunction with Annexin V to help distinguish between early and late stage apoptosis. B. Kit Control Cells: Compensation and Assay Validation. Control Cells (Kit component) were stained or left unstained as indicated using the reagents in the CytoGLO™ Annexin V-FITC Apoptosis Detection Kit.*

A). The CytoGlo™ Annexin V-FITC Apoptosis Kit is designed for detecting cell death in unfixed cell populations by flow cytometry. The inclusion of stable, positive control cells in our Kit (Fig 1 B) makes it unique among other commercially available Annexin V Kits. These cells are useful for setting compensation as well as assay validation, thereby sparing precious experimental sample.

Annexin V Assay Principles

One of the earliest features of apoptosis is the translocation of PS from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external environment (Fig 1A). Annexin V binds to PS exposed on the cell surface and identifies cells at an earlier stage of apoptosis than assays based on DNA fragmentation. The appearance of PS on a cell surface is a general indicator of apoptosis, and binding of dye-labeled Annexin V to PS is a widely used standard technique for detecting apoptosis by flow cytometry and fluorescence microscopy. The Annexin V assay is straightforward and quick. A protocol overview is shown in Figure 2, and Figure 1B illustrates the use of the control cells for compensation and assay validation.

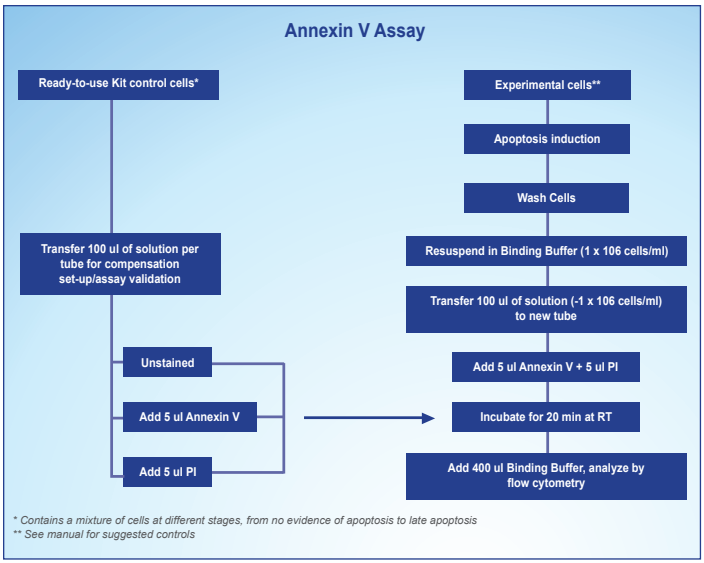

Figure 2. CytoGlo™ Annexin V-FITC Apoptosis Detection Kit: protocol overview.

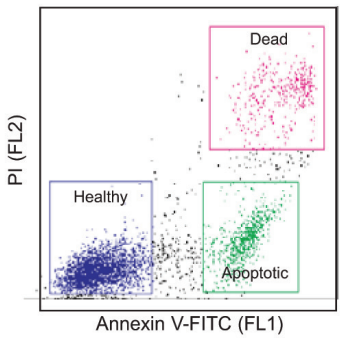

Figure 3. Sample data using the CytoGlo™ Annexin V-FITC Apoptosis Detection Kit. *Adherent RAW cells were treated with 0.05 ug/ml actinomycin-D for 17 hr to induce apoptosis. Cells were detached with Accutase and stained with the CytoGLO™ Annexin V-FITC Apoptosis Detection Kit.*

Translocation of PS to the external cell surface (PS exposure) is not unique to apoptosis, but also occurs during necrosis. Both late stage apoptosis and necrosis are associated with leaky or permeable cell membranes. Therefore, the measurement of Annexin V binding to the cell surface is typically performed in conjunction with the vital dye propidium iodide (PI) to distinguish between non-apoptotic (Annexin-FITC negative/PI negative), early apoptotic (Annexin V-FITC positive/PI negative) and late apoptotic necrotic or dead cells (Annexin V-FITC positive/PI positive) (Fig 3).

In addition to apoptosis and necrosis, PS exposure has also been found in anoikis, caspase-independent apoptosis, and autophagy (reviewed in Melino et al 2005). The Annexin V Assay was first described by Vermes et al. in 1995 (Vermes et al. 1995) and has since been cited in thousands of references for detecting apoptosis in a broad spectrum of cell types, species, and physiologic and pathologic conditions (PubMed: www.ncbi.nih.gov). Figure 4 shows an example of a publication citing the CytoGlo™ Annexin V-FITC Kit (NBP2-29373).

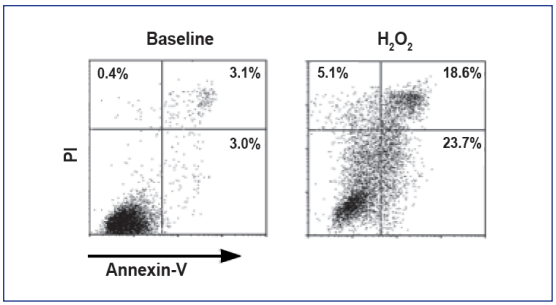

Figure 4. Analysis of H_20_2 induced apoptosis in mutant SOD1 overexpressing cells using the CytoGlo™ Annexin V-FITC Kit (NBP2-29373). *Cells were left untreated or treated with 225 μM H_20_2 for 48 hr and stained with Annexin V-FITC and PI. The data shows a low basal level of early apoptotic (Annexin V-FITC +/PI-), late apoptotic (Annexin V-FITC +/PI+) and dead or necrotic (Annexin V-/PI-) cells. These levels increased significantly following H_20_2 treatment. Source: Lu et al (2009).*

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Caspase Antibody Assays

Apoptosis, or programmed cell death, is a common property of all multicellular organisms. The current dogma of apoptosis suggests that the components of the core cell-death machinery are integral to cells and are widely conserved across species. Caspases, a family of cysteinyl aspartate-specific proteases, are integral components of the cell death machinery (reviewed in Li and Yuan, 2008). They play a central role in the initiation and execution of apoptotic cell death and in inflammation. Caspases are typically divided into three major groups depending on the structure of their prodomain and their function; Group I: inflammatory caspases (caspases 1, 4, 5, 11, 12, 14). Group II: initiator of apoptosis caspases (caspases 2, 8, 9). Group II: effector caspases (caspases 3, 6, 7).

Caspases are synthesized as zymogens (inactive pro enzyme precursors which require a biochemical change to become ac-

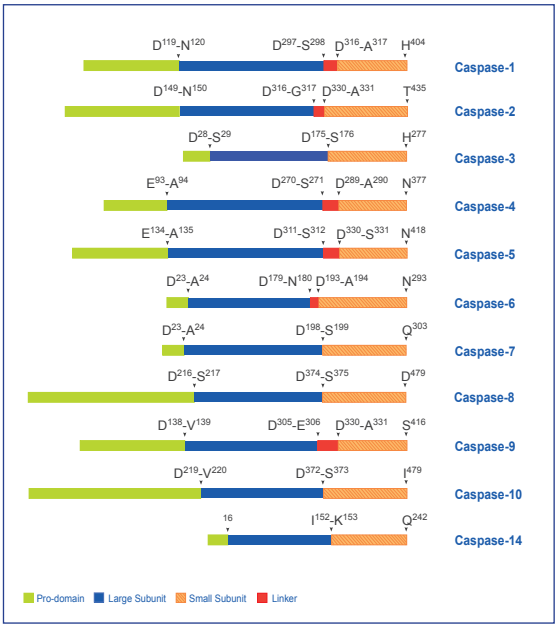

Figure 1. Structure of caspases

tive enzymes) with an N-terminal prodomain of variable length followed by a large subunit (p20) and a small subunit (p10). Caspases are activated through proteolytic cleavage at specific asparagine residues that are located within the prodomain, the p10, and p20 subunits. Activation results in the generation of mature active caspases that consist of the heterotetramer $p20_2$-$p10_2$. Active caspases mediate cell death and inflammation through cleavage of particular cellular substrates that are involved in these processes. See Figure 1 for a schematic of caspase structure and cleavage sites.

Caspase Abs are classical tools for detecting inactive (pro) and active (cleaved) forms of the enzymes. The presence of the large or small subunits in western blots is a considered to be a marker of caspase activation (Fig 2). Likewise, nuclear immunostaining of caspase-3 is also considered be an indication of active/cleaved caspase-3 (Fig 3). Caspase-3 activation is considered to be a hallmark of apoptosis (Figs 2 and 3) and there are literally thousands of references citing caspase-3 and other caspase antibodies for detecting apoptosis (PubMed: www.ncbi.nlm.nih.gov).

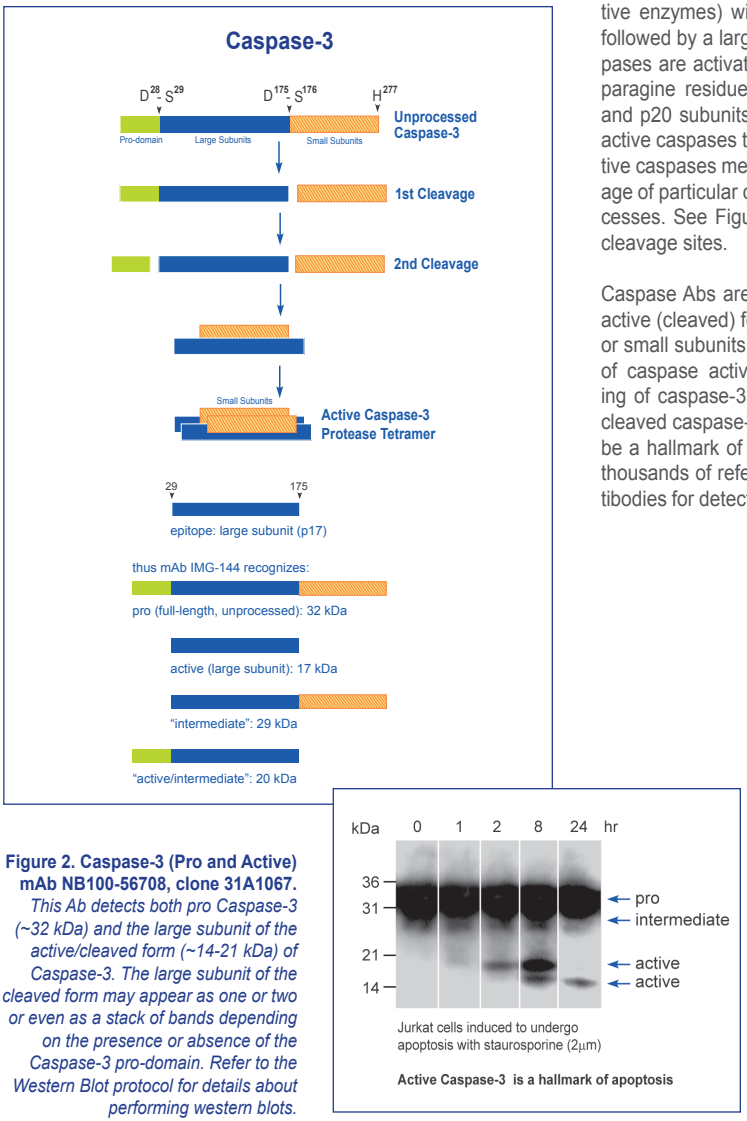

Figure 2. Caspase-3 (Pro and Active) mAb NB100-56708, clone 31A1067. *This Ab detects both pro Caspase-3 (~32 kDa) and the large subunit of the active/cleaved form (~14-21 kDa) of Caspase-3. The large subunit of the cleaved form may appear as one or two or even as a stack of bands depending on the presence or absence of the Caspase-3 pro-domain. Refer to the Western Blot protocol for details about performing western blots.*

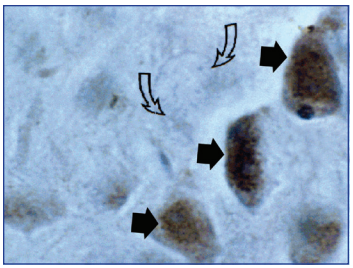

Figure 3. Analysis of formalin-fixed paraffin-embedded dog ischemic brain tissue using an Active/Cleaved Caspase-3 pAb (NB100-56113). *This Ab preferentially detects active caspase-3 (large subunit: ~14-21 kDa, and small subunit: ~10 kDa) in immunohistochemical staining. Staining is seen in the nuclei of dying neurons (black arrows) but not in the morphologically normal nuclei (open arrows). The presence of Caspase-3 in the nucleus is considered to be an indication of active Caspase-3, a hallmark of apoptosis.*

It is interesting to note that the non-apoptotic roles for caspases are increasingly being highlighted. For example, it is increasingly being recognized that Caspase-8 has roles in embryonic development, monocyte differentiation, T and B cell proliferation and as well as the activation of TLR and NF-κB signaling (Reviewed in Maelfait and Beyaet, 2008). Other caspases have been found to have non-apoptotic roles as well. For example, caspases 2, 3, and 9 have also been found to have essential non-apoptotic roles in hematopoietic cell differentiation (reviewed in Droin et al, 2008). Undoubtedly, knowledge of the apoptotic and non-apoptotic roles of caspases in TLR and NF-κB signaling, as well as in cell differentiation, will continue to unfold in the coming years. Novus is proud to have the largest portfolio of caspase antibodies and related reagents worldwide to help caspase related research and we encourage researchers to visit our website for additional information (www.novusbio.com). Refer to the Immunohistochemistry protocol for details about performing immunohistochemistry assays.

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Cell Extraction Kits

Endoplasmic Reticulum Enrichment Kit:
NBP2-29482, 50 Tests (0.5 gram tissue/test)

The Endoplasmic Reticulum (ER) is a membranous labyrinth that extends throughout the cytoplasm of the cell as a single continuous network of flattened sacs and tubules. The ER accounts for more than half the total membrane in eukaryotic cells and 10% of a cell's total volume, thus representing one of the largest organelles. It plays a critical role not only in lipid and protein biosynthesis but is central to protein modification, folding, and assembly. The ER is generally characterized as being devoid of membrane-bound ribosomes (smooth ER), or studded with ribosomes (rough ER); each differing in structure, appearance, and function.

The Endoplasmic Reticulum Enrichment Kit is designed for enrichment of ER from tissues. This kit can be used for the enrichment of total ER (rough and smooth ER microsomes), rough ER microsomes, or both (Fig 1) It's easy-to-use, gentle enrichment method is ideal where isolated ER microsomal fractions are desired, but density gradient purified preparations are not required. A protocol overview is shown in Figure 2.

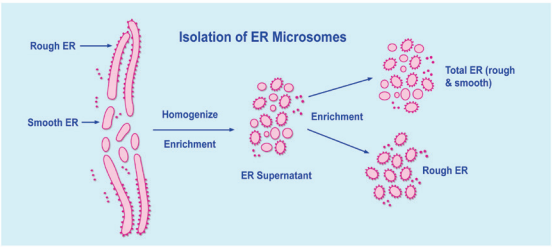

Figure 1. The ER Kit is designed for enrich for both total ER and rough ER. *Both assays can be performed by dividing the ER supernatant obtained after the initial enrichment .*

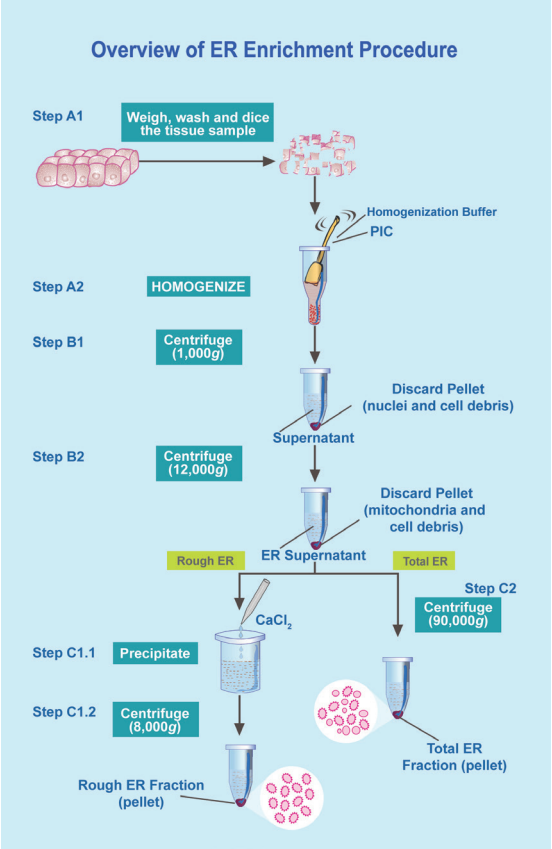

Figure 2. ER protocol overview. *Researchers can use the kit to enrich for both total ER and rough ER. Alternatively, they can select to enrich for only total ER or only rough ER, depending on the needs of their experiments.*

Isolated ER microsomal fractions may be used in understanding biochemical processes associated with the ER such as protein synthesis, subcellular localization, lipid synthesis, unfolded protein response, cellular stress, and more. Figure 3 shows an example of ER enrichment results.

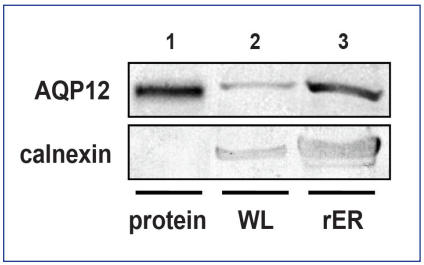

Figure 3. Western blot analysis of rat pancreas whole tissue and rER. *Lane 1 in vitro translated aquaporin 12 (AQP12). Lane 2, WL: whole tissue lysates. Lane 3, rER. Calnexin is an ER protein. The data shows the AQP12 was enriched in the rER.*

Reference

Pancreas-specific aquaporin 12 null mice showed increased susceptibility to caerulein-induced acute pancreatitis. Ohta E, T Itoh, T Nemoto, J Kumagai, SBH Ko, K Ishibashi, M Ohno, K Uchida, A Ohta, E Sohara, S Uchida, S Sasaki, T Rai. *Am J Physiol. doi*:10.1152/ajpcell.00117.2009 (2009).

Novus product cited: Endoplasmic Reticulum (ER) Enrichment Kit (NBP2-29482): WB (rat pancreas), Fig 9. The results show that APQ12 was enriched in the rough ER.

Mitochondrial Extraction Kit:
NBP2-29448, 100 (cell) or 10 (tissue) Tests

The Mitochondrial Extraction Kit is designed for isolation of intact mitochondria from cells and tissues. The isolation procedure is based on the principle of differential centrifugation. The high density nuclei are first removed by low-speed centrifugation on a sucrose cushion. The supernatant containing the mitochondria is then subjected to high speed centrifugation to retrieve the mitochondria. During this process both mitochondrial and cytosolic fractions are obtained. The fractions can be used or protein analysis by western blotting, ELISA, or other assays. Additionally, many mitochondria are still intact prior to the final certrifugation and lysis steps, and may retain respiration properties. Mitochondrial integrity can be observed by microscopy and intact mitochondria can also be used for a variety of studies. A protocol overview is shown in Figure 1, and sample western blot results are shown in Figure 2.

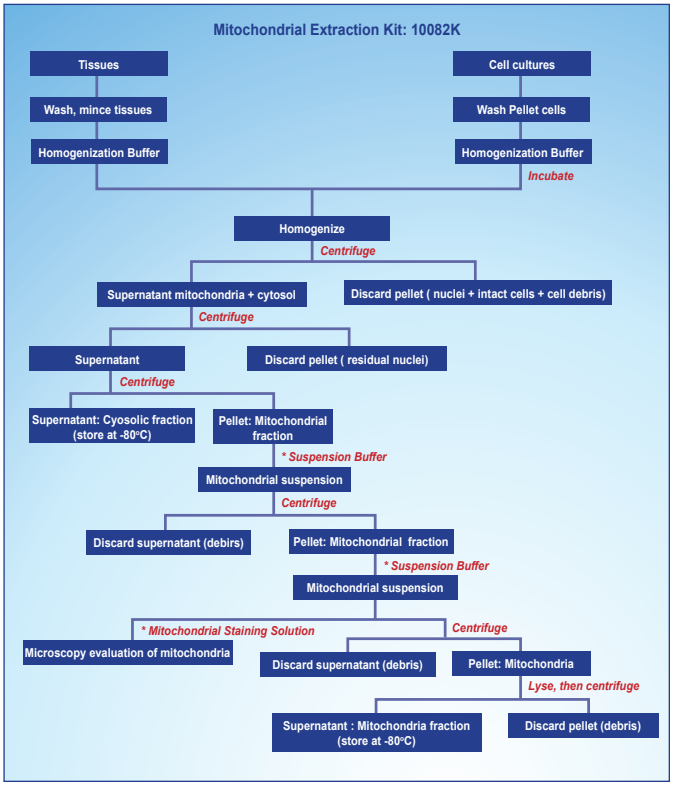

Figure 1. Mitochondrial Extraction Kit protocol overview. *The protocol is based on the principle of differential centrifugation. The high density nuclei are first removed by low-speed centrifugation on a sucrose cushion. The supernatant containing the mitochondria is then subjected to high speed centrifugation to retrieve the mitochondria.*

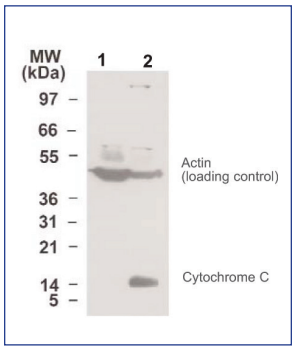

Figure 2. Western blot analysis of cytosolic and mitochondrial fractions using Cytochrome C (NB100-56503) and β-actin (NB100-56874) Abs. *Cytochrome C is a mitochondrial protein and β-actin is a loading control. Mouse liver tissue was fractionated using Novus' Mitochondrial Extraction Kit. 20 ug of cytosolic (lane 1) and mitochondrial (lane 2) extracts were probed with Cytochrome C mAb (NB100-56503), and β-actin pAb (loading control, NB100-56874). The results show that Cytochrome C was detectable in the mitochondrial, but not the cytosolic fraction indicating that the kit effectively fractionated the mitochondria.*

Representative Novus Product Citations

Ayala JE, DP Bracy, BM Julien, JN Rottman, PT Fueger, and DH Wasserman. Chronic treatment with sildenafil improves energy balance and insulin action in high fat-fed conscious mice. Diabetes 56:1025-1033 (2007). **Isolation of mitochondria from intacapsular brown fat (mice) using NBP2-29448, Fig 3: Mitochondrial extracts were used for western blot.**

Diao J, EM Allister, V Koshkin, SC Lee, A Bhattacharjee, C Tang, A Giacca, CB Chan, and MB Wheeler. PNAS 105:12057-12062 (2008). UPC2 is highly expressed in pancreatic alpha-cells and influences secretion and survival. PNAS 105:12057-12062 (2008). **Isolation of mitochondrial and cytosolic fractions from mouse pancreatic cell lines, alpha-TC6 and MIN6 using NBP2-29448: Fig 2, mitochondrial and cytosolic fractions were used for western blot.**

Nuclear Extraction Kit:
NBP2-29447, 100 (cell) or 10 (tissue) Tests

The Nuclear Extraction Kit provides a simple and convenient method for the isolation of nuclear and cytoplasmic extracts from mammalian cells and tissue samples. This procedure is relevant to monitoring translocation of cell signaling molecules from the cytoplasm to the nucleus. Examples include translocation of NF-κB molecules to the nucleus in TLR ligand or TNFα treated cells, and translocation of mitogen-activated protein kinase to the nucleus in growth factor treated cells. The Nucelar Extraction Kit is a component of the NF-κB/p65 ActivELISA Kit™ (NBP2-29661), where it is referred to as the Lysate Preparation Module.

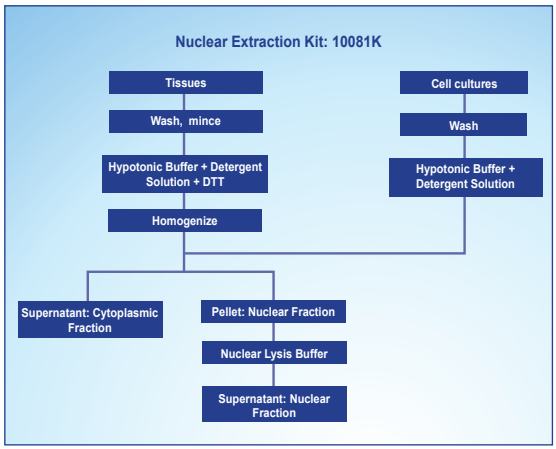

Figure 1. Nuclear Extraction Kit (10081K) protocol overview.

The Nuclear Extraction Kit, aka Lysate Preparation Module, can be used to prepare fractionated lysates for western blotting, Electrophoretic Mobility Assays (EMSA) and preparative purification of nuclear proteins. A protocol overview is shown in Figure 1. As shown in the protocol, the kit can be used to isolate cytosolic and nuclear fractions from the same cell or tissue preparation. Sample data is shown in Figure 2. See the section on the NF-κB/p65 ActivELISA Kit (NBP2-29661) for additional data. The data shows that TLR signaling is activated with LPS (TLR4 ligand) and nuclear extracts are used in an NF-κB/p65 readout assay.

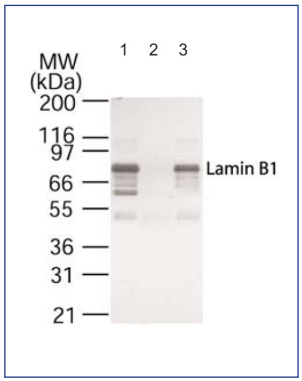

Figure 2. HeLa cells fractionated using the Nuclear Extraction Kit (10081K) *and analyzed for Lamin B1 (NB100-56403) expression by western blot using IMG-5133A. 20 ug/protein per lane was used. Lane 1. Nuclear extract, Lane 2. Cytoplasmic extract, Lane 3. Total cell lysate. Lamin B1, a nuclear protein, was detected in the total cell lysate and nuclear extract.*

ChIP Assays

Chromatin Immunoprecipitation (ChIP) is a powerful method used to identify regions of the genome associated with specific proteins (reviewed in Collas, 2008). Protein-DNA associations are crucial for vital cellular functions including gene transcription, DNA replication and recombination, repair, segregation, chromosomal stability, cell cycle progression, and epigenetic silencing. The ChIP assay is used to study both histones and non-histone proteins, such as transcription factors, within the context of the cell. Transcription factors and other DNA binding proteins have a weaker affinity for DNA than histones, which generally are tightly associated with the chromatin complex. To avoid dissociation of non-histone proteins from the chromatin binding site, a cross-linking step is incorporated into the assay.

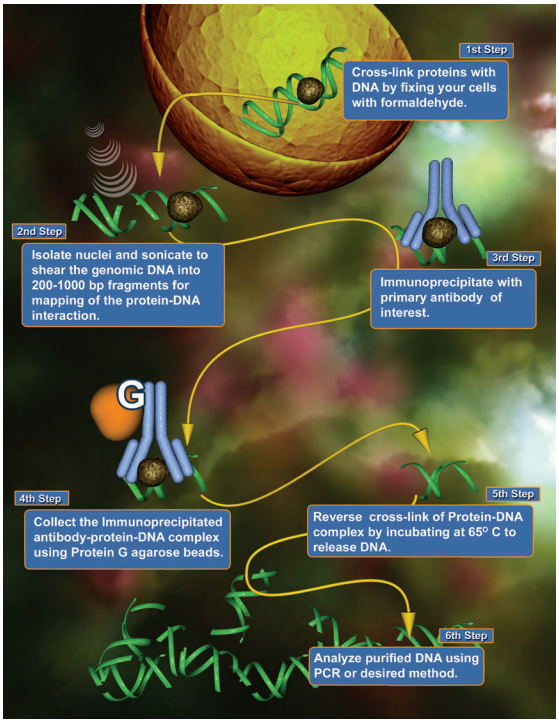

Figure 1. Overview of the Novus QuikChIP™ Assay

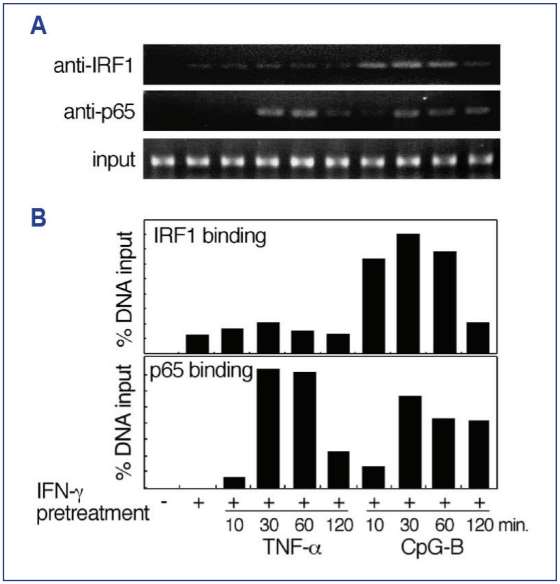

Figure 2. ChIP analysis of IRF1 and p65 using the QuikChIP™ Kit (NBP2-29902). *RAW264.7 cells were stimulated with IFN-γ and then with TNF-α or the TLR9 agonist CpG-B.*
A. The results of the ChIP analysis were quantified by measuring the band intensities with a densitometer. The intensity of the band corresponding to the IRF1 or p65 antibodies binding to the promoter was compared with that corresponding to input DNA.
B. The densitometry analysis shows that strong recruitment of the NF-κB component p65 to the IL-12/p35 promoter was observed for both TNF-α and CpG-B stimulated cells. In contrast, a strong recruitment of IRF1 was observed only for CpG-B and not TNF-α stimulated cells. It is interesting to note that the kinetics of recruitment of p65 and IRF1 were similar during CpG-B stimulation. Source: Negishi et al (2006).

QuikChIP™ Kits:
NBP2-29902 & NBP2-29497, 25 assays

Novus' QuikChIP™ (NBP2-29902) and Novus' QuikChIP™ + Controls (NBP2-29497) Kits are optimized for analyzing both histone and non-histone proteins. See Figure 1 for an overview of the the QuikChIP™ assay. In this assay, cells are fixed with formaldehyde to cross-link non-histone proteins to DNA. Alternatively, the cross-linking steps are not needed for histone proteins, which are already tightly bound to DNA. The chromatin is released from the

nuclei and sheared into 200-1000 bp fragments. The chromatin is then immunoprecipitated with antibodies specific to the proteins of interest. During this process, DNA sequences cross-linked to the antibody-bound proteins co-precipitate as part of the chromatin complexes. The DNA/Chromatin/Protein/Antibody complex precipitates are isolated using Protein A/G Agarose. The associated DNA is then released from the complexes by reverse cross-linking and ready for analysis, typically by PCR, qPCR, sequencing or microarray technology.

Figure 2 shows an example of using the QuikChIP™ Kit (NBP2-29902) for analysis of NF-κB and IRF1 transcriptional activation following TLR9 (CpG treatment) or TNFR (TNFα treatment) activation.

References

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Flow Cytometry Kits

Proteins are expressed on the cell surface, intracellularly or both. Flow cytometry detection methods are based on where the protein is thought to be expressed.

Novus offers both TLR and general cell surface and intracellular staining flow kits :

1. Cell Surface Toll-like Receptor Staining Flow Kit (NBP2-26247)
2. Cell Surface Staining Kit (NBP2-29481)
3. Intracellular Toll-like Receptor Staining Flow Kit (NBP2-26248)
4. Intracellular Staining Flow Kit (NBP2-29450)

Benefits of the kits

Simple – optimized protocols for fast results
Complete – contains necessary reagents to fix and/or permeabilize cells
Flexible – suitable for both conjugated and unconjugated mono and polyclonal antibodies.

The TLR kit protocols follow, please see the web site (www.novusbio.com) for additional flow cytometry kits, reagents, and protocols.

Cell Surface TLR Staining Flow Kit: NBP2-26247

Novus' Cell Surface TLR Staining Flow Kit is a convenient way to quickly and efficiently prepare samples for flow cytometric analysis (Fig 1).

Kit Contents (Store at 4°C)

KC-136	Staining buffer, 1X	3 X 60 ml
KC-125	Paraformaldehyde 10%	1 X 10 ml

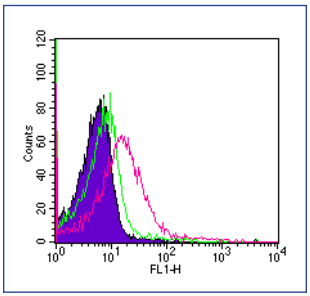


Figure 1. Cell surface flow cytometric analysis of TLR4 in mouse splenocytes using a TLR4 mAb (IMG-5031AF488) and the Cell Surface TLR Staining Kit (10099K). *The shaded histogram represents cells alone; green represents isotype control staining; red represents TLR4 mAb staining.*

1. Determine the number of cells required for staining. For each test sample, a final concentration of 1 x 10^6 cells in 50 µl of staining buffer will be needed. The following controls are suggested: a) unstained cells [no primary or secondary antibody staining], b) cells with an isotype control antibody, c) cells with a positive control Ab.
2. Harvest the cells and spin down to a pellet at 200 x g for 10 min; decant supernatant.
3. Depending on the size of the pellet, resuspend in 2-3 ml of 1X PBS. An exact volume is not necessary at this step.
4. Count the cells with a hemocytometer. Remove 1 x 10^6 cells for each sample (including controls) to be tested to a clean conical centrifuge tube. Add 1 ml of 1X PBS to make the decanting easier.

5. Spin down cells to a pellet at 200 x g for 10 min and decant supernatant.
6. Tap the conical tube gently to loosen the pellet.
7. Resuspended pellet in an appropriate volume (50 µl per 1 x 10^6 cells) of Staining buffer. Aliquot 50 µl of cell suspension to individual flow cytometer compatible tubes, one aliquot for each sample to be tested.
8. To wash cells, add 1 ml of Staining buffer to each tube, spin down cells to a pellet at 200 x g for 10 min and decant supernatant. Prepare primary Ab solutions during centrifugation.
9. Dilute each Ab to be tested to the desired concentration in 50 ul of staining buffer. Resuspend each cell pellet with the appropriate primary Ab. Pipette up and down to thoroughly mix the Ab/cell suspension.
10. Incubate on ice for 30 min (protect from light if using a fluorescent labeled primary Ab).
11. Centrifuge at 200 x g for 10 min and decant supernatant. Note: If using a fluorescent-labeled primary Ab, skip Steps 12-14.
12. Wash the cells by adding 2 ml of staining buffer, spinning at 200 x g for 10 min, and decanting supernatant. While centrifuging, dilute secondary Ab (FITC, PE or Biotin labeled) in 50 µl of Staining buffer per sample.
13. Resuspend cells in diluted secondary Ab solution.
14. Incubate on ice (protected from light) for 30 min. Centrifuge at 200 x g for 10 min and decant supernatant.
15. Wash cells twice by adding 2 ml of Staining buffer, centrifuging and decanting after each wash step.
16. After the final wash, add 1 ml of Staining buffer to each tube. Note: If not analyzing on the same day, resuspend cells in 1% paraformaldehyde in Staining buffer to "fix" cells, and store overnight at 4°C. The Fixation buffer can be removed and the cells prepared for analysis by repeating step 15 and adding 1 ml of Staining buffer to each tube.
17. Test samples on a flow cytometer following manufacturer recommendations.

Figure 1. Intracellular flow cytometric analysis of TLR3 in human PBMCs using a TLR3 mAb (NBP2-24902) and the Intracellular TLR Staining Flow Kit (NBP2-26248). *The shaded histogram represents cells without Ab; green represents isotype control (20106) staining; red represents TLR3 mAb staining.*

Intracellular TLR Staining Flow Kit: NBP2-26248

Novus' Intracellular TLR Staining Flow Kit is optimized for intracellular staining of cells in flow cytometric (Fig 1) or immunocytochemical applications. It is designed and optimized to minimize non-specific staining while maximizing signal-to-noise ratio for clear and consistent data.

Kit Contents (Store at 4°C)

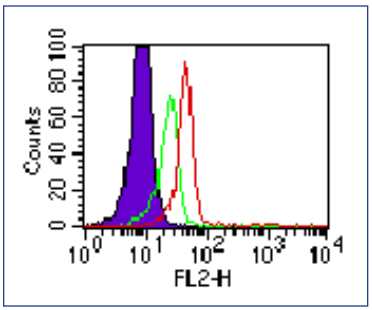
KC-136	Staining buffer, 1X	2 X 60 ml
KC-137	Fixation buffer, 1X	1 X 60 ml
KC-138	Permeabilization buffer, 10X	2 X 60 ml

Background Information

Fixation buffer is used to "fix" the cells/cellular proteins prior to permeabilization.

Permeabilization buffer permeabilizes the cell membrane, allowing the detecting Abs access to intracellular proteins. It is supplied as a 10X solution and can be diluted with deionized water to its final 1X working concentration.

1. Determine the number of cells required for staining. For each test sample, a final concentration of 1 x 10^6 cells in 50 µl of staining buffer will be needed. The following controls are suggested: a) unstained cells [no primary or secondary Ab staining], b) cells with an isotype control Ab, c) cells with a positive control Ab.



2. Harvest the cells and spin down to a pellet at 200 x g for 10 min; decant supernatant.
3. Depending on the size of the pellet, resuspend in 2-3 ml of 1X PBS. An exact volume is not necessary at this step.
4. Count the cells with a hemocytometer. Remove 1 x 10^6 cells for each sample (including controls) to be tested to a clean conical centrifuge tube. Add 1 ml of 1X PBS to make the decanting easier.
5. Centrifuge cells at 200 x g for 10 min and decant supernatant.
6. Tap the conical tube gently to loosen the pellet.
7. Resuspend pellet in the appropriate volume (50 µl per 1 x 10^6 cells) of Fixation buffer. Incubate at room temperature for 30 min
8. Centrifuge cells at 200 x g for 10 min and decant supernatant.
9. Resuspend pellet with the appropriate volume (50 µl per 1 x 10^6 cells) of 1X Permeabilization buffer.
10. Dispense 1x10^6 cells (50 µl) to the desired number of flow cytometer compatible test tubes and centrifuge cells at 200 x g for 10 min. Carefully aspirate the supernatant. During centrifugation, the primary Abs to be used can be diluted to the required concentration in 50 µl of Permeabilization buffer.
11. Resuspend each cell pellet with the appropriate primary Ab. Pipette up and down to thoroughly mix the antibody/cell suspension.
12. Incubate at room temperature for 30 min (protect from light if using a fluorescent-labeled primary Ab).
13. Centrifuge at 200 x g for 10 min and carefully aspirate supernatant.
    Note: If using a fluorescent-labeled primary Ab, skip Steps 14-17.
14. Wash the cells by resuspending each cell pellet with 2 ml of Permeabilization buffer, centrifuge at 200 x g for 10 min., and decant supernatant. While centrifuging, dilute secondary Ab (FITC, PE or Biotin labeled) in 50 µl of Permeabilization buffer per sample.
15. Resuspend cells in diluted secondary Ab.
16. Incubate at RT (protected from light) for 30 min.
17. Centrifuge at 200 x g for 10 min and carefully aspirate supernatant.
18. Wash cells twice with 2 ml of Permeabilization buffer, centrifuging and decanting after each wash step.
19. After the final decanting, add 1 ml of Staining buffer to each tube.
    Note: If not analyzing on the same day, samples can be stored overnight, in the dark, at 4°C.
20. Test samples on a flow cytometer following manufacturer recommendations.

TLR Phenotyping: A Working Model of Expression Patterns in Cell Subsets

TLRs function not only as key components of the innate immune system, but also as regulators of adaptive immunity through their activation of APCs. Among APCs, DCs are the primary APCs that activate and differentiate T lymphocytes into Th1, Th2 and CTL effector cells. Different subsets of DCs seem to differentially express TLRs. For example, human plasmacytoid (pDCs) express TLR7 and TLR9, whereas human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6, TLR8 and TLR10 (Hornung et al, 2002; Jarrosay et al, 2001; Kadowaki et al, 2001).

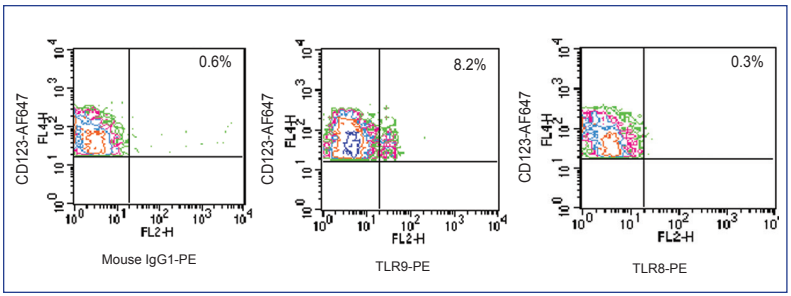


Figure 1. Dendritic Cell & TLR Phenotyping. Differential expression patterns of TLRs in human plasmacytoid dendritic cells (pDC: CD123+, CD14low/-) which express TLR6, TLR7 and TLR9 but not TLR8. *Data shown illustrate human PBMCs which were monocyte-gated and then subgated for pDC (CD123+, CD14low/-). The antibodies shown are CD123 (IMG-6483AF647) CD14 (NBP2-27238) TLR9 (NBP2-24907) TLR8 (NBP2-24817) and Mouse IgG1 (NBP2-24976). Results illustrate expression of TLR9 but not TLR8 in pDCs.*

With respect to lymphocytes, a number of studies have collectively shown that mRNA expression is detectable for most TLRs (Bourke et al, 2003; Hornung et al, 2002; Zarember, 2002). However, functional expression of each TLR in specific subsets of T or B lymphocytes seem to be selective. For instance, TLR8 is functionally expressed in CD4+CD25+ Tregs but not in naïve T (CD4+/CD25-) cells, whereas TLR5 is functionally expressed in both CD4+CD25+ Treg and naïve T cells (Crellin et al, 2005, Peng et al, 2005). Similarly, TLR1 and TLR9 are apparently expressed in B lymphocytes, but their expression seems to be biased towards CD19+/CD5+ B cells (Dasari et al, 2005).

However, comprehensive knowledge about TLR expression patterns with respect to cell lineage markers does not yet exist. Yet, the ability to phenotype TLR expression is critical for advancing our knowledge of TLR signaling. To address this, we have developed a working model from information ferreted out of the published literature (Table 1).

Most of the TLR expression information in Table 1 is based on ligand responsiveness, although TLR Abs were used in some of the studies, eg. Crellin et al, 2005. The relative absence of TLR protein expression data in phenotyping models is due in part to the heretofore paucity of TLR-conjugated flow cytomety antibodies. Novus is developing an increasing number of fluorescently labeled TLR Abs and Kits to enable TLR phenotyping within the context of cell lineage markers. Data examples are shown in Figures 1 and 2. These figures illustrate phenotyping of plasmacytoid dendritic cells (pDCs) and immature dendritic cells (iDCs).

The ability to phenotype TLR expression will be invaluable as the field of TLR biology moves forward. The working model presented in this chapter will undoubtedly be expanded and reshaped in the years to come.

Table 1. TLR expression patterns in human immune cells.

	T cells				B cells			Monocyte	Dendritic Cells		
Activation				Anti-CD3 & Anti-CD28			Anti-CD40 and/or Anti-μ				GM-CSF & IL-4
Subtype					B-1	B-2			mDC	pDC	Differentiated iDC
Markers	**CD3+ CD4+**	**CD3+ CD8+**	**CD4+ CD25-**	**CD4+ CD25+**	**CD19+ CD5+**	**CD19+ CD5-**	**CD19+ CD80+ CD86++**	**CD14+**	**CD123- CD11b+ CD11c+**	**CD123+ CD11c- CD14-**	**CD80+ CD86+ CD83+ CD14-**
TLR1					++	+		++	++	+	++
TLR2	+/-	+		+	+/-	+/-	+/-	++	++	-	++
TLR3					+/-	+/-		-	++	-	++
TLR4	+/-	+/-		+	+/-	+/-	+/-	++	+/-	-	++
TLR5	+		+	+				++	+	-	+/-
TLR6								++	++	++	++
TLR7	+				-		+	+/-	+/-	++	-
TLR8			-	+				++	++	-	++
TLR9					++	+	++	-	-	++	-
TLR10							+	-	+	+	

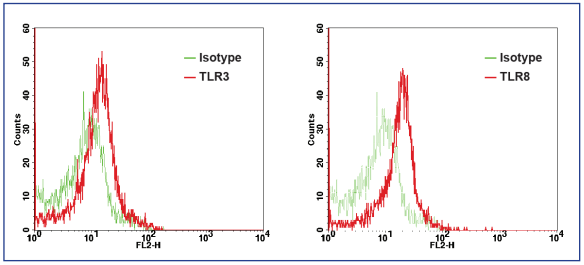


Figure 2. Positive expression patterns of TLR3 and TLR8 in differentiated human immature dendritic cells (iDC: CD83^{+}/CD14$^{low/-}$). *The cells were generated from human PBMC monocytes treated with GM-CSF and IL-4 for 7 days. The antibodies used are CD83-FITC, CD14 (NBP2-27253), TLR3 (NBP2-24902) and TLR8 (NBP2-24817).*

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Protocols for Dendritic Cell (DC) Flow Analysis

These protocols utilize the Intracellular Toll-like Receptor Staining Flow Kit (NBP2-26248). The KC components listed in the protocols are components of this kit, i.e., Staining Buffer (KC-136), Fixation Buffer (KC-137), and Permeabilizaton Buffer (KC-138).

I. Plasmacytoid DC (pDC) Flow

Cell purification and staining

1. Isolate human peripheral blood mononuclear cells (PBMC) from human blood samples by Ficoll gradient centrifugation.
2. Resuspend PBMC in RPMI media containing 10% heat-inactivated fetal bovine serum (RPMI/10% FBS) and count cells.
3. Aliquot cells into a 15-ml tube and wash once with Staining Buffer (KC-136) by centrifugation at 200 x g for 10 min.
4. Resuspend cells in Staining Buffer at 2x10^{7} cells/ml (50 µl per million cells).
5. Aliquot 1x10^{6} cells into each flow tube.
6. Add cell surface antibodies to cells (0.25 µg anti-hCD14-AF488, 0.05 µg anti-hCD123-AF647 and incubate on ice for 30 min.
7. Wash cells by adding 2 ml of Staining Buffer to each tube and spinning for 10 min at 200 x g.
8. (Skip Steps 8-11 for surface TLRs) Resuspend cells in 200 µl Fixation Buffer (KC-137) and leave on ice for 20 min.
9. Wash cells by adding 2 ml of 1 Permeabilization Buffer (KC-138) to each tube and spinning at 200 x g for 10 min.
10. Add 1.0 µg intracellular hTLR antibodies (anti-hTLR8-PE, anti-hTLR9-PE or Isotype-PE) and incubate on ice for 30 min.

11. Wash cells by adding 2 ml of Staining Buffer to each tube and spinning for 10 min at 200 x g.
12. Resuspend cells in 500 µl of Staining Buffer and perform flow cytometry.

Flow analysis

1. Gate the monocyte population as R1 upon a dot plot of FSC vs. SSC.
2. Gate the CD123+/CD14- population of R1 as R2 upon a dot plot of FL1 vs. FL4.
3. Read TLRs from the R2 population upon a density or contour plot of CD123 (FL4) vs. TLR or Isotype (FL2).

Note: An example is illustrated in Figure 1.

II. Differentiated immature DC (iDC) Flow

Cell purification and staining

1. PBMC (purification is identical to that described for pDC) are plated in a 10-cm cell culture plate ($2x10^7$ cells/plate) and incubated for 2 h at 37°C.
2. Remove non-adherent cells and gently rinse the plate twice with 5 ml RPMI/10% FBS.
3. Add 15 ml RPMI/10% FBS containing 1000 U/ml GM-CSF and 500 U/ml IL-4, and incubate for 7 days at 37°C.
4. Remove culture media, add PBS containing 0.2% EDTA, and incubate for 15 min at 4°C.
5. Harvest the adherent iDCs and wash twice with RPMI/10% FBS by spinning at 200 x g for 10 min.
6. Count cells and aliquot $1x10^6$ cells into each flow tube.
7. Stain cells with the fluorescent antibodies (anti-hCD83-FITC/CD14-AF647/TLR3-PE or TLR8-PE) according to the procedures described for pDC Flow earlier.

Flow analysis

1. Gate the live cell population as R1 upon a dot plot of FSC vs. SSC.
2. Gate the CD83+/CD14- population of R1 as R2 upon a dot plot of FL1 vs. FL4.
3. Read TLRs or isotype from the R2 population upon a histogram of FL2.

Note: An example is illustrated in Figure 2.

Histo-Array™ Tissue Microarray Slides

Novus' Histo-Array™ tissue microarray slides (TMAs) are a simple, powerful, and highly efficient method for expression analysis or localization studies of molecular targets at the protein as well as DNA and RNA levels (Fig 1). Refer to the Immunohistochemistry Protocols section for recommended protocols.

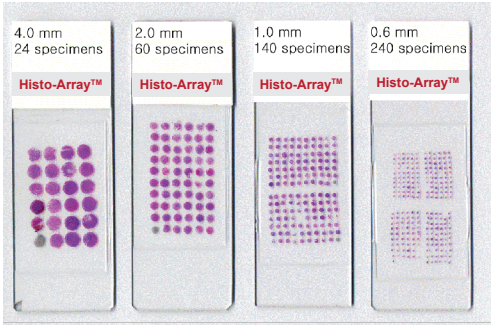
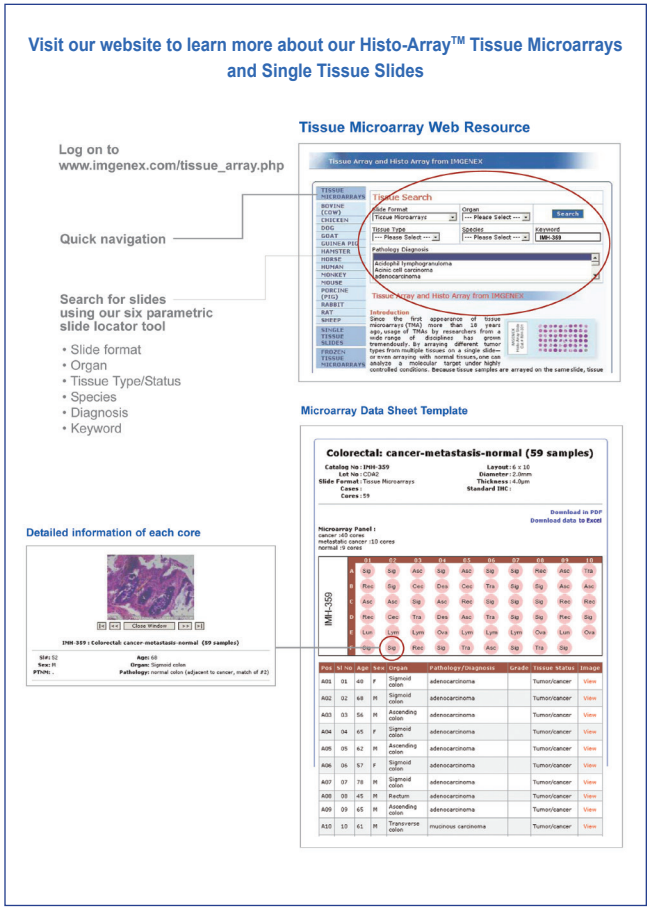
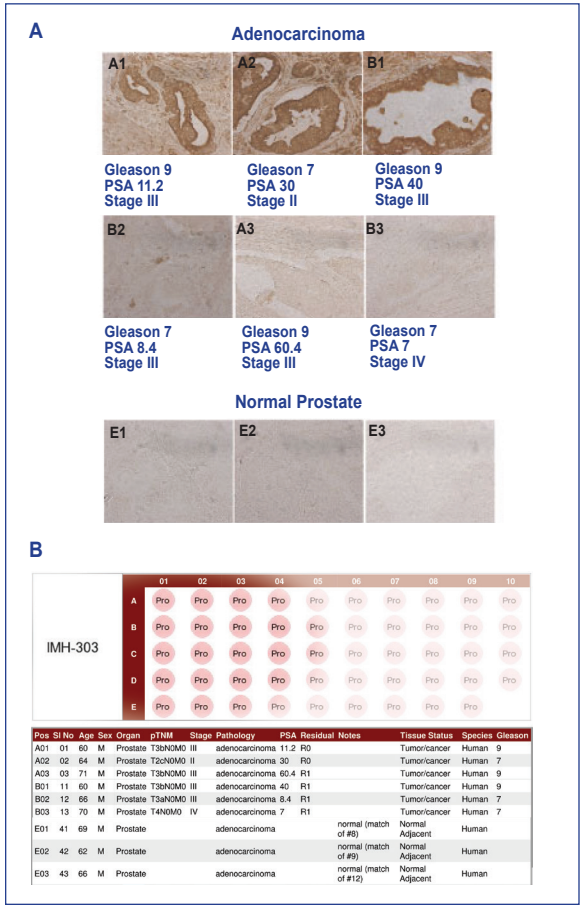


Figure 1. Representative TMAs. *Over ninety different human or animal TMAs are available, with tissue sample densities ranging from 24 to 146 spots per slide, corresponding to tissue sample sizes of 4.0 mm down to 0.6 mm.*

Slides are available with different cancerous, normal, normal adjacent, or diseased tissues of different organs as well as matched tissue samples for certain slides. The Tissue Microarray Web Resource is an easy to use navigation tool for identifying slides that meet your needs (Fig 2). Each slide includes patient data on tissue samples including age, sex, diagnosis, and any applicable staging codes. A representative slide data sheet (Colorectal: IMH-359) is shown in Figure 2. The microarray template shown for IMH-359 applies to all Histo-Array™ microarray slides beginning with the Cat no. "IMH".

A literature citation example is shown in Figure 3. In this figure, the data obtained is shown in A, and B indicates the cores on IMH-303 used to generate the data and the corresponding patient data.





Pos	Sl No	Age	Sex	Organ	pTNM	Stage	Pathology	PSA	Residual	Notes	Tissue Status	Species	Gleason
A01	01	60	M	Prostate	T3bN0M0	III	adenocarcinoma	11.2	R0		Tumor/cancer	Human	9
A02	02	64	M	Prostate	T2cN0M0	II	adenocarcinoma	30	R0		Tumor/cancer	Human	7
A03	03	71	M	Prostate	T3bN0M0	III	adenocarcinoma	60.4	R1		Tumor/cancer	Human	9
B01	11	60	M	Prostate	T3bN0M0	III	adenocarcinoma	40	R1		Tumor/cancer	Human	9
B02	12	66	M	Prostate	T3aN0M0	III	adenocarcinoma	8.4	R1		Tumor/cancer	Human	7
B03	13	70	M	Prostate	T4N0M0	IV	adenocarcinoma	7	R1		Tumor/cancer	Human	7
E01	41	69	M	Prostate			adenocarcinoma			normal (match of #8)	Normal Adjacent	Human	
E02	42	62	M	Prostate			adenocarcinoma			normal (match of #9)	Normal Adjacent	Human	
E03	43	66	M	Prostate			adenocarcinoma			normal (match of #12)	Normal Adjacent	Human	

Figure 2. TMA Web Resource and Data Sheet Template.
Novus' TMAs have been widely published. For example, Figure 3 shows the analysis of TLR expression in prostate adenocarcinoma and normal adjacent tissues using a Prostate Cancer Histo-Array™ (NBP2-30169).

Figure 3. Immunohistochemical analysis of TLR9 expression in a Prostate Cancer Histo-Array™ (NBP2-30169) using a TLR9 mAb. *A. The data shows that variable expression of TLR9 was seen in adenocarcinomas whereas little or no TLR9 was detected in normal adjacent tissue samples. Novus offers a TLR9 mAb (NBP2-24729) for immunohistochemistry of formalin-fixed, paraffin embedded tissue sections. This antibody is also useful for other applications, including flow cytometry, western blot, IHC (frozen) and IF/ICC. See their respective product sheets on the web site (www.novusbio.com) for additional information. Source: Ilvesaro et al, 2007.*

B. TMA cores utilized to generate the data in 3A by Ilvasaro et al (2007). See the following link for additional Novus TMA product citations: www.novusbio.com. Representative publications are also listed in the TMA product citation list.

Histo-Array™ TMA product citations (selected list)

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2. Krop, I, A Marz, H Carlsson, X Li, N Bloushtain-Qimron, M Hu, R Gelman, MS Sabel, S Schnitt, S Ramaswamy, CG Kleer, C Enerback, and K Polyak. A putative role for psoriasin in breast tumor progression. *Cancer Res* 65:11326-11334. (2005). **Novus products cited 1. Breast: cancer-metastasis-normal TMA (NBP2-30152). Fig 3D [IHC (paraffin)] 2. Psoriasin/HID5/S100A7 mAb (NB100-56559), Fig 1 (WB, IHC (paraffin)], Fig 3D [IHC (paraffin)]**

3. O'Toole J, K Rabenau, K Burns, D Lu, V Mangalampalli, P Balderes, N Covino, R Bassi, M Prewett, K Gottfredsen, M Thobe, Y Cheng, Y Li, D Hicklin, Z Zhu, S Waltz, M Hayman, D Ludwig, D Perira. Therapeutic implications of a human neutralizing antibody to the macrophage-stimulating protein receptor tyrosince kinase (RON), a c-MET family member. *Cancer Res* 66: 9162-9170 (2006). **Novus products cited for IHC (paraffin), Fig 1: 1. Breast: cancer-metastasis-normal TMA (NBP2-30152). 2. Colon and rectal cancer TMA (NBP2-30256). Prostate cancer TMA (NBP2-30169). 4. Stomach cancer TMA (IMH-316).**

4. Wang J, N Simonavicius, X Wu, G Swaminath, J Reagan, H Tian, L Ling. Kynurenic acid as a ligand for orphan G Protein-coupled receptor GPR35 *JBC* 281: 22021-22028 (2006). **Novus product citation: Mouse normal organs TMA (NBP2-30224): In situ hybridization, Fig 8B-D.**

5. Zhang S, K MacDonal, M Baguma-Nibasheka, L Geldenhuys, A Casson, P Murphy. Alternative splicing and differential subcellular localization of the rat FGF antisense gene product. *BMC Mol Biol* 9:1-15 (2008). **Novus product citation: Rat normal organs TMA (NBP2-30227): IHC (Paraffin), Fig 5 a-h.**

Immunohistochemistry Protocols

The Immunohistochemistry protocols are a guide for using Novus Histo-Array™ tissue microarray slides and antibodies. Protocols for *in situ* hybridization on tissue slides and DNA isolation from tissue slides are also included. Please note that modifications may be necessary depending on the target gene(s), antibodies, or nucleic acid probes, and researchers should optimize for their own model system.

De-Paraffinization and Hydration Protocol for Slides

Materials Required

- Tissue array slide
- 100%, 95%, and 75% ethanol
- Xylene

Method

1. Incubate in a dry oven at 62oC for 1 hour. Slides should be maintained in a vertical orientation to allow complete removal of the paraffin.
2. Dewax slides in xylene for 5 x 4 minutes.
3. Hydrate slides in 100%, 95%, and 75% ethanol for 2 x 3 minutes each.
4. Immerse slides in tap water for 5 minutes.

Suggested Antigen Retrieval Protocol

The following procedure are a suggestion only. Other protocol can be used on the array slides.

Materials Required

- Tissue array slide
- Phosphate buffered saline (pH 7.6)
- Citrate buffer (0.01 M, pH 6.0)
- Microwave oven (700 W)

Method 1 (Microwave)

1. Immerse slides into citrate buffer (0.01 M, pH 6.0).
2. Microwave (700 W or high) for 5 min, add citrate buffer if necessary.

3. Microwave (medium) for 5 min, add citrate buffer if necessary.
4. Microwave (low) for 5 min.
5. Immerse in cold PBS.

Method 2 (Autoclave/Pressure Cooker)

1. Immerse slides in citrate buffer.
2. Incubate in a pressure cooker for 2 min at 95°C.
3. Cool to room temperature.
4. Wash slides in PBS for 3 x 5 min.

Method 3 (Enzyme treatment)

1. Incubate slides with pronase [0.05% (w/v) in PBS] or trypsin [0.05% (v/v) in PBS] or pepsin [0.05% (v/v) in 2 N HCl] at 37°C (incubation time should be adjusted according to the antibody).
2. Wash slides in PBS for 3 x 5 min.

Method 4 (Hot bath)

1. Heat citrate buffer (1mM EDTA, pH8.0 or 0.01M sodium citrate buffer, pH6.0) to about 95°C.
2. Place slides in the buffer for 10-15 min.

Standard Protocol for Immunohistochemistry

Materials Required

1. Slides
2. Phosphate buffered saline (pH 7.6)
3. Hydrogen peroxide
4. Primary antibody
5. Blocking serum (normal serum)
6. Biotinylated secondary antibody
7. ABC reagent (6, 7, and 8 are included in Vectastain Elite ABC kit)
8. Diaminobenzidine
9. Meyer's hematoxylin
10. Permount

Method

1. Incubate in a dry oven at 62oC for 1 hour. Slides should be maintained in a vertical orientation to allow complete removal of the paraffin.
2. Dewax slides in xylene for 5 x 4 minutes.
3. Hydrate slides in 100%, 95%, and 75% ethanol for 2 x 3 minutes each.
4. Immerse slides in tap water for 5 minutes
5. Antigen retrieval method (optional).
6. Quenching of endogenous peroxidase (optional)
    a. immerse slides in 3% hydrogen peroxide solution for 6 minutes.
    b. wash slides in PBS for 3 x 5 minutes.
7. Incubate slides with blocking serum (1:50) for 30 min.*
8. Blot excess serum from section, and incubate with primary Ab. Suggested incubation time (may vary between antibodies): mAb 2 hours at room temperature or overnight at 4°C. pAb: 1 ~ 1.5 hours at room temperature.
9. Wash slides in PBS for 3 x 5 minutes.
10. Incubate slides with biotin-conjugated secondary Ab for 30 min.*
11. Wash slides in PBS for 3 x 5 minutes.
12. Incubate slides with Avidin-Biotin Complexes for 30 min.*
13. Wash slides in PBS for 3 x 5 minutes.
14. Incubate slides in fresh DAB solution for 2 minutes. (We use DAB solution in Vector DAB/Ni substrate kit).**
15. Stop the reaction by washing in tap water.
16. Counterstain in Meyer's hematoxylin for 10 seconds.
17. Dehydrates slides in 75%, 80%, 95% and 100% ethanol
18. Clear slides in xylene 4 X 5 minutes.
19. Mount cover slide with Permount.

* Blocking serum, secondary antibody and avidin-biotin-peroxidase complexes are included in most of the immunostaining kit. Our lab uses the ABC kit from Vector Lab (Vectastain Elite ABC kit).

** We use DAB solution in Vector/DAB/Ni substrate kit (Vector Labs., Cat. SK-4100).

Standard Protocol for *In Situ* Hybridization

The following instruction is provided as a guideline for using digoxigenin-labeled probe. Other labeled probes may be used with tissue array slides.

Part I. The design and generation of probes with digoxigenin

1. Template preparation step:

   1a) for cDNA insert in transcription vector including pro moter for SP6, T7, or T3 RNA polymerase, linearize cDNA with restriction enzyme, and purify linearized cDNA by ethanol precipitation.
   1b) for PCR-generated fragments:
   - prepare primers, such as SP6, T7, or T3 promoter sequence.
   - generate PCR fragments using PCR reaction.
   - purify PCR fragments using a commercially available kit.

2. Probe construction step:

   Prepare cRNA probe reaction mixture as follows:

- template (1- 2 ug)	1 ul
- appropriate RNA polymerase	2 ul
- 10X transcription buffer	2 ul
- 10X DIG RNA labeling mix	2 ul
- sterile RNase free water	13 ul
Total volume	20 ul

3. Incubate reaction mixture for 2 hours at 37°C, add 2 ul DNase I.

4. Incubate for 15 minutes at 37°C, add 2 ul 0.2 M EDTA.

Part II. In Situ Hybridization

1. De-paraffinization and hydration step:

   1a) Incubate in a dry oven at 62°C for 1 hour. Slides should be maintained in a vertical orientation to allow complete removal of the paraffin.
   1b) Dewax slides in xylene for 5 x 4 minutes.
   1c) Hydrate slides in 100%, 95%, and 75% ethanol for 2 x 3 minutes each.
   1d) Immerse slides in DEPC-treated PBS for 5 minutes.

2. Post-fixation step:

   2a) Immerse slides into 4% buffered paraformaldehyde for 10 minutes.

3. Proteinase digestion step:

   3a) Immerse slides in 10 ug/ml proteinase K solution for 20 minutes at RT.

4. Quenching step of endogenous alkaline phosphatase:

   4a) Immerse slides in 0.2 N HCl for 1 hour.
   4b) Wash slides in DEPC-PBS for 5 minutes.

5. Electrostatic interaction inhibition step:

   5a) Immerse slides in 0.1 M TEA for 10 minutes.
   5b) Immerse slides in 0.1 M TEA including 0.25% acetic acid for 10 minutes.
   5c) Wash slides in DEPC-PBS for 5 x 2 minutes.
   5d) Dry slides on air.

6. Hybridization step:
   6a) Prewarm hybrisol for 20 minutes at 50°C prior to mix probe. Mix DIG-labeled probe (2 ug) with 100 ul prewarmed hybrisol.
   6b) Incubate slides with probe for 16 hours at 50°C on HYBrite instrument.

7. Washing step:
   7a) Immerse slides in 2 x SSC/50% formamide for 15 minutes at 50oC water bath.
   7b) Remove coverslip from slides.
   7c) Wash slides in 2 x SSC/50% formamide for 2 x 30 minutes at 50oC water bath.
   7d) Wash slides in 2 x SSC for 20 minutes at room temperature.
   7e) Wash slides in washing buffer (or DIG1 solution) for 5 minutes at room temperature.

8. Incubate slides with blocking solution (or DIG2 solution) for 2 hrs.

9. Blot excess blocking solution from sections, and incubate with anti-DIG anti body for 2 hrs at room temperature or overnight at 4oC.

10. Wash slides in washing buffer (or DIG1 solution) including Tween-20 for 4 x 30 minutes on shaker.

11. Detection step:
   11a) Incubate slides in NBT/BCIP solution with 2 M MgCl2 and 1 mM levamisole for 4.5 ul NBT and 3.5 ul BCIP solution in 1 ml DIG3 solution with 25 ul MgCl2 and 1 mM levamisole.
   11b) Stop the reaction by washing in DIG4 solution for 10 minutes.
12. If needed, counterstain with methyl green.

13. Mount the slides with glycerol gels.

Protocol for DNA Isolation from Tissue Array Slide

A. Deparaffinization

1. Incubate tissue array slides in a dry oven at 60oC for 1 h.
2. Dewax slides in Xylene for 4 min, 5x.
3. Remove Xylene in 100% ethanol, 3x.
4. Air dry.

B. Tissue Collection

1. Wet the tissue with 20% glycerol buffer (5 ul for a single core).
   - glycerol buffer: glycerol 20% in 1X TE (pH 8.0).
2. Scratch the tissue with 26G syringe needle.
3. Collect the cores from 2-10 slides into 1.5 ml eppendorf tube (use laser capture microdissection if necessary).
4. Add 200 ul DNA extraction buffer.
   - DNA extraction buffer: 100 mM NaCl, 0.5% SDS (pH 8.0).
5. Add 5 ul of 20 mg/ml protein kinase solution.
6. Incubate at 55oC overnight with shaking.

C. Extraction

1. Add equal volume of phenol/chloroform/isoamyl alcohol (25:24:1).
2. Vortex for 5 min.
3. Centrifuge 14,000 rpm for 10 min.
4. Transfer aqueous layer into new tube (150 ul).

D. Precipitation

1. Add half volume of 7.5 M ammonium acetate (75 ul).
2. Add 2.5 volume of 100% cold ethanol (560 ul).
3. Store at -20oC for 30 min.
4. Centrifuge 14,000 rpm at 0oC for 10 min.
5. Wash with 500 ul of 70% ethanol, centrifuge again.
6. Dry at RT.
7. Dissolve the DNA pellet in TE (pH 8.0).

* We usually collect the cores of 2 to 10 slides, and get 200 ng DNA.

Lysates (Cell & Tissue)

Novus offers a number of specialized tissue and cell line derived products for protein expression screening (Fig 1).These products are useful for a wide variety of applications ranging from positive western blot controls (Fig 2) to bridging the gaps between basic research and translational medicine (Fig 3). For example, Figure 2 shows the application of the 40153 Intestine Tissue Lysate as a positive control for detecting TLR4 expression. The expression of TLRs can be elusive and vary between donors, and physiological or cell culture states. Hence, positive western blot controls where TLR expression has been documented are particularly useful.

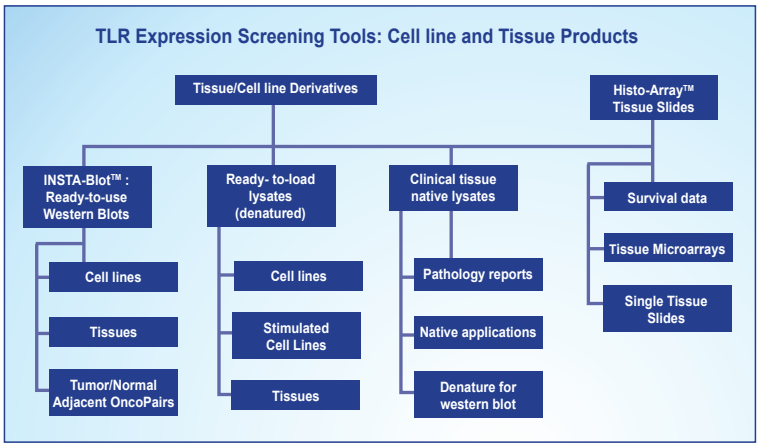


Figure 1. Protein expression screening tools: Novus' cell line and tissue products.

In addition to tumor cell line and normal tissue lysates, Novus offers comprehensive collections of ready-to-use human clinical tissue lysates. These matched tumor and normal adjacent lysates are derived from pathology-defined clinical specimens collected under IRB and HIPAA approved protocols from hospitals around the world (Fig 4). High quality lysates are developed from specimens that have been flash frozen at the collection sites within minutes of removal, maintained in liquid nitrogen, and then processed using protocols optimized for extracting proteins from tissue. Tissue lysates include detailed pathology reports (Fig 5) and are useful for a wide range of proteomic studies.

An example of TLR expression screening in clinical tissue lysates in shown in Figure 6. The data shows that TLR3 was detected in all of the samples (Fig 6A). This data may suggest that TLR3 expression is ubiquitous in colon tumors/normal adjacent tissue. However, screening additional patient donors and tumor types would be needed to determine the significance of this finding.

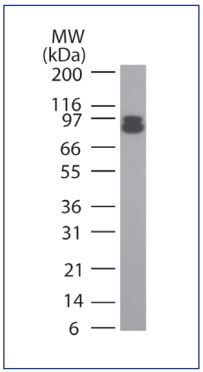


Figure 2. Western blot analysis of TLR4 using mAb clone 76B357.1 (NB100-56566) in 40 µg/lane of human Intestine Tissue Lysate (NBP2-25040). *The lysate is packaged denatured and ready-to-load for western blot analysis.*

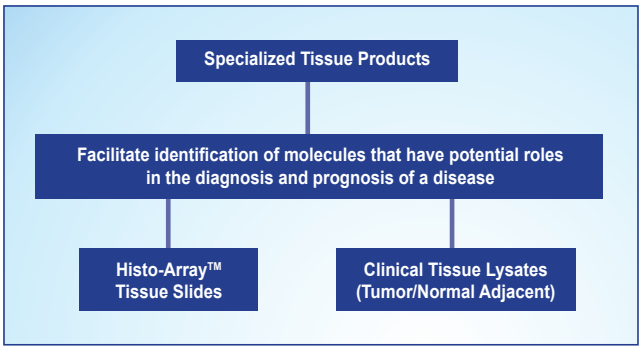


Figure 3. Bridging the gap between basic research and translational medicine.

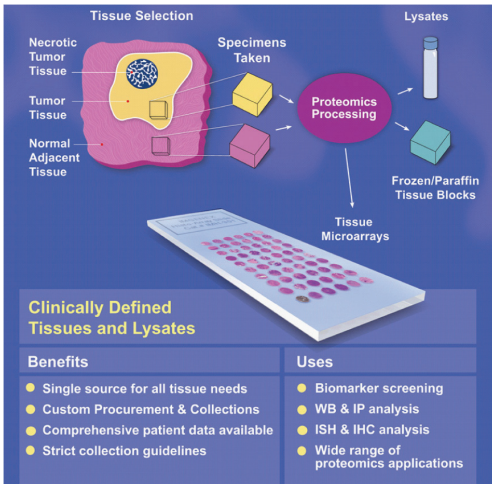


Figure 4. Proteomics pathway: Specialized tissue derived products.

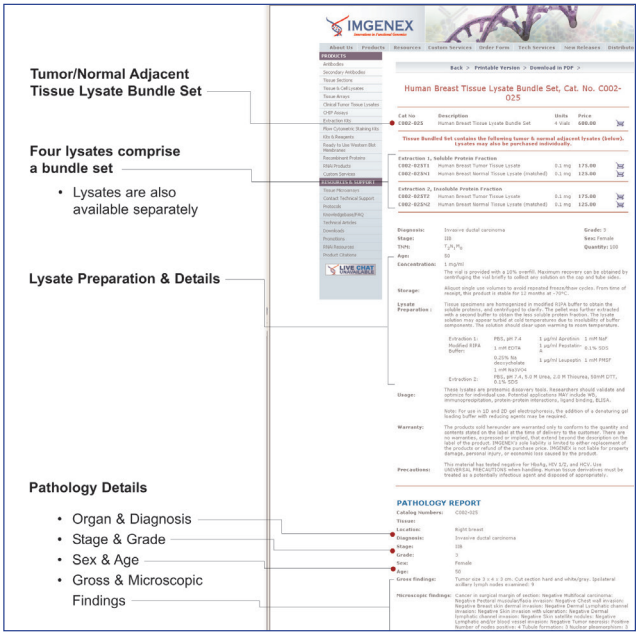
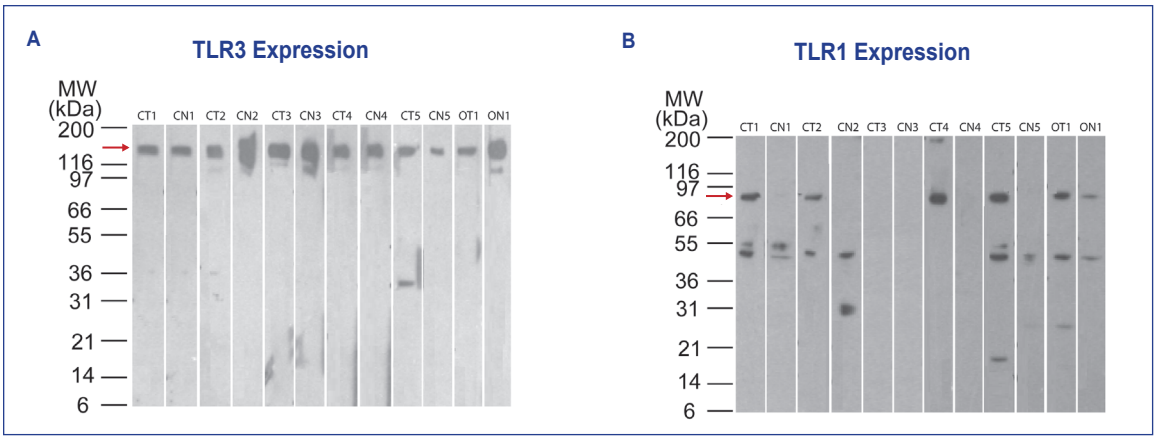


Figure 5. Clinical tissue lysates: Patient donor details can be viewed at www.imgenex.com.



In contrast to TLR3, TLR1 was differentially expressed (Fig 6B). For example, TLR1 was detected in the colon tumor (CT5) but not the normal adjacent (CN5) sample of Donor #5, and not in either sample of Donor #3 (neither CT3 nor CN3).

Please refer to the Immunohistochemistry and Western Blot sections for additional information on tissue/cell derived products including tissue microarray slides, ready-to-use western blots (INSTA-Blots™) and protocols.

Figure 6. Western blot analysis of TLR expression in clinical tissue tumor /normal adjacent tissue lysates. *A, TLR3 mAb (clone 40C1285.6: NBP2-24875). B, TLR1 pAb (NB100-56563).*

C = colon; O = ovary., T = Tumor, N = Normal adjacent. Six patient donor pairs are shown. The tissue specimens were prepared as illustrated in Figure 4. The clinical tissue lysates are packaged native and should be denatured prior to SDS-PAGE and western blot analysis. Denatured packaging is available upon request. The variable banding pattern of TLR1 may result from TLR cleavage products or alternate expression forms in the samples, however this has not been characterized.

TLR Engineered Stable Cell Lines

Novus' engineered TLR Stable Cell Lines (Fig 1) express human TLRs and are emerging as important research tools for a wide array of applications. Most of the cell lines express functional TLRs enabling researchers to screen for compounds that have TLR agonist or antagonist activities, and to study the mechanisms involved in TLR signaling.

All of the Stable Cell Lines can be used for TLR flow cytometric calibration, an important application that is being widely adopted as a positive control for TLR expression. Researchers whose samples have unknown, low, questionable, or undetectable levels of endogenous TLRs are finding that cell lines are particularly useful for assay and antibody validation.

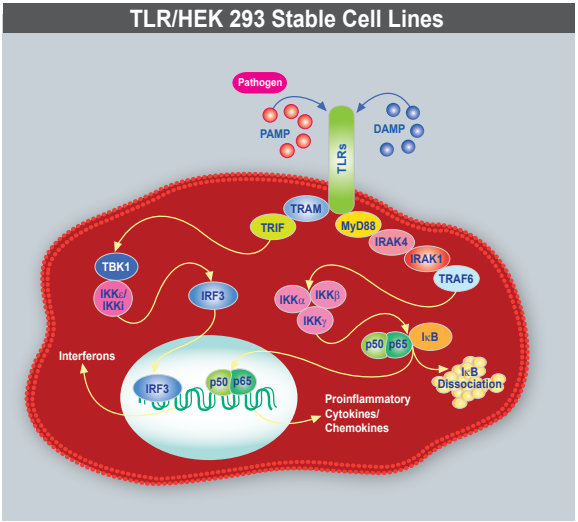


Figure 1A. HEK 293 cells stably transfected with unique and original plasmids for human TLRs. Designed for studying TLR expression and functional analysis, the TLR1, 2, 3, 4, 5, 6, 7, 8 and 9 cell lines have been validated with extensive functional testing. These products are the most optimized and advanced cell lines currently available.

Features

- Optimized TLR Expression
- Extensive Functional Validation
- Flexible and Advanced Research Tools
- Convenience

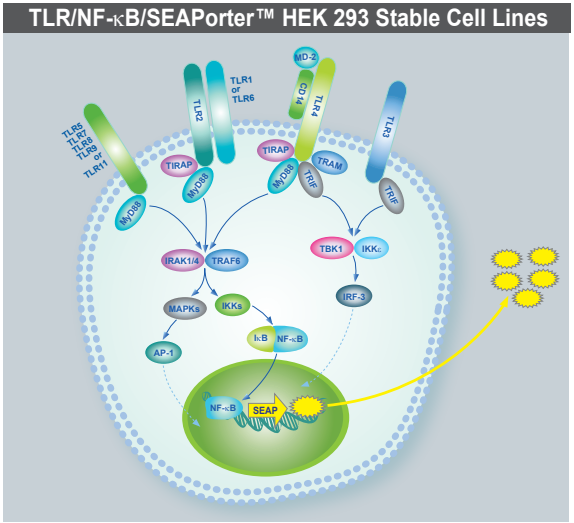


Figure 1B. HEK 293 cells stably co-transfected with human Toll-like Receptor (TLR) and NF-κB reporter genes. These cell lines are designed for sensitivity, broad working range dynamics and applicable for High-Throughput Screening for TLR agonists and antagonists.

Features

- Optimized TLR & NF-κB Expression
- Validated Cell Line Efficacy
- Fast
- Flexible
- High-Throughput
- Convenience

The TLR Stable Cell Lines (Fig 1) are engineered in HEK293 cells and are available in two complimentary formats

1. TLR gene: (Fig 1A)

Transfected TLR	Cat No
TLR1	NBP2-26264
TLR2	NBP2-26266
TLR3	NBP2-26267
TLR4	NBP2-26268
TLR5	NBP2-26269
TLR6	NBP2-26293
TLR7	NBP2-26270
TLR8	NBP2-26271
TLR9	NBP2-26272
Negative Control Vector	NBP2-26259

2. TLR gene + NF-κB/ SEAPorter™ Reporter gene: (Fig 1B)

Product	Cat No
NF-kB/SEAPorter™	NBP2-26260
TLR2/NF-kB/SEAPorter™	NBP2-26274
TLR3/NF-kB/SEAPorter™	NBP2-26275
TLR4/NF-kB/SEAPorter™	NBP2-26276
TLR5/NF-kB/SEAPorter™	NBP2-26277
TLR7/NF-kB/SEAPorter™	NBP2-26278
TLR8/NF-kB/SEAPorter™	NBP2-26279

The NF-κB/SEAPorter™ reporter gene expresses SEAP (secreted alkaline phosphatase) under the control of the NF-κB promoter in the IML-100 series. NF-κB is a major pathway activated by TLR signaling and in TLR/NF-κB/SEAPorter™ cells, NF-κB activation leads to the production of SEAP into the culture supernatant. SEAP is then quantified using the SEAPorter™ Assay Kit, which is based on a quick and easy colorimetric detection method. Refer to the section 'NF-κB SEAPorter™ Assay Kit for additional information.

TLR/HEK 293 Cells

TLR1/HEK 293 Cell Line: (NBP2-26264)

The TLR1/HEK 293 cell line can be used for TLR1 flow cytometric calibration and detection control.

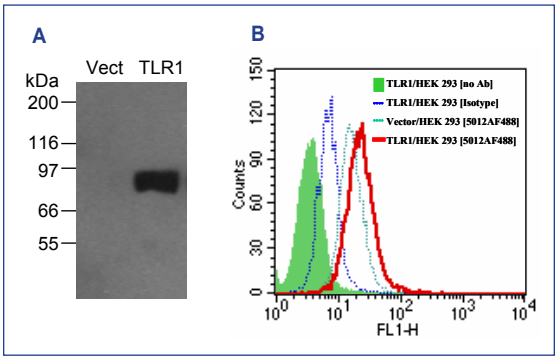


Figure A. Western blot analysis of TLR1 expression in the TLR1/HEK 293 cell line using an HA antibody (20 μg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259); TLR1: TLR1/HEK 293 (NBP2-26264). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR1 expression in the TLR1/HEK 293 cell line. *Cell surface expression of TLR1 in TLR1/HEK 293 (NBP2-26264) cells were analyzed by flow cytometry using Alexa Fluor® 488-conjugated TLR1 antibody (NBP2-24984) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' rabbit IgG isotype control (NBP2-24982) and Cell Surface TLR Staining Flow Kit (NBP2-26247) were used for this test. *Note: An endogenous level of TLR1 is present in HEK 293 cells.*

TLR2/HEK 293 Cell Line: (NBP2-26266)

The TLR2/HEK 293 cell line can be used for TLR2 flow cytometric calibration and detection control as well as TLR2-dependent functional assays.

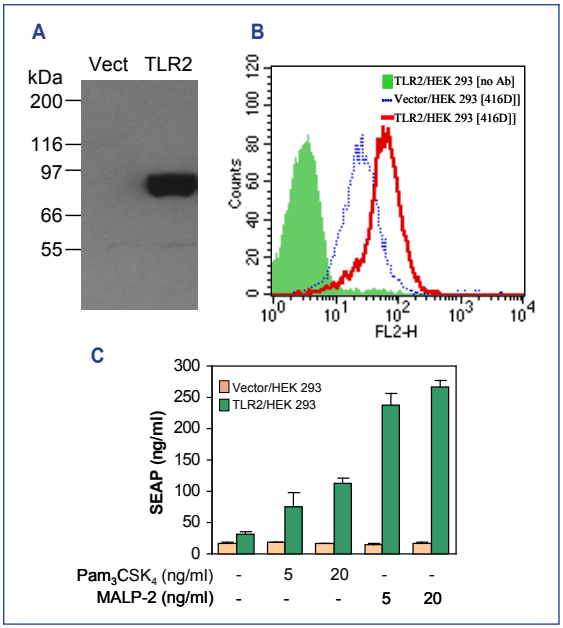


Figure A. Western blot analysis of TLR2 expression in the TLR2/HEK 293 cell line using an HA antibody (20 µg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259); TLR2: TLR2/HEK 293 (NBP2-26266). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR2 expression in the TLR2/HEK 293 cell line. *Cell surface expression of TLR2 in TLR2/HEK 293 (NBP2-26266) cells was analyzed by flow cytometry using PE-conjugated TLR2 antibody (NBP2-24909) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' Cell Surface TLR Staining Flow Kit (NBP2-26247) was used for this test.*

Figure C. Functional analysis of the TLR2/HEK 293 cell line. The assay was performed using the NF-κB SEAPorter™ Assay Kit (NBP2-25286). *The Vector/HEK 293 (NBP2-26259) and TLR2/HEK 293 (NBP2-26266) cell lines were transfected with NF-κB/SEAP reporter plasmid for 16 h. Cells were stimulated with Pam_3CSK_4 (NBP2-25297) or MALP-2 (NBP2-26219) for 24 h followed by SEAP assay. Note: The TLR2/HEK 293 cell line can be activated by either Pam_3CSK_4 or MALP-2 since endogenous levels of TLR2 and TLR6 are present in HEK 293 cells.*

TLR3/HEK 293 Cell Line: (NBP2-26267)

The TLR3/HEK 293 cell line can be used for TLR3 flow cytometric calibration and detection control as well as TLR3-dependent functional assays.

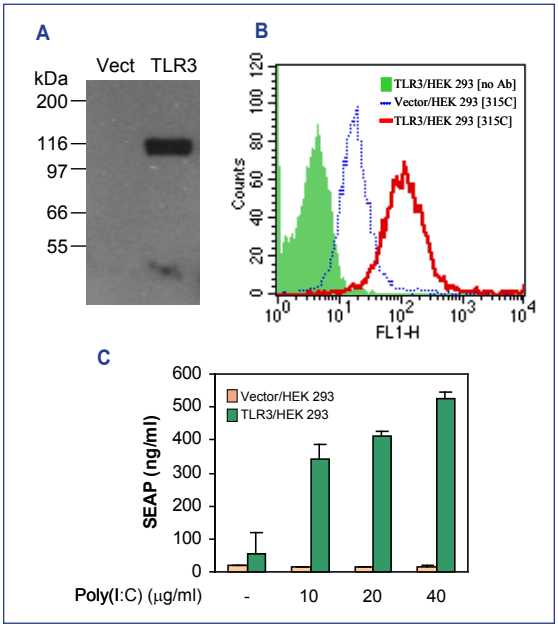


Figure A. Western blot analysis of TLR3 expression in the TLR3/HEK 293 cell line using an HA antibody (20 µg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259); TLR3: TLR3/HEK 293 (NBP2-26267). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR3 expression in the TLR3/HEK 293 cell line. *Intracellular expression of TLR3 in TLR3/HEK 293 (NBP2-26267) cells was analyzed by flow cytometry using a FITC-conjugated TLR3 antibody (NBP2-24899) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' Intracellular TLR Staining Flow Kit (NBP2-26248 was used for this test.*

Figure C. Functional analysis of the TLR3/HEK 293 cell line. The assay was performed using the NF-κB SEAPorter™ Assay Kit (IMK-515). *The Vector/HEK 293 (NBP2-26259) and TLR3/HEK 293 (NBP2-26267) cell lines were transfected with NF-κB/SEAP reporter plasmid for 16 h. Cells were stimulated with Polyinosinic-polycytidylic acid (Poly(I:C)) (NBP2-25288) for 24 h followed by SEAP assay.*

TLR4/HEK 293 Cell Line: (NBP2-26268)

The TLR4/HEK 293 cell line can be used for TLR4 flow cytometric calibration and detection control as well as TLR4-dependent functional assays.

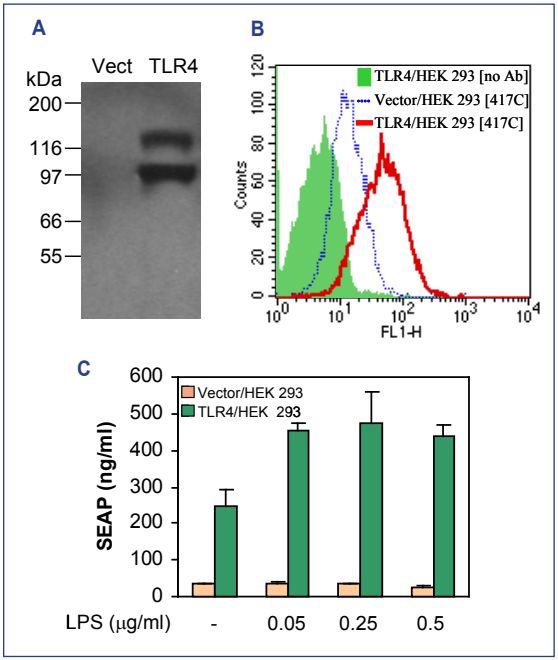


Figure A. Western blot analysis of TLR4 expression in the TLR4/HEK 293 cell line using an HA antibody (20 μg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259); TLR4: TLR4/HEK 293 (NBP2-26268). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR4 expression in the TLR4/HEK 293 cell line. *Cell surface expression of TLR4 in TLR4/HEK 293 (NBP2-26268) cells was analyzed by flow cytometry using a FITC-conjugated TLR4 antibody (NB100-56059) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' Cell Surface TLR Staining Flow Kit (NBP2-26247) was used for this test.*

Figure C. Functional analysis of the TLR4/HEK 293 cell line. The assay was performed using the NF-κB SEAPorter™ Assay Kit (IMK-515). *The Vector/HEK 293 (NBP2-26259) and TLR4/HEK 293 (NBP2-26268) cell lines were co-transfected with NF-κB/SEAP reporter plasmid and MD-2 expression plasmid for 16 h. Cells were stimulated with LPS (NBP2-25295) for 24 h followed by SEAP assay.*

TLR5/HEK 293 Cell Line: (NBP2-26269)

The TLR5/HEK 293 cell line can be used for TLR5 flow cytometric calibration and detection control as well as TLR5-dependent functional assays.

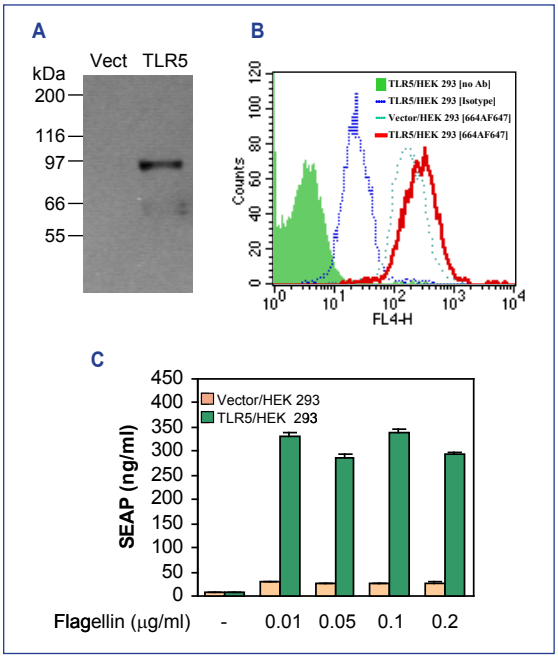


Figure A. Western blot analysis of TLR5 expression in the TLR5/HEK 293 cell line using an HA antibody (20 μg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259; TLR5: TLR5/HEK 293 (NBP2-26269). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR5 expression in the TLR5/HEK 293 cell line. *Cell surface expression of TLR5 in TLR5/HEK 293 (NBP2-26269) cells was analyzed by flow cytometry using an Alexa Fluor® 647-conjugated TLR5 antibody (NBP2-24785) and compared with the Vector/HEK 293 cell line (NBP2-26259). A mouse IgG2a isotype control and Novus' Cell Surface TLR Staining Flow Kit (NBP2-26247) were used for this test. *Note: An endogenous level of TLR5 is present in HEK 293 cells.*

Figure C. Functional analysis of the TLR5/HEK 293 cell line. The assay was performed using the NF-κB SEAPorter™ Assay Kit (IMK-515). *The Vector/HEK 293 (NBP2-26259) and TLR5/HEK 293 (NBP2-26269) cell lines were transfected with NF-κB/SEAP reporter plasmid for 16 h. Cells were stimulated with Flagellin (NBP2-25289) for 24 h followed by SEAP assay.*

TLR6/HEK 293 Cell Line: (NBP2-26293)

The TLR6/HEK 293 cell line can be used for TLR6 flow cytometric calibration and detection control.

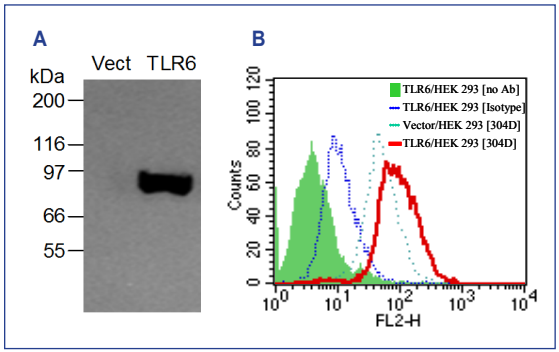


Figure A. Western blot analysis of TLR6 expression in the TLR6/HEK 293 cell line using an HA antibody (20 µg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259); TLR6: TLR6/HEK 293 (NBP2-26293). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR6 expression in the TLR6/HEK 293 cell line. *Cell surface expression of TLR6 in TLR6/HEK 293 (NBP2-26293) cells was analyzed by flow cytometry using a PE-conjugated TLR6 antibody (NBP2-24969) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' mouse IgG1 isotype control (NBP2-24976) and Cell Surface TLR Staining Flow Kit (NBP2-26247) were used for this test. *Note: An endogenous level of TLR6 is present in HEK 293 cells.*

TLR7/HEK 293 Cell Line: (NBP2-26270)

The TLR7/HEK 293 cell line can be used for TLR7 flow cytometric calibration and detection control as well as TLR7-dependent functional assays.

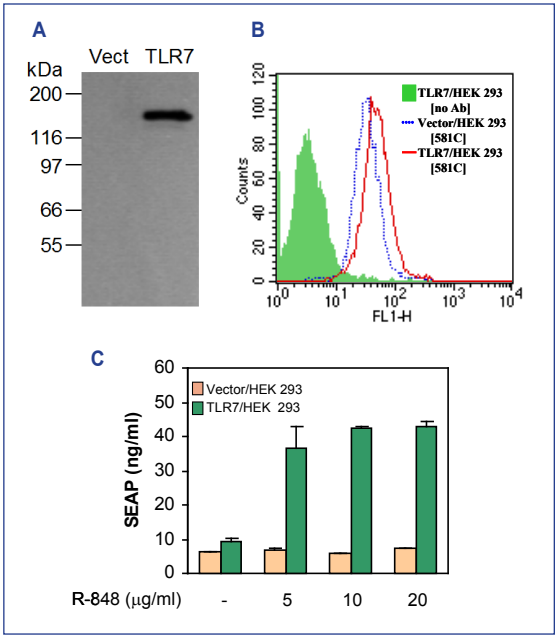


Figure A. Western blot analysis of TLR7 expression in the TLR7/HEK 293 cell line using an HA antibody (20 µg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259); TLR7: TLR7/HEK 293 (NBP2-26270). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR7 expression in the TLR7/HEK 293 cell line. *Intracellular expression of TLR7 in TLR7/HEK 293 (NBP2-26270) cells was analyzed by flow cytometry using a FITC-conjugated TLR7 antibody (NBP2-24777) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' Intracellular TLR Staining Flow Kit (NBP2-26248) was used for this test.*

Figure C. Functional analysis of the TLR7/HEK 293 cell line. The assay was performed using the NF-κB SEAPorter™ Assay Kit (IMK-515). *The Vector/HEK 293 (NBP2-26259 and TLR7/HEK 293 (NBP2-26270) cell lines were transfected with NF-κB/SEAP reporter plasmid for 16 h. Cells were stimulated with Imidazoquinoline resiquimod R-848 (NBP2-26231) for 24 h followed by SEAP assay.*

TLR8/HEK 293 Cell Line: (NBP2-26271)

The TLR8/HEK 293 cell line can be used for TLR8 flow cytometric calibration and detection control as well as TLR8-dependent functional assays.

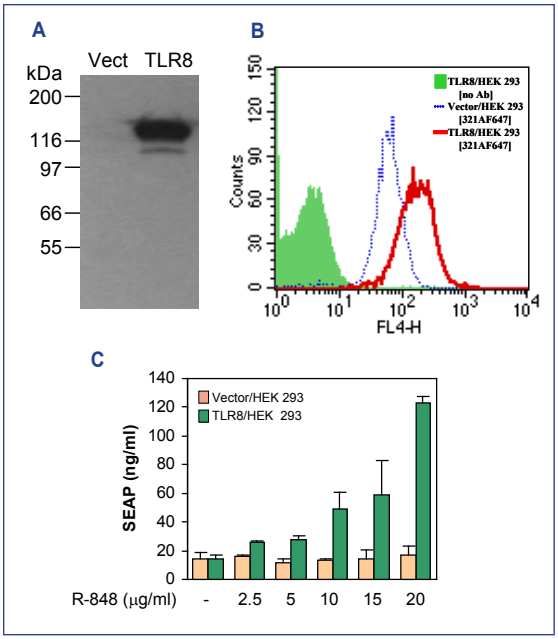


Figure A. Western blot analysis of TLR8 expression in the TLR8/HEK 293 cell line using an HA antibody (20 μg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259) TLR8: TLR8/HEK 293 (NBP2-26271). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR8 expression in the TLR8/HEK 293 cell line. *Intracellular expression of TLR8 in TLR8/HEK 293 (NBP2-26271) cells was analyzed by flow cytometry using an Alexa Fluor® 647-conjugated TLR8 antibody (IMG-321AF647) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' Intracellular TLR Staining Flow Kit (NBP2-26248) was used for this test.*

Figure C. Functional analysis of the TLR8/HEK 293 cell line. The assay was performed using the NF-κB SEAPorter™ Assay Kit (IMK-515). *The Vector/HEK 293 (NBP2-26259) and TLR8/HEK 293 (NBP2-26271) cell lines were transfected with NF-κB/SEAP reporter plasmid for 16 h. Cells were stimulated with Imidazoquinoline resiquimod R-848 (NBP2-26231) for 24 h followed by SEAP assay.*

TLR9/HEK 293 Cell Line: (NBP2-26272)

The TLR9/HEK 293 cell line can be used for TLR9 flow cytometric calibration and detection control as well as TLR9-dependent functional assays.

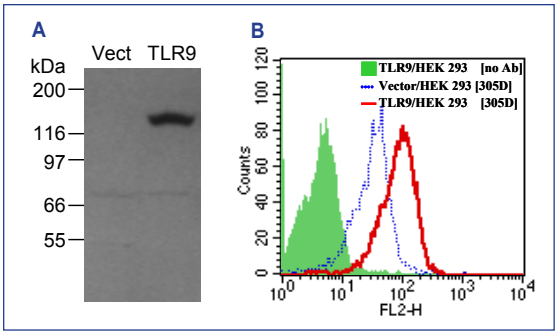


Figure A. Western blot analysis of TLR9 expression in the TLR9/HEK 293 cell line using an HA antibody (20 μg total protein/lane). *Legend. Vect: Vector/HEK 293 (Novus, NBP2-26259); TLR9: TLR9/HEK 293 (NBP2-26272). Western blot analysis with the HA tag antibody was used to assess protein expression.*

Figure B. Flow analysis of TLR9 expression in the TLR9/HEK 293 cell line. *Intracellular expression of TLR9 in TLR9/HEK 293 (NBP2-26272) cells was analyzed by flow cytometry using a PE-conjugated TLR9 antibody (NBP2-24907) and compared with the Vector/HEK 293 cell line (NBP2-26259). Novus' Intracellular TLR Staining Flow Kit (NBP2-26248) was used for this test.*

TLR/NF-κB/SEAPorter™ HEK 293 Cells

NF-κB/SEAPorter™ HEK 293 Cell Line: (NBP2-26260)

The NF-κB/SEAP stable HEK 293 cell line is designed to be used to measure NF-κB activation using SEAP protein secreted to the culture media as a read-out with SEAPorter Assay kit (Novus, Cat. No. NBP2-25285). The NF-κB/SEAP stable cells are not only useful in helping with the identification of pro-or anti-inflammatory substances, but also can help to assay for proteasome activity since the activation of NF-κB results in the degradation of IkB through the proteasome-dependant pathway.

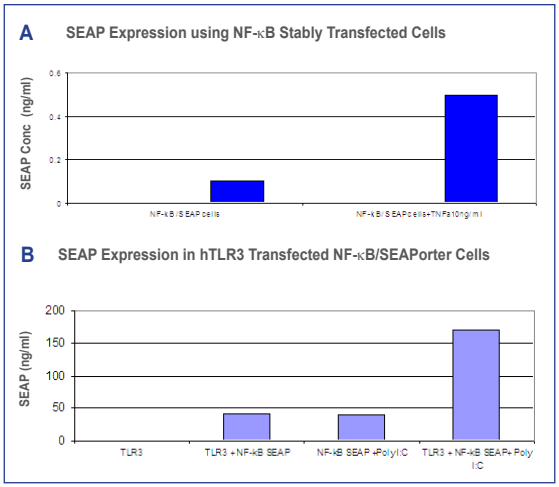


Figure A. NF-κB/SEAP stably transfected HEK 293 cells were plated into a 6 well tissue culture plate. *The next day, hTNFα (10 ng/ml) was added. After 20 hrs supernatant was collected and SEAP assay was performed using SEAPorter™ Assay Kit (Novus, Cat. No. NBP2-25285). An increase in SEAP indicates an increase in NF-κB activation.*

Figure B. NF-κB/SEAP stably transfected HEK 293 cells were transfected with hTLR3 plasmid. *After 48 hrs of transfection, Poly (I:C) was added. After 20 hrs supernatant was collected and SEAP assay was performed using SEAPorter™ Assay Kit (Novus, Cat. No. NBP2-25285). NF-κB was activated, only in the presence of TLR3 and Poly (I:C).*

TLR2/NF-κB/SEAPorter™ HEK 293 Cell Line: (NBP2-26274)

The TLR2/NF-κB/SEAPorter™ HEK 293 cell line can be used for TLR2-dependent functional assays as well as screening of TLR2 agonists or antagonists.

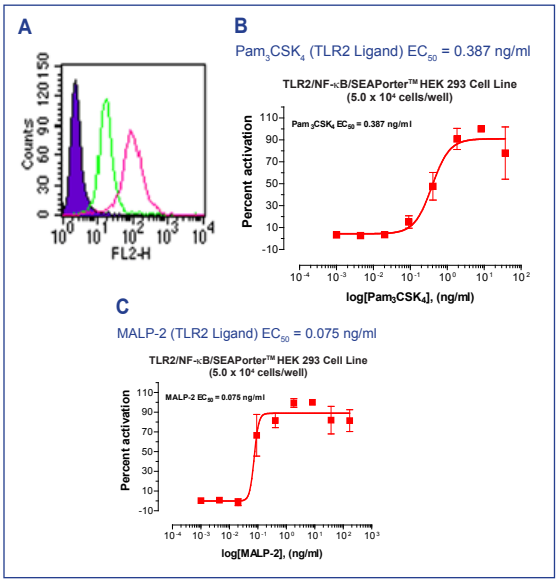


Figure A. Flow cytometric analysis. *Cell surface expression of TLR2 in TLR2/NF-κB/SEAPorter™ HEK 293 (Novus, NBP2-26274) cells was analyzed by flow cytometry using a PE-conjugated TLR2 antibody (NBP2-24909). Flow samples were prepared using the Cell Surface TLR Staining Flow Kit (NBP2-26247). Purple: Cells without antibody; Green: NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26260) stained with anti-TLR2-PE (NBP2-24909); Red: TLR2/NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26274) stained with anti-TLR2-PE (NBP2-24909).*

Figure B. Ligand dose response evaluation. *TLR2/NF-κB/SEAPorter™ cells (Novus, NBP2-26274) were plated in 96-well plates at 5.0 x 10⁴ cells/well. After 16 h, cells were stimulated with various amounts of Pam_3CSK_4 for 24 h. SEAP was analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285). Dose-responsive percent activation of each sample well was calculated to yield the ligand EC_{50} value.*

Figure C. Ligand dose response evaluation. *TLR2/NF-κB/SEAPorter™ cells (Novus, NBP2-26274) were plated in 96-well plates at 5.0 x 10⁴ cells/well. After 16 h, cells were stimulated with various amounts of MALP-2 for 24 h. SEAP was analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285). Dose-responsive percent activation of each sample well was calculated to yield the ligand EC_{50} value.*

TLR3/NF-κB/SEAPorter™ HEK 293 Cell Line: (NBP2-26275)

The TLR3/NF-κB/SEAPorter™ HEK 293 cell line can be used for TLR3-dependent functional assays as well as screening of TLR3 agonists or antagonists.

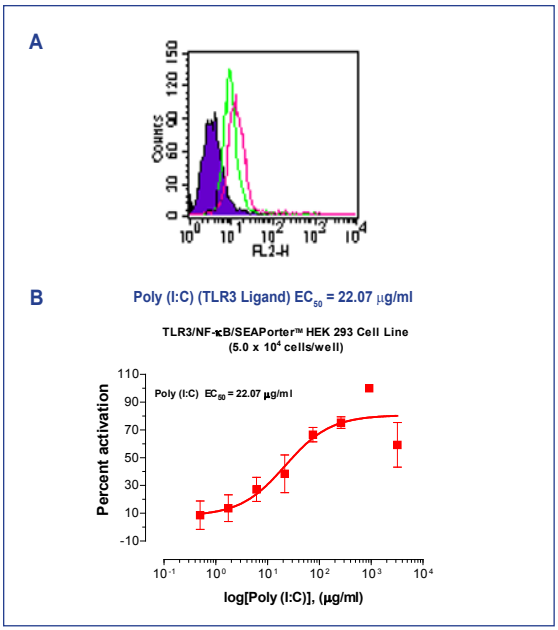


Figure A. Flow analysis of TLR3. Intracellular staining of *TLR3 inTLR3/NF-κ B/SEAPorter™ HEK 293 (Novus, NBP2-26275) cells was analyzed by flow cytometry using a PE-conjugated anti-TLR3 antibody (NBP2-24902). Flow samples were prepared using the Intracellular TLR Staining Flow Kit (10098K). Purple: Cells without antibody;Green: NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26260) stained with 1ug anti-TLR3-PE (NBP2-24902); Red: TLR3/NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26275) stained with 1 ug anti-TLR3-PE (NBP2-24902).*

Figure B. Ligand dose response evaluation. *TLR3/NF-κB/SEAPorter™ cells (NBP2-26275) were plated in 96-well plates at 5.0x 104 cells/well. After 16 h, cells were stimulated with various amounts of Polyinosinic-polycytidylic acid (Poly(I:C)) (NBP2-25288) for 24 h. SEAP was analyzed using SEAPorter™ Assay Kit (NBP2-25285). Dose-responsive percent activation of each sample well was calculated to yield the ligand EC_{50} value.*

TLR4/NF-κB/SEAPorter™ HEK 293 Cell Line: (NBP2-26276)

The TLR4/NF-κB/SEAPorter™ HEK 293 cell line can be used for TLR4-dependent functional assays as well as screening of TLR4 agonists or antagonists.

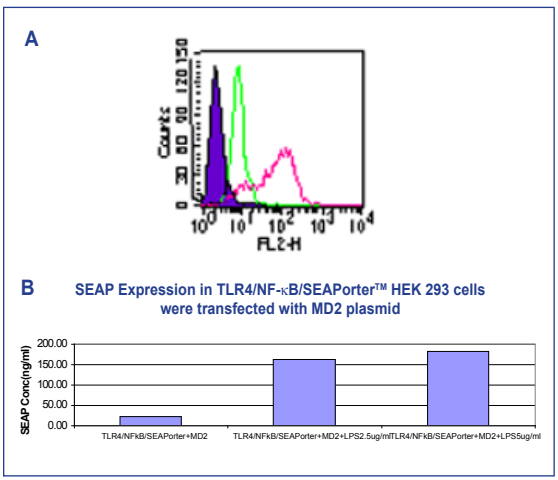


Figure A. Flow cytometric analysis. *Cell surface staining of TLR4 inTLR4/NF-κB/SEAPorter™ HEK 293 (Novus, NBP2-26276) cells was analyzed by flow cytometry using a PE-conjugated anti-TLR4 antibody (NBP2-24909). Flow samples were prepared using the Cell Surface TLR Staining Flow Kit (NBP2-26247). Purple: Cells without antibody; Green: NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26260) stained with 1 ug anti-TLR4-PE (NBP2-24909); Red: TLR4/NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26276) stained with 1 ug anti-TLR4-PE (NBP2-24909).*

Figure B. Functional analysis of TLR4. *The assay was performed using the SEAPorter™ Assay Kit (NBP2-25285). TLR4/NF-κB/SEAPorter™ HEK 293 (NBP2-26276) cells were transfected with MD-2 expression plasmid for 16 h. Cells were stimulated with LPS (NBP2-25295) for 24 h followed by SEAP assay. An increase in SEAP expression indicates an increase in NF-κB activation.*

TLR5/NF-κB/SEAPorter™ HEK 293 Cell Line: (NBP2-26277)

The TLR5/NF-κB/SEAPorter™ HEK 293 cell line can be used for TLR5-dependent functional assays as well as screening of TLR5 agonists or antagonists.

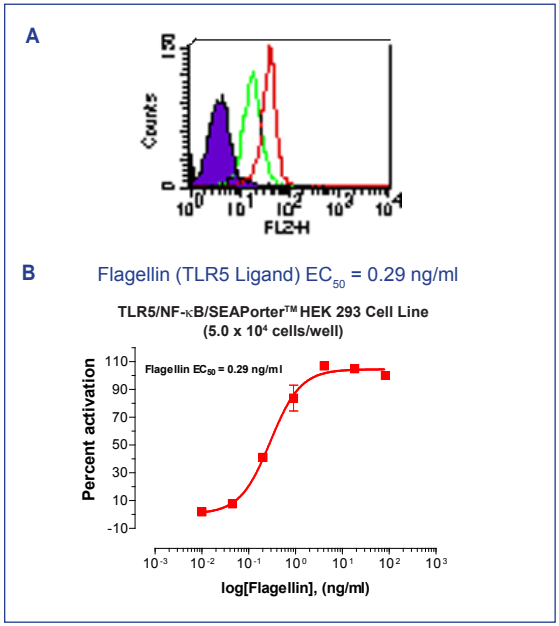


Figure A. Flow cytometric analysis. *Cell surface staining of TLR5 inTLR5/NF-κB/SEAPorter™ HEK 293 (Novus, NBP2-26277) cells was analyzed by flow cytometry using a PE-conjugated anti-TLR5 antibody (NBP2-24783). Flow samples were prepared using the Cell Surface TLR Staining Flow Kit (NBP2-26247). Purple: Cells without antibody; Green: NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26260) stained with 1 ug anti-TLR5-PE (NBP2-24783); Red: TLR5/NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26277) stained with 1 ug anti-TLR5-PE (NBP2-24783).*

Figure B. Ligand dose response evaluation. *TLR5/NF-κB/SEAPorter™ cells (Novus, NBP2-26277) were plated in 96-well plates at 5.0 x 10⁴ cells/ well. After 16 h, cells were stimulated with various amounts of flagellin for 24 h. SEAP was analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285). Dose-responsive percent activation of each sample well was calculated to yield the ligand EC_{50} value.*

TLR7/NF-κB/SEAPorter™ HEK 293 Cell Line: (NBP2-26278)

The TLR7/NF-κB/SEAPorter™ HEK 293 cell line can be used for TLR7-dependent functional assays as well as screening of TLR7 agonists or antagonists.

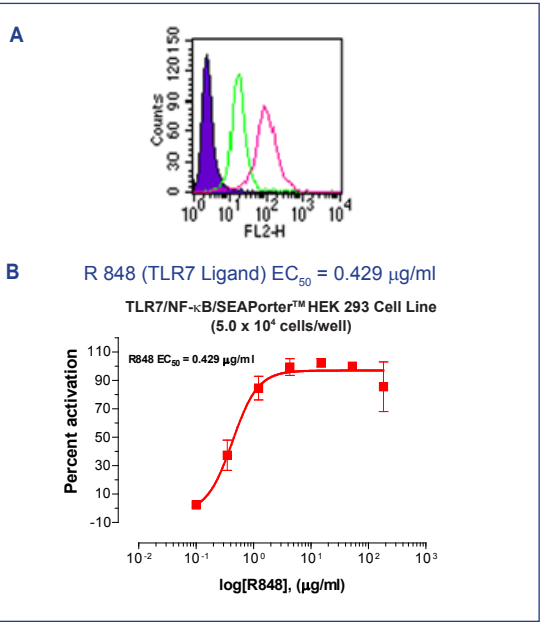


Figure A. Flow cytometric analysis. *Intracellular staining of TLR7 in TLR7/NF-κB/SEAPorter™ HEK 293 (Novus, NBP2-26278) cells was analyzed by flow cytometry using PE-conjugated anti-TLR7 antibody (NBP2-24761). Flow samples were prepared using the Intracellular TLR Staining Flow Kit (NBP2-26248). Purple: Cells without antibody; Green: NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26260) stained with 2 ug anti-TLR7-PE (NBP2-24761). Red: TLR7/NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26278) stained with anti-TLR7-PE (NBP2-24761).*

Figure B. Ligand dose response evaluation. *TLR7/NF-κB/SEAPorter™ cells (Novus, NBP2-26278) were plated in 96-well plates at 5.0 x 10⁴ cells/well. After 16 h, cells were stimulated with various amounts of R848 (NBP2-26231) for 24 h. SEAP was analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285). Dose-responsive percent activation of each sample well was calculated to yield the ligand EC_{50} value.*

TLR8/NF-κB/SEAPorter™ HEK 293 Cell Line: (NBP2-26279)

The TLR8/NF-κB/SEAPorter™ HEK 293 cell line can be used for TLR8-dependent functional assays as well as screening of TLR8 agonists or antagonists.

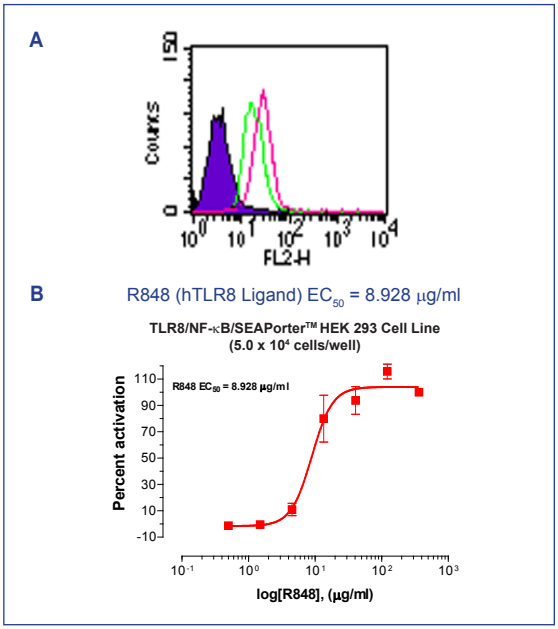


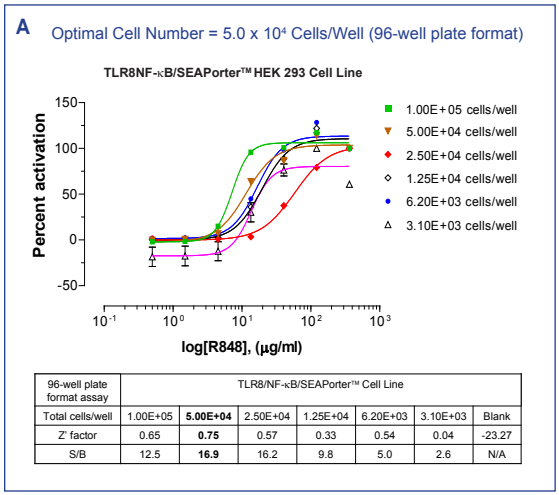
Figure A. Flow analysis of TLR8. *Intracellular staining of TLR8 in TLR8/NF-κB/SEAPorter™ HEK 293 (Novus, NBP2-26279) cells was analyzed by flow cytometry using a PE-conjugated anti-TLR7 antibody (NBP2-24817). Flow samples were prepared using the Intracellular TLR Staining Flow Kit (NBP2-26248). Purple: Cells without antibody; Green: NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26260) stained with 2 ug anti-TLR8-PE (NBP2-24817). Red: TLR8/NF-κB/SEAPorter™ HEK 293 cell line (NBP2-26279) stained with anti-TLR8-PE (NBP2-24817).*

Figure B. Ligand dose response evaluation. *TLR8/NF-κB/SEAPorter™ cells (Novus, NBP2-26279) were plated in 96-well plates at 5.0 x 10^4 cells/well. After 16 h, cells were stimulated with various amounts of R848 (NBP2-26231) for 24 h. SEAP was analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285). Dose-responsive percent activation of each sample well was calculated to yield the ligand EC_{50} value.*

Representative 96-well Plate Titration & Validation Data

General titration and validation data for the 96-well plate format has established using the TLR8/NF-κB/SEAPoter™ HEK 293 cell line (IML-108) and is applicable to all of the cell lines in the IML-100 Series. This standardized data (Figs A-C) should be used as a guideline for optimizing cell number, cell stimulation time, and DMSO tolerance levels when using the IML-100 Series in the 96-well plate format. Researchers however should optimize and establish parameters for other high throughput screening formats such as 384,1536 and 3450-well plate formats.

Cell Number Optimization



96-well plate format assay	TLR8/NF-κB/SEAPorter™ Cell Line						
Total cells/well	1.00E+05	**5.00E+04**	2.50E+04	1.25E+04	6.20E+03	3.10E+03	Blank
Z' factor	0.65	**0.75**	0.57	0.33	0.54	0.04	-23.27
S/B	12.5	**16.9**	16.2	9.8	5.0	2.6	N/A

Figure A. TLR8/NF-κB/SEAPorter™ cells (Novus, NBP2-26279) were plated in 96-well plates at different concentrations of cells (100 µl/well) as noted in the figure. *After 16 h, cells were stimulated with various amounts of R848 (NBP2-26231) for 24 h. Secreted alkaline phosphatase (SEAP) was analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285). Z' factor and Signal vs. Background (S/B) ratio were evaluated for each cell concentration set.*

Time Course Optimization Assay

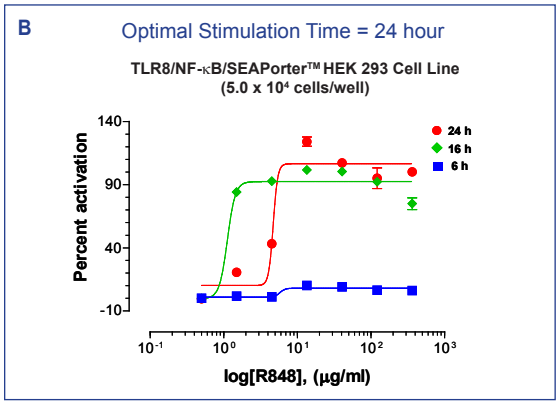


Figure B. Optimal cell stimulation time evaluation. *TLR8/NF-κB/SEAPorter™ cells (Novus, NBP2-26279) were plated in 96-well plates at 5.0 x 10⁴ cells/well. After 16 h, cells were stimulated with various amounts of R848 (NBP2-26231) for 6 h, 16 h or 24 h. SEAP was then analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285).*

DMSO Tolerance Assay

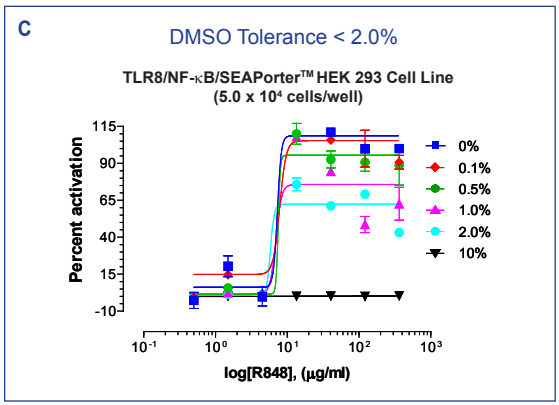


Figure C. DMSO tolerance evaluation. *TLR8/NF-κB/SEAPorter™ cells (Novus, NBP2-26279) were plated in 96-well plates at 5.0 x 10⁴ cells/well. After 16 h, cells were stimulated with various amounts of R848 (NBP2-26231) in the presence of different amounts of DMSO as noted for 24 h. SEAP was then analyzed using Novus' SEAPorter™ Assay Kit (NBP2-25285).*

TLR Stable Cell Line Handling Protocol

Note: Please read the entire data sheet before thawing the cells. Product data sheets accompany each product and are also available at www.novusbio.com. It is recommended that users follow good cell culture practice when using the cells. The cells are sterile and all work should be performed under sterile conditions.

1. Prepare a sterile 15-ml tube with 9 ml complete growth medium pre-warmed at 37°C.
2. Thaw the frozen cell vial quickly in a 37°C water bath, keeping the cap portion out of the water to avoid any possible contamination.
3. Upon thawing, take the vial out of the water and clean it with 70% ethanol to decontaminate.
4. Transfer contents to the 15-ml tube (Step 1) and mix with medium by gentle inversion of tube.
5. Centrifuge at 1,000 RPM for 5 minutes.
6. Remove supernatant and resuspend cells in 10 ml of fresh medium.
7. Transfer cells into a 25-cm^2 tissue culture flask and incubate at 37°C in a 95% air-5% CO_2 mixture.
8. Maintain the cells by changing with fresh complete growth medium every 2-3 days.

Complete Growth Media

TLR/HEK 293 Cell Lines

DMEM + 10% FBS + 4 mM L-glutamine + 1 mM sodium pyruvate + 100 units/ml penicillin + 100 µg/ml streptomycin + 10 µg/ml blasticidin.

TLR/NF-κB/SEAPorter™ HEK 293 Cell Lines

DMEM + 10% FBS + 4 mM L-glutamine + 1 mM sodium pyruvate + 100 units/ml penicillin + 100 µg/ml streptomycin + 10 µg/ml blasticidin + 500 µg/ml G418 (Geneticin).

NF-κB/SEAPorter™ HEK 293 Cell Lines

DMEM + 10% FBS + 1.5 g/L sodium bicarbonate + 1.0 mM sodium pyruvate + penicillin and streptamycin + 500 µg/ml G418 antibiotic.

Vector/HEK 293 Cell Lines

DMEM + 10% FBS + 4 mM L-glutamine + 1 mM sodium pyruvate + 100 units/ml penicillin + 100 µg/ml streptomycin + 10 µg/ml blasticidin.

TLR Screening Sets for Optimizing Antibody Selection

Choosing among the various antibodies that are commercially available is one of the most critical steps in obtaining success in immunochemical assays. However, antibodies can behave dramatically different in different techniques and model systems, and it may be difficult a priori to determine the optimal antibody. In the TLR field, researchers are increasingly turning to antibody screening as a strategy for identifying antibodies best suited for their assay platforms.

TLRs have multiple isoforms, post-translational modifications, and cleavage forms as well as other nuances. Utilizing antibodies that have different properties, such as recognizing different epitopes, can help to identify optimal antibodies for a particular experimental model system (Fig 1). Additionally, TLRs are typically expressed at low levels which can make their detection challenging. As a result, many researchers are also finding that using more than one antibody against a given TLR target facilitates interpretation and validation of results.

Novus is pleased to announce the availability of new TLR Antibody Screening Sets for screening multiple antibodies against the same TLR target in cells or tissues. Antibody applications include Flow Cytometry, Western Blot and Immunohistochemistry. The Screening Sets enable researchers to efficiently and cost-effectively identify and select antibodies best suited for their model systems.

Novus' leading edge portfolio of reagents specific for TLRs and related areas of Inflammation/Immunology Signaling Pathways includes: Antibodies, Kits, Agonists, Inhibitors and Reporter Cell Lines.

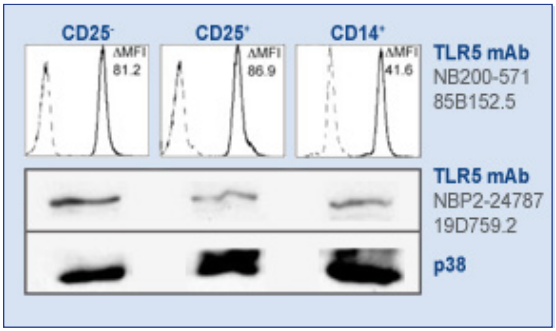


Figure 1. Flow cytometry (intracellular) and western blot analysis of TLR5 in human CD4+ T cell subsets employing a different Novus TLR5 specific mAb for each technique, clone 85B152.5 (NB200-571) and 19D759.2 (NBP2-24787) respectively. *Cell subsets were purified from normal human PBMCs. Subsets were fixed and permeabilized for flow cytometry: TLR5-FITC (solid lane) and isotype control (dotted line). Whole cell subset lysates were used for western blots, a p38 Ab was used as a loading control.*

TLR5 expression was detected in all three subsets, and the validity of the results was supported with complimentary techniques and TLR5 antibodies.

Source: Crellin et el. J Immunol 175:8051-8059 (2005).

TLR Screening Sets

Specificity	Cat No
TLR Antibody Screening Set	NBP2-25083
TLR (Cell Surface) Screening Set	NBP2-25086
TLR (Intracellular) Screening Set	NBP2-25085
TLR2 Antibody Screening Set	NBP2-25083
TLR3 Antibody Screening Set	NBP2-25083
TLR4 Antibody Screening Set	NBP2-25083
TLR5 Antibody Screening Set	NBP2-25083
TLR7 Antibody Screening Set	NBP2-25083

TLR/NF-κB Activation Screening Assays

NF-κB is a major transcription factor that regulates genes responsible for innate and adaptive immune responses, including responses activated by TLR signaling. In unstimulated cells, NF-κB dimers are held in the cytoplasm by IκBs that mask the nuclear localization signals of NF-κB. Upon cell stimulation, which leads to IκBα phosphorylation and degradation, NF-κB quickly translocates to the nucleus and activates various genes that have DNA-binding sites for NF-κB. IκBα phosphorylation (IMK-501), NF-κB nuclear localization (NBP2-29661), and NF-κB gene activation (NBP2-25286) are all indicators of NF-κB signaling pathway activation.

Novus has developed a number of readout assay kits to measure NF-κB activation:

1. **IMK-501: Phospho-IκBα ActivELISA™ Kit**
2. **NBP2-29661: NF-κB/p65 ActivELISA™ Kit**
3. **NBP2-25286: NF-κB SEAPorter™ Assay Kit (NBP2-25286)**

Information about the kits is presented in their respective sections, and complete information can be found in our online manuals at www.novusbio.com.

Phospho-IκBα ActivELISA™ Kit:
IMK-501, 2 x 96 well format (192 tests)

Phosphorylated IκBα is marker of NF-κB activation because various stimuli that activate NF-κB, including TLR signaling, cause IκBα Ser32/Ser36 phosphorylation. This happens as signals propagate through NF-κB signaling pathway nodes. The mechanism functions as follows: In its inactive form, NF-κB is sequestered in the cytoplasm, bound by members of the IκB family of inhibitors including IκBα. IKK activation is a major mechanism of NF-κB activation. Activated IKK phosphorylates IκB proteins. When IκBα is phosphorylated at Ser32/Ser36, it is rapidly ubiquitinated and then is degraded by the 26S proteosome. During this process, NF-κB is released from its inhibitory IκBα hold. This results in exposure of the nuclear localization signals (NLS) on NF-κB subunits, and NF-κB translocates to the nucleus and activates gene transcription.

Hence, IκBα phosphorylation is an upstream activation marker of NF-κB nuclear translocation as illustrated in Figure 29 on page 78. The IMK-501 kit measures IκBα phosphorylation, whereas the IMK-503 kit measures NF-κB translocation which is another good marker of NF-κB. By using both the IMK-501 and IMK-503 kits, researchers can map aspects of NF-κB signaling nodes and activation time courses in their experimental system.

Additionally, the use of these Kits can help researchers define the type of NF-κB signaling in their system. For example, IKK activation and IκBα Ser32/Ser36 phosphorylation occurs in the classical NF-κB pathway. In the alternative signaling pathway there can an absence of IκBα Ser32/Ser36 phosphorylation in NF-κB activation. For example, NF-κB translocation without

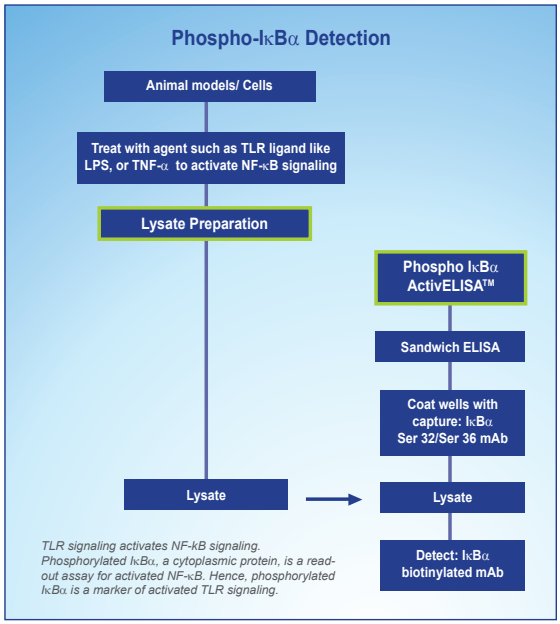


Figure 1. Phospho-IκBα ActivELISA™ protocol overview.

IκB degradation has been reported to occur during H202, UV, pervanadate, hypoxia, NGF, and HER-2 treatment in certain model systems. If researchers discover that NF-κB translocation occurs in the absence of IκBα phosphorylation, it may suggest that signals are proceeding through alternative, rather than classical NF-κB signaling mechanisms. This may have implications for translational medicine and other treatments designed to manipulate signaling. The IMK-501 kit is based on a sandwich ELISA protocol whereby a phosphospecific IκBα (Ser32/Ser36) mAb coated plate captures phosphorylated IκBα from cell extracts. The amount of bound phospho-specific IκBα is detected by adding a second pan IκBα biotinylated mAb, followed by Streptavidin-HRP luminescence detection with a luminometer. Figure 1 shows a protocol overview and sample results are shown in Figure 2. Please see the NF-κB/p65 ActivELISA™ Kit (IMK-503) section for a schematic of sandwich ELISA technology.

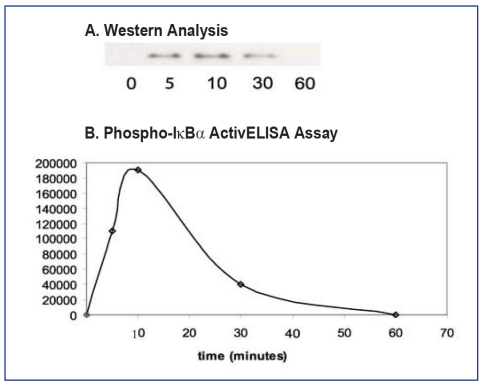


Figure 2. Analysis of IκBα phosphorylation in TNFα treated Jurkat cells. *Jurkat cells were treated with TNFα (1 nm). Cell lysates were prepared at various timepoints using IMK-501 Phospho-IκBα ActivELISA™ Kit components. A. Western blot analysis of lysates using a phospho-IκBα mAb, clone 39A1413 mAb (IMG-156). B. Sandwich ELISA analysis phospho-IκBα in the cell lysates. The data shows that untreated cells lacked phospho-IκBα expression. Phospho-IκBα expression rapidly increased following TNFα treatement, peaking just after 10 min and then rapidly declining to undetectable levels by 60 min.*

NF-κB/p65 ActivELISA™ Kit:
NBP2-29661, 2 x 96 well format (192 tests)

The NF-κB/p65 ActivELISA™ Kit measures free p65 in the nucleus of cells or tissues, a marker of NF-κB activation. p65 is a component of the NF-κB signaling complex which translocates to the nucleus during activation. Hence, the level of p65 in the nucleus is considered to correlate in a positive manner with the activation of the of NF-κB pathway. An advantage of this kit, compared to many other kits measuring NF-κB activation, is that it does not involve radioactivity and can be completed in one day. It is based on a nuclear extraction methodology to isolate nuclear p65, and a sandwich ELISA protocol to quantify p65 in the nuclear extract. A protocol overview is shown in Figure 1, and Figure 2 details the concept of the sandwich ELISA assay. In the ELISA assay, the p65 Ab coated plate captures free p65 and the amount of bound p65 is detected by adding a second p65 Ab followed by alkaline phosphatase (AKP) -conjugated secondary antibody using colorimetric detection in an ELISA plate reader.

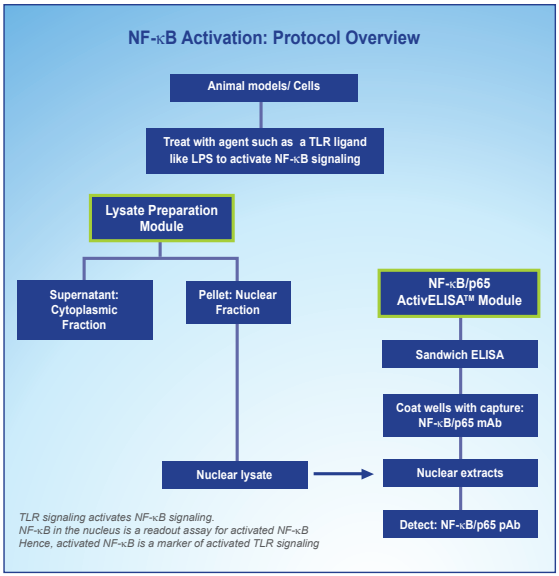


Figure 1. NF-κB/p65 ActivELISA™ protocol overview.

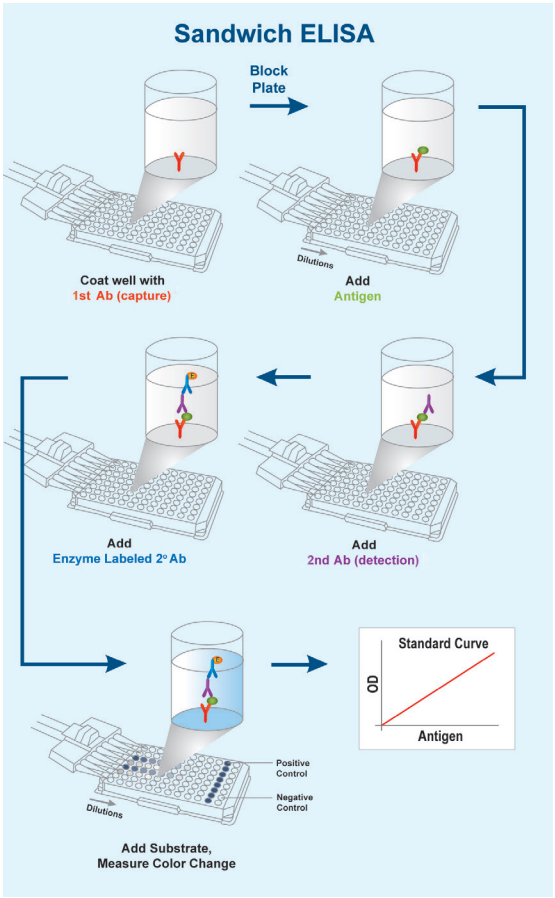


Figure 2. Sandwich ELISA technique overview.

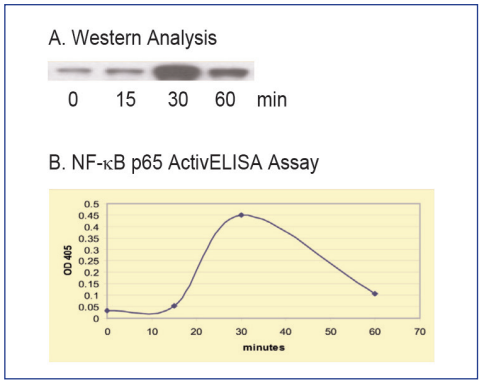


Figure 3. Analysis of nuclear p65 in LPS treated Jurkat cells. Jurkat cells were treated with LPS (15 ug/ml). *Nuclear extracts were prepared at various timepoints using the Lysate Preparation Module of the NBP2-29661 NF-κB/p65 Active ELISA™ Kit [this Module is also available separately as the Nuclear Extraction Kit (NBP2-29447)]. A. Western blot analysis of nuclear extracts using a p65 mAb, clone 112A1021 (NB100-56712). B. Sandwich ELISA analysis of nuclear extracts using the NF-κB/p65 ActivELISA™ Module of the NBP2-29661 Kit. The data shows that there was a basal level of p65 which increased by 30 min and decreased by 60 min following LPS treatment.*

Table 1. Analysis of p65 translocation in LPS + bromelain treated THP-1 cells

	Untreated	LPS (1 ug/ml)	LPS + 50 ug/ml Bromelain	LPS + 100 ug/ml Bromelain
NF-κB (pg/ml)	38 +/-4	65 +/-7	50 +/-6	40 +/-5

Table 1. Analysis of p65 translocation in LPS + bromelain treated THP-1 cells. THP-1 cells were left untreated or stimulated with LPS (1 ug/ml) in the presence or absence of bromelain for 24 hr. 1 x 10^6 cells were used for each analysis, and data represents the mean from at least four separate experiments. Bromelain comprises a group of proteases extracted from the stem of pineapple and is known for its anti-inflammatory effects. Note: Prior to treatment, THP-1 cells were differentiated by treatment with 200 nM PMA for 48 h, followed by a 24 hr rest incubation. The data shows that LPS activated NF-κB signaling and that bromelain attenuated this activation. Source: Huang et al, 2008.

Figure 3 shows an example of an analysis of NF-κB activation in LPS-treated Jurkat cells using the NBP2-29661 kit. In another example, Huang et al (2008) used the NBP2-29661 kit to show that LPS activated NF-κB in THP-1 cells, and that bromelain reduced activation in a dose dependent manner (Table 1).

Novus Product Citation

Huang J-R, C-C Wu, RC-W Hou, K-C Jeng. Immunological Investigations. 37:263-277 (2008). Bromelain inhibits lipopolysaccharide-induced cytokine production in human THP-1 monocytes via the removal of CD14: **NF-κB/p65 ActivELISA™ Kit (NBP2-29661). ELISA (human THP-1 cells): Table 2. Note: Cells were treated with LPS (TLR4 ligand) which induced p65 translocation an indication of TLR activation and downstream activation of NF-κB. Bromelain, an anti-inflammatory agent, inhibited NF-κB activation.**

NF-κB SEAPorter™ Assay Kit: NBP2-25286

The NF-κB SEAPorter™ Assay Kit contains the pNF-κB/SEAP reporter plasmid, which expresses SEAP protein (secreted alkaline phosphatase) under the control of the NF-κB promoter. The reporter gene system is the most commonly used method for monitoring gene expression within cells. SEAP is a particularly useful reporter because the heat stable SEAP reporter protein is secreted into the culture medium (reviewed in Torisawa et al, 2006). The cellular secretion of SEAP is directly proportional to the changes in the intracellular SEAP mRNA. This feature allows the continuous quantification of gene expression.

The NF-κB SEAPorter™ Assay Kit is designed to measure NF-κB activation using SEAP protein secreted to the culture media as a read-out (Fig 1). SEAP activity is sensitively detected by a chemiluminescent assay with reagents included in the kit (Fig 2). Plasmids included in the kit are sufficient for one 96 well plate transfection and other reagents included in the kit are sufficient for four 96 well plate SEAP assays. However, users can replicate the plasmid to generate enough material for as many transfections as desired. The NF-κB SEAPorter™ Kit is useful for analyzing NF-κB activation in a wide variety of model systems. For example, the NF-κB promoter can be activated or suppressed with ligands or inhibitors as shown in Figure 3. This example shows NF-κB activation following Pam3CSK4 (TLR1/2) ligand treatment and

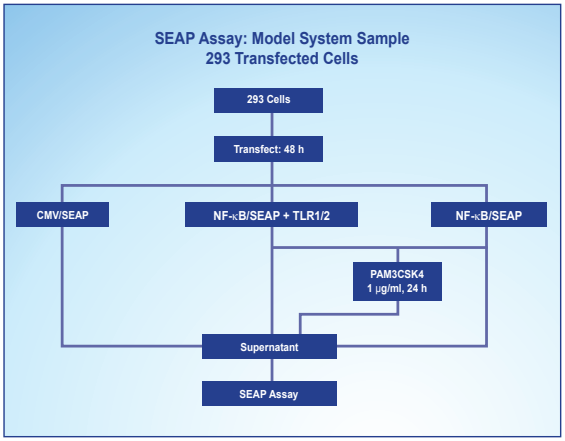


Figure 1. NF-κB SEAPorter™ Assay Kit (NBP2-25286): conceptual overview. *The SEAP ELISA assay quantitatively detects NF-κB activation by measuring SEAP in the culture media.*

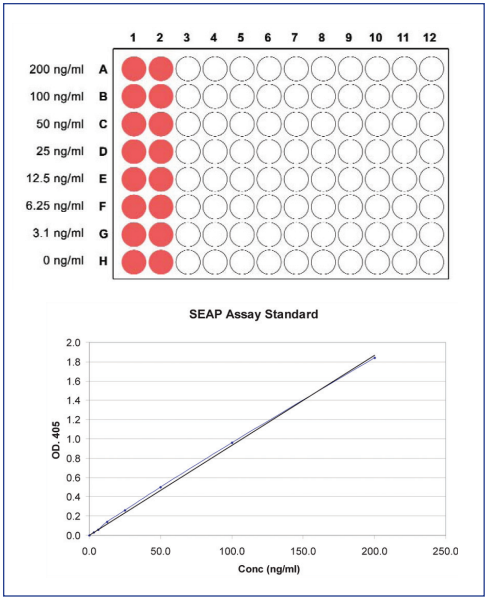


Figure 2. NF-κB /SEAP assay methods. *The assay is quantitative and quick. Samples and the SEAP protein standard are loaded onto ELISA plates and incubated at 65°C to obliterate endogenous AP (alkaline phosphatase). The PNPP substrate is added and incubated at room temperature. Results are read at 405 nm after 30 and 60 min.*

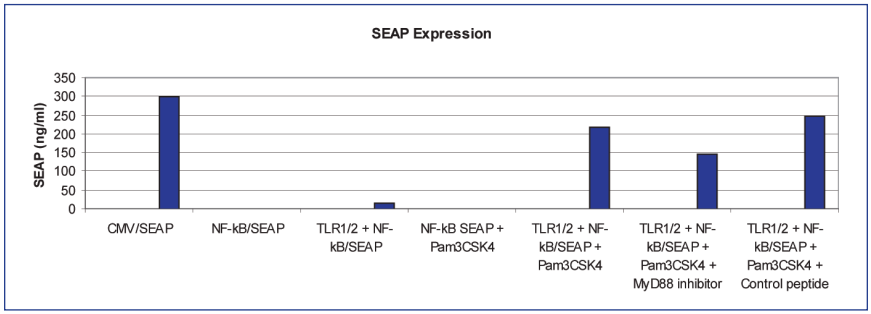
partial inhibition with the MyD88 inhibitory, but not the control peptide where the kit was used to measure NF-κB activation following treatment with the Pam3CSK4.

Reference

Torisawa Y, N Ohara, K Nagmine, S Kasai, T Yasuka, H Shiku, T Matsue. Electrochemical monitoring of cellular signal transduction with a secreted alkaline phosphates reporter system. *Anal Chem* 78:7625-7631 (2006).

Figure 3. Sample NF-κB /SEAP experimental model system. *293 cells were transfected with CMV/SEAP, NF-κB/SEAP or NF-κB/SEAP + TLR1/2 plasmids as shown in Figure 1. The pNF-κB/SEAP and CMV/SEAP (control) plasmids are supplied in the NF-κB SEAPorter™ Assay Kit: NBP2-25286. After 48 hr of transfection, 100 nmole/ml of MyD88 inhibitory peptide or control peptide (NBP2-29328: MyD88 Peptide Set) was added as shown to two of the cultures and incubated at 37°C. After 24 hr, 1 ug/ml of Pam3CSK4 TLR1/2 ligand (NBP2-25297) was added as shown to four of the cultures and incubated at 37°C for 24 hours. Transfectant supernatant was collected and analyzed using the Kit reagents.*

The results show that CMV/SEAP transfected (positive control) cells had high levels of SEAP expression. Cells co-transfected with NF-κB/SEAP + TLR1/2 plasmids, and TLR signaling activated with PAM3CSK4 also had high levels of expression. The MyD88 inhibitor, but the not control inhibitor, partially inhibited ligand induced NF-κB activation, as indicated by a reduction in SEAP activity when the inhibitor was added prior to ligand treatment. As expected, the MyD88 control peptide, however, did not inhibit ligand induced activation. Other combinations had only low levels of SEAP activity, representing a basal level of NF-κB activation. *CMV is used as a positive control promoter because it is constitutively transcribed at high levels and therefore CMV/SEAP transfected cells will express high levels of SEAP.*



TLR/NF-κB Pathway Perturbation Tools

Curcumin: A Natural Plant-Based Inhibitor

Curcumin, the active ingredient of the spice turmeric, is a highly pleiotropic, complex molecule which interacts with a number of signaling targets involved in inflammation and other biological processes (Fig 1). Many signaling molecules ultimately signal through NF-κB. Whereas many pharmacological activities have been ascribed to curcumin, the inhibition of NF-κB signaling has been a key focus (Fig 2). In this regard, data from Novus' laboratories, as well as from other model systems in the scientific literature, provide supporting evidence for curcumin as an extremely powerful research tool for inhibiting NF-κB activation *(Epstein et al, 2009; Jurenka et al, 2010)*.

NF-κB is a ubiquitous eukaryotic transcription factor that plays a key role in regulating a number of cellular processes including inflammation, cellular proliferation, transformation and tumorigenesis. In unstimulated cells, NF-κB dimers are held in the cytoplasm by IκBs which mask the nuclear localization signals (NLS) of NF-κB required for nuclear translocation and activation. Following cell stimulation, IκBα is phosphorylated and degraded. This results in NLS exposure, thereby enabling NF-κB to translocate to the nucleus and activate

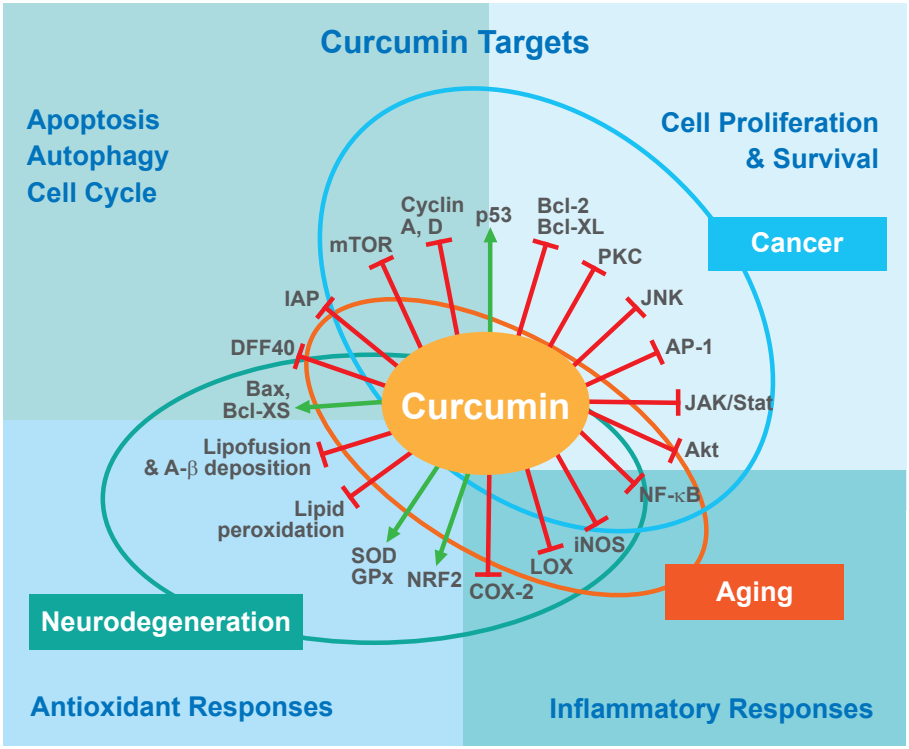


Figure 1. Curcumin: An NF-κB and multiple pathway signaling inhibitor. *Curcumin is primarily recognized as a signaling inhibitor, however it does activate certain signaling pathways: red arrows indicate inhibition and green arrows activation. The molecular targets of curcumin can be grouped by biological actions (blue lettering) or processes (colored circles).*

transcription. Evidence suggests that curcumin suppresses NF-κB activation and subsequent proinflammatory gene expression by blocking IκB phosphorylation.

Novus' Curcumin (Fig 2, Cat. No. NBP2-26243) is a highly purified assay validated extract from the turmeric plant Curcuma longa especially designed for research use. The research interest in curcumin is expected to continue to increase because of data suggesting that curcumin holds promise as both a health-promoting and therapeutic agent as well as the strong public research interest in naturally occurring plant-based remedies.

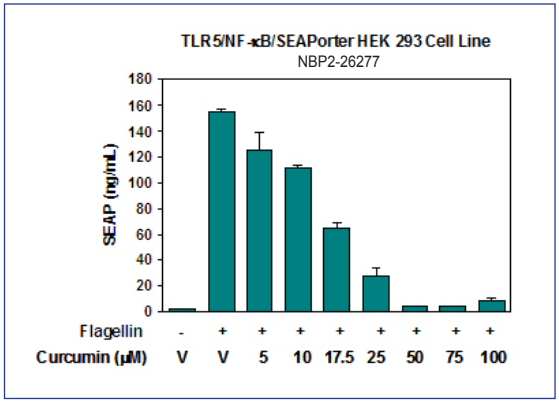


Figure 2. Curcumin inhibition of ligand activated TLR/NF-κB signaling. *TLR5/NF-kB/SEAPorter™ HEK 293 (NBP2-26243) cells were plated in 12-well plates (0.5 x 10^6 cells/well) for 16 h. Cells were preincubated with increasing concentrations of curcumin (NBP2-26243) for 2 h or a DMS0 vehicle (V) control. Cells were then stimulated with the TLR5 ligand Flagellin (10 ng/ml, NBP2-25289) for 24 h. The SEAPorter™ Assay Kit (NBP2-25285) was used to measure SEAP, the readout assay for measuring NF-kB activation in TLR5/NF-κB cells. The results show that the cells had a minimal basal level of NF-κB activity which was dramatically increased by Flagellin. They also show that curcumin decreased Flagellin-activated NF-kB signaling in a dose-dependent manner.*

Peptide Inhibitors

Cell permeable inhibitory peptides are research tools for manipulating signal transduction pathways. Their mechanism of action is based on blocking signaling events through decoy action. A decoy is a realistic replica used as a lure or bait. Each peptide employs a specific decoy mechanism that blocks the propagation of downstream signaling pathways. Mechanisms are depicted by "STOP" signs in Figure 1 which illustrates various TLR and NF-κB peptide inhibitors. Novus offers a growing portfolio of peptide inhibitors which can be broadly categorized into the TLR, NF-κB, Protein Kinase and Caspase signaling pathways. Representative TLR/NF-κB pathway inhibitors and their catalog numbers are shown in Figure 1, please see the website (www.novusbio.com) for a complete listing.

MyD88, a central player in TLR signaling, is used as an example to provide additional detail about the concept of "decoy" inhibitory mechanisms. As shown in Figure 2, the MyD88 inhibitory peptide (IMG-2005) inhibits IRAK/MyD88/TLR complex formation. The inhibitory mechanism is based on preventing or blocking MyD88 homodimerization as detailed in Figure 3. Specifically, the inhibitory peptide binds to MyD88 monomers, acting as a MyD88 monomer "decoy" because it contains MyD88 binding site sequences. Hence, the MyD88 monomer thinks the peptide is actually another MyD88 monomer, but it is only a decoy, i.e., a mere amino acid sequence not authentic MyD88! To clarify, the peptide bound monomer is unable to bind to another MyD88 monomer because its MyD88 binding sites are already bound to the peptide. MyD88 homodimers can not form and since MyD88 homodimerization is a requirement for TLR MyD88-dependent signaling, TLR signaling is blocked or inhibited as depicted in Figures 1 and 2.

Sample data using the MyD88 peptide is shown in Figure 4. This study is based on LPS activation and MyD88 inhibition of TLR4 signaling. LPS induces inflammatory cytokine responses via TLR4-mediated NF-κB activation. MyD88 homodimerization is required for MyD88-TLR binding (TLR and MyD88 TIR-TIR domain interactions) and IRAK recruitment and activation. Hence the inhibitory peptide is a mechanism to block TLR signaling and downstream NF-κB activation.

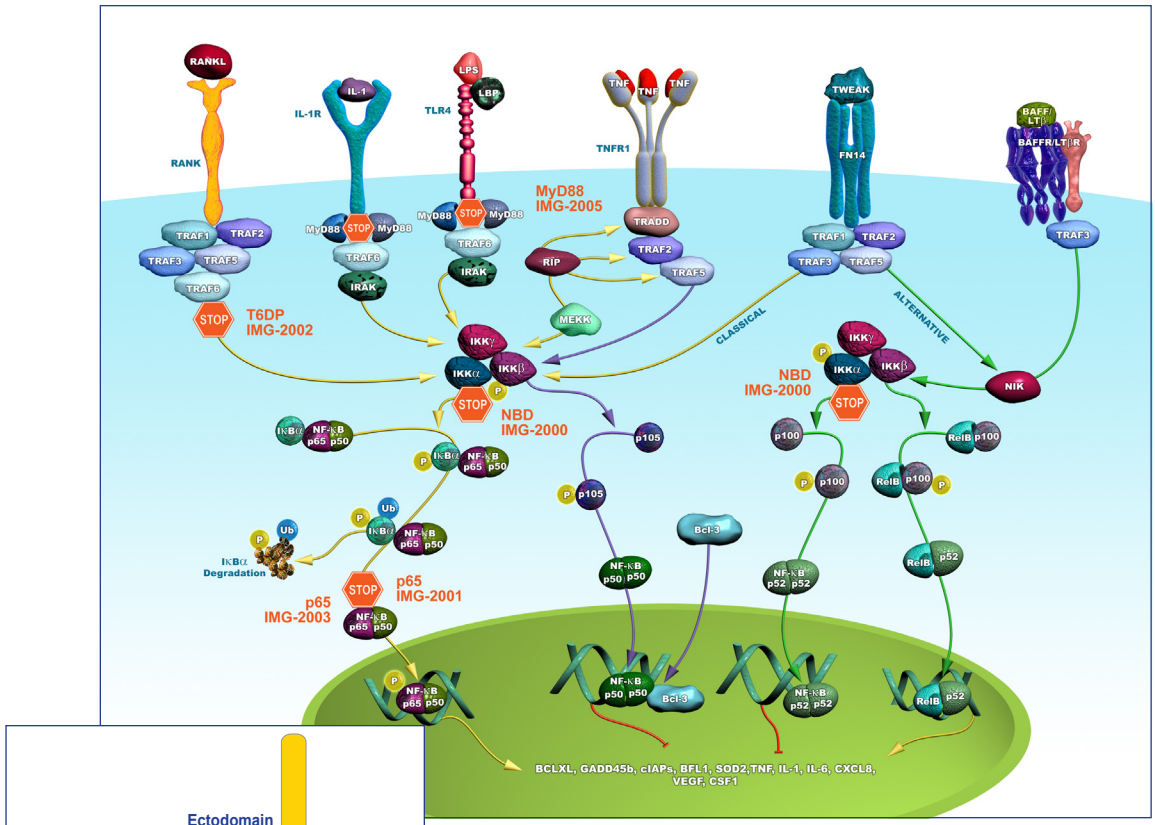


Figure 1. Inhibiting TLR and NF-κB signaling with cell permeable peptides.
Peptide inhibitor are tools for manipulating signal transduction pathways.

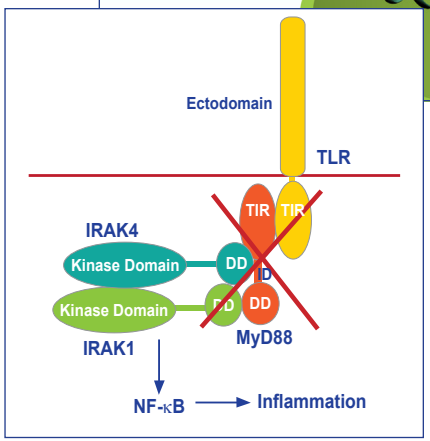


Figure 2. Concept of inhibition of IRAK/MyD88/TLR complex formation with the MyD88 inhibitory peptide (NBP2-29328).
MyD88 homodimerization is required for MyD88-TLR binding (TLR and MyD88 TIR-TIR domain interactions) and IRAK recruitment and activation. Hence, the inhibitory peptide (red cross bar) is a mechanism to block TLR signaling.

MyD88 Homodimerization Inhibitory Peptide: IMG-2005

- MyD88 homodimerization: critical for TLR/IL-1R signaling
  MyD88 recruited to TLR/IL-1Rs as a homodimer
- MyD88 inhibitory peptide sequence
  DRQIKIWFQNRRMKWKKRDVLPGT
  Sequence required for MyD88 homodimerization
  a.k.a MyD88 'decoy' **sequence**
  Binds to MyD88 monomer, blocking homodimerization
  Protein transduction **sequence** for cell permeability
- Control peptide: **DRQIKIWFQNRRMKWKK**

Figure 3. Cell permeable inhibitory features. The MyD88 inhibitory peptide is shown as an example. All of the inhibitory peptides have a "decoy" sequence that blocks a specific node in their respective signaling pathways. They also have a cell permeable translocation sequence, which in of itself is supplied with peptide sets as a control.

This study by Barnford et al (2007) underscores the complexity of TLR pathways and how inhibitor pertubation can help define elusive signaling mechanisms. For example, the MyD88 inhibitor was instrumental in showing that different types of LPS utilize different adaptor mechanisms to activate TLR4, i.e. MyD88-dependent (*E. coli* and *B. cenocepacia*) versus MyD88-independent (*B. multivorans*) pathways. This could have significant implications when designing pharmaceuticals to specifically block various strains of infectious agents.

Representative Novus Product Citation

Bamford S, H Ryley, S Jackson. Highly purified lipopolysaccharides from Burkholderia cepacia complex clinical isolates induce inflammatory cytokine responses via TLR4-mediated MAPK signaling pathways and activation of NF-κB. *Cellular Microbiology* 9: 532-543 (2007). **MyD88 Inhibitory Peptide (NBP2-29328): MM6 human monocytic cell line stimulated with LPS, and inhibited with NBP2-29328: Fig 4A-C. Note: TNFα production was used as a read-out assay to measure the effect of MyD88 inhibition.**

TLR/NF-κB Inhibitory Peptides: General Protocol

Researchers can study the effect of inhibitory peptides using a variety of treatments, cell types and read-out assays. Cells are typically preincubated with inhibitory or control peptides, or no peptide, for 12-24 hr and then treated with TLR or NF-κB activating agents. Optimal peptide concentrations and incubation times vary between model systems and should be determined empirically by users.

Likewise, read-out-assays will vary depending on the model system. Read-out assays should be validated. For example, Figure 4 shows that LPS induced TNFα production in the monocytic cell line model system, hence TNFα production was a valid assay for measuring the effect of peptide inhibition. Read-out assays should be performed on a time course to monitor the effect of peptide inhibition. Refer to individual data sheets for additional details. Additionally, researchers are encouraged to review the inhibitor product citations to see how researchers have used and published with Novus' Inhibitors.

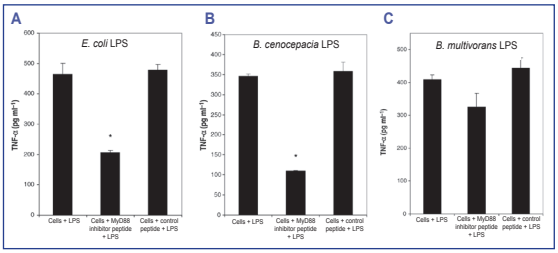


Figure 4. LPS stimulation of MyD88-dependent and –independent pathways of TLR4 activation. *ELISA data of TNFα production from MM6, a human monocytic cell line, in the presence and absence of the NBP2-29328 MyD88 inhibitor or control peptide. Cells stimulated with LPS from E. coli (A), B. cenocepacia (B) or B. multivorans (C).*

*Representative data is shown for each LPS type (mean +SD) from three independent experiments. *P < 0.001 versus cells treated with control peptide and LPS. Source: Bamford et al, 2007.*

TLR Ligands

TLR ligands are standard tools for activating specific TLRs. Refer to the section "TLRs: Structure, Ligands, and Expression" for additional information on the concept of ligand activation and TLRs.

TLR ligands can be used in cell culture and in animal models, and protocols should be established according to the experimental design. Assay amounts are guidelines only and obtained from the published literature and studies carried out at Novus' laboratories. Ligands are provided in formats that enable researchers to optimize protocols and ligand treatment parameters for their own model system. See the Novus' website (www.novusbio.com) for a complete listing and product details.

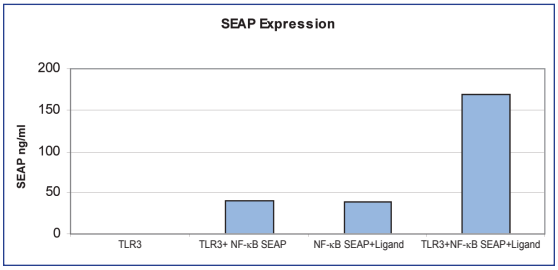


Figure 1. Poly(I:C) (NBP2-25288) activation of TLR3. *Poly (I:C) is a synthetic analog of dsRNA that binds directly to TLR3 where it activates TLR3 signaling. dsRNA is a PAMP associated with viral infection and is produced during the viral replicative life cycle.*

293T cells were co-transfected with the pNF-κB/SEAP plasmid (NBP2-25286 component) and a pCMV/TLR3 plasmid. Single transfections with either pCMV/TLR3 or pNF-κB /SEAP plasmids were included as controls. After 48 hr, 10 ug/ml of Poly(I:C) was added to some of the cultures, and cells were incubated at 37°C for 24 hr. Cell supernatant was collected and analyzed using the NF-κB SEAPorter™ Assay Kit (NBP2-25286). Controls included single transfections with either TLR3 or pNF-κB /SEAP, and co-transfected cells not treated with the Poly(I:C) ligand.

The results indicate a lack of NF-κB activity in the TLR3 transfected cells and low levels in both the TLR3 + NF-κB SEAP and the Poly(I:C) treated NF-κB/SEAP cells. However, the level of NF-kB was significantly increased in cells transfected with TLR3 + NF-κB SEAP and treated with Poly(I:C) ligand. This is because Poly(I:C) binds to TLR3, thereby activating TLR3 signaling, which in turn activates NF-κB signaling. When NF-κB is activated in the NF-κB/SEAP transfected cells, SEAP is produced and secreted into the media where it can be measured. See the 'TLR Signaling' Chapter for details regarding TLR3 activated NF-κB signaling.

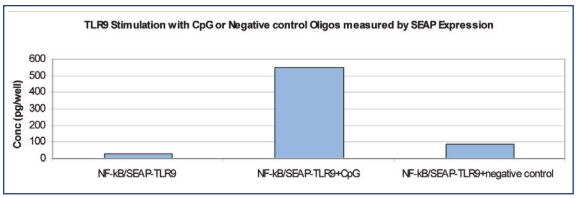


Figure 2. CpG ODN 2006 Type B (NBP2-26232) activation of TLR9 in NF-κB/SEAP stable HEK 293 cells (NBP2-26260). *CpG ODNs are synthetic oligonucleotides containing unmethylated CpG dinucleotides or motifs. These CpG motifs are relatively common in bacterial and viral DNA, but are suppressed and methylated in vertebrate DNA (reviewed in Vollner, 2009). The disparity in methylation status is a key distinction between bacterial/viral and vertebrate DNA. Unmethylated CpG motifs are considered PAMPs due to their abundance in microbial genomes, but rarity in vertebrate genomes.*

The CpG PAMP is recognized by TLR9 which is constitutively expressed in B cells and pDCs in humans and other primates. TLR9 agonists, like CpG ODN, induce pDC activation and maturation and enhance the differentiation of B cells into antibody-secreting plasma cells. There are five unofficial classes of CpG ODN (Fig 3) based on their sequence, secondary structures and effects on human PBMCs (reviewed in Vollner, 2009).

NF-κB/SEAP stable HEK 293 cells were transiently transfected with a pCMV/TLR9 plasmid. After 48 hr, 10 µg of CpG ODN or GpC ODN [negative control oligo (included in NBP2-26232)] was added, or cells were left untreated. Cells were incubated at 37°C for 12 hrs, and cell supernatant was collected and analyzed using the NF-κB SEAPorter™ Assay Kit (IMK-515). The data showed that CpG ODN upregulated SEAP activity more than 5 fold compared to the negative control GpC ODN. Both GpC ODN and untreated cultures had a low level of SEAP activity, which may represent basal activation of TLR9 or NF-κB.

Figures 1 and 2 are examples of Poly(I:C) (NBP2-25288) and CpG ODN (NBP2-26232) activation in TLR3 and TLR9 transfected cell lines, respectively. In both of these examples, TLR activation was measured using the NF-κB SEAPorter™ Assay Kit (NBP2-25286). This kit measures NF-κB activation using a SEAP reporter system and is an analysis of TLR activation occurring through the NF-κB signaling pathway.

Reference

Vollmer J and AM Krieg. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Advanced Drug Delivery Rev* 61:195-2004 (2009).

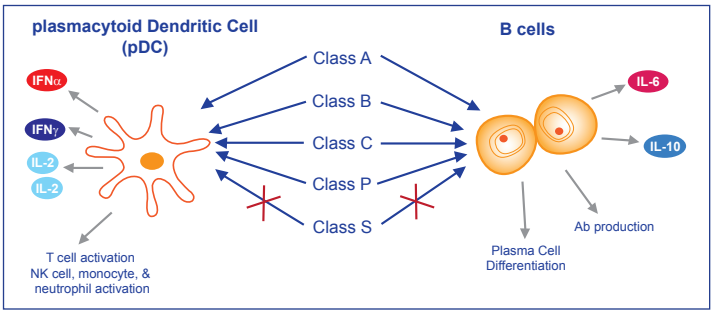


Figure 3. Different classes of CpG ODN elicit different responses in pDC and B cells. *S-class ODNs do not contain classical CpG motifs and may act as antagonists that directly interfere with the binding of CpG DNA to TLR9. Adapted from Vollmer and Krief, 2009.*

Western Blot

Western blot analysis continues to be among the most widely used proteomic technique to evaluate protein expression. The majority of Novus' antibodies are evaluated by western blot during the development, manufacturing, and quality control processes. Novus manufactures ready-to-use western blots, INSTA-Blots™, from the same tissue and cell lysates used in our laboratories. Custom Western blot services are also available. INSTA-Blots™ and Western blot protocols can be found at the end of this section.

Ready-to-Use INSTA-Blots™

INSTA-Blots™ are ready-to-use PVDF membranes containing denatured protein from cells or tissue lysates (Figure 1). The INSTA-Blot™ product line provides a simple and cost-effective solution for screening the expression of TLRs and other proteins in different tissues and cells. Novus has streamlined the western blotting procedure by eliminating the sample aquisition, preparation, SDS-PAGE and electro-blotting steps. With INSTA-Blots™, immunoblotting is an easy six-step procedure: blocking, primary antibody incubation, wash, secondary antibody incubation, wash, and develop. Additionally, most of the lysates used to manufacture INSTA-Blots™ are available separately, enabling researchers to obtain additional or bulk material for tissues or cell lines of particular interest.

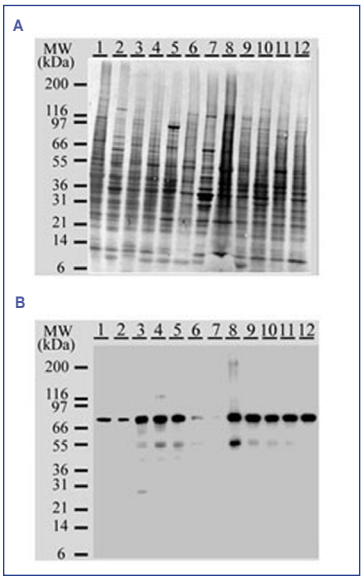


Figure 1. From INSTA-Blot™ to Western blot analysis. *A. Tissue or cell lysates are separated by SDS-page, transferred onto an Immobilon PVDF membrane and stained with Amido Black. The INSTA-Blots™ are vacuum packed to ensure freshness. B. Researchers probe INSTA-Blots™ with antibodies of their choice, the amido black stain automatically washes out during the blocking and antibody incubation steps.* *See protocol on page 159.*

OncoPair INSTA-Blots™

OncoPairs are specialized INSTA-Blots™ that are manufactured from Novus' extensive Human Clinical Tissue Lysate collection. See Figures 1-6 in the Tissue & Cell Lysates section for additional information about these lysates. Each OncoPair INSTA-Blot™ blot contains 14 lanes of alternating tumor (T) and matched normal adjacent (NA) tissue lysates from 7 patient donors. Incorporating both T and NA on the blot enables protein expression analysis of the tumor and microenvironment. The tumor microenvironment is increasingly being recognized as a major factor in influencing malignant progression and metastatic potential. Cancer is a heterogeneous disease and the presence of multiple donors on a single blot enables rapid screening of protein expression variability between T/NA from different individuals and at different disease stages. Clinical diagnosis and histopathology reports from board-certified pathologists are available for every patient sample.

The OncoPair INSTA-Blots™ are particularly useful tools for biomarker analysis. Biomarkers have enormous potential for revolutionizing the diagnosis and treatment of disease, and western blot analysis is an integral technique for biomarker profiling. While key for biomarker discovery, the existing body of western blot data has primarily been defined from studies of tumor, immortal, and primary cells growing *in vitro*. Collectively, results obtained over decades have been integral to the dogma that up- and down-regulation of proteins can be leveraged as biomarkers of normal development, homeostasis, and disease. However, data from human tissue-derived products is key for generating biomarker validation data and progressing the most promising biomarker targets towards the clinic.

The OncoPair INSTA-Blot™ product line addresses the increasing demand for the inclusion of human tissue-derived products in biomarker profiling. Figure 2 shows an example of a prototypical OncoPair INSTA-Blot™ probed for expression of the common tumor biomarker p53 and several loading controls. Figure 6 on page 148 shows these same patient screened for TLR1 and TLR3 expression. The OncoPair INSTA-Blot™ lysates are also useful in conjunction with the Cell Line and Normal Tissue INSTA-Blots™ for profiling antibody reactivity and protein expression patterns across species and between tissues and cells lines.

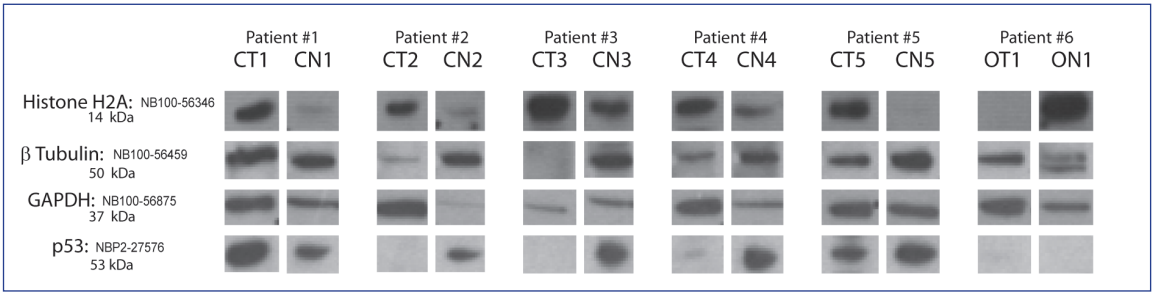


Figure 2. Western blot analysis of several proteins in OncoPair INSTA-Blot™ tissue lysates. *This prototypical INSTA-Blot™ contains 5 colon (C) and 1 ovary (O) patient sets of tumor (T) /normal adjacent (N) tissue lysates. The data shows differential expression of the common tumor marker p53. Likewise, there is also differential expression of histone H2A between samples.*

The expression of standard loading control proteins such as β-tubulin and GAPDH can vary between tumor and normal lysates, as well as between tissue types as illustrated by the data. Hence it is often advantageous to use more than one loading control when assessing protein expression relative to "housekeeping proteins."

Representative INSTA-Blot™ Product Citations

1. Zhou M, L Xia, J Wang. Drug Metabolism and Disposition *DOI*:10.1124/dmd.107.01549 (2007). Metformin transport by a newly cloned proton-stimulated organic transporter (Plasma membrane monoamine transporter) expressed in human intestine. **Novus products cited for WB (human small intestine, Fig 6B):**
   1. GAPDH
   2. INSTA-Blot™ containing human small intestine tissue lysate (NBP2-30113, NBP2-30118)
2. Yuan Z, A Tie, M Tarnopolsky, M Bakovic. *Physiol Genomics*, 26: 76-90 (2006). Genomic organization, promoter activity, and expression of the human choline transporter-like protein 1. **Novus products cited:**
   1. QuikChIP™ Kit (NBP2-29902): Human breast cancer MCF-7 cells and mouse C2C12 myoblast cells, Fig. 5.
   2. INSTA-Blot™ Multi-species Brain, Testis, and Ovary (NBP2-30115): Fig 8, WB.
   3. INSTA-Blot™ Multi-species Liver, Lung, and Spleen (NBP2-30117): Fig 8, WB.
   4. INSTA-Blot™ Multi-species Skeletal muscle, Heart, and Kidney (NBP2-30116): Fig 8, WB.

INSTA-Blot™ Protocol

INSTA-Blot™ Ready-to-Use Western Blot Detection System
INSTA-Blot™ ready-to-use membranes offer a simple and fast solution for screening the expression of a particular protein in human, mouse, and rat tissues plus cell lines. INSTA-Blot™ ready-to-use membranes eliminate the preparation of lysates, gel electrophoresis and transfer to PVDF membrane. With INSTA-Blot™, immunoblotting is a simple six-step procedure: blocking, primary antibody incubation, washing, secondary antibody incubation, washing, and develop.

1. Wet the blots with methanol and then wash with TBST (25 mM Tris-Cl, pH 8.0; 125 mM NaCl; 0.1% Tween 20) twice to remove residual methanol.
2. Incubate the blots for 1 hour with 5% Carnation nonfat dry milk in TBST to block non-specific antibody binding.
3. Incubate the blots with primary antibody in 1% milk/TBST for 1-2 hours at room temperature or overnight at 4°C.
4. After incubation with the primary antibody, wash the blots five times in TBST and then incubate with a secondary antibody conjugated to horse radish peroxidase (HRPO; 1:1000-1:10000 dilution; Novus) for 1 hour at RT. Sodium azide is an inhibitor of HRPO and should not be added to the secondary buffer.
5. After five washes with TBST, develop the blots for 5 minutes using PicoTect™ Western Blot Chemiluminescent Substrate (Cat. No.10087K).
6. Expose the blots to photographic film for an appropriate time period. We normally use Hyper-film™-ECL films (Amersham Life Science Inc.) and expose for various periods ranging from 10 seconds to 20 minutes to visualize the chemiluminescence signal corresponding to the specific antibody-antigen reaction.

Western Blot Protocol

1. Equal amounts (10-50 µg/lane) of protein samples are resolved by SDS-PAGE and electro-blotted using the Bio-Rad mini-gel transfer system (Bio-Rad Laboratories, Cambridge, MA) onto Immobilon P membranes (Millipore Corporation, MA).
2. The blots are stained with amido black for 1 min and destained with 10% methanol plus 10% acetic acid. Amido black helps to monitor the efficiency of transfer without interfering with subsequent immuno-reaction. The blot can be blocked immediately or allowed to air dry until the color fades.
3. If dry, wet the blots in 100% methanol, rinse with TBST, and then block for 1 hour with 5% Carnation nonfat dry milk in TBST (25 mM Tris-Cl, pH 8.0; 125 mM NaCl; 0.1% Tween 20).
4. The blots are incubated with primary antibody in 1% milk/TBST overnight at 4°C. The primary antibody should be used at the recommended concentration/dilution, and diluted with 1% Carnation nonfat dry milk in TBST.
5. After incubation with the primary antibody, the blots are washed five times in TBST and then incubated with the proper secondary antibody conjugated to horse-radish peroxidase (HRPO; 1:1000-1:10000 dilution; Novus) for 60 minutes at RT. Dilute with 1% milk/TBST without sodium azide (an inhibitor of HRP).

6. After five washes with TBST, the blots are developed for 5 minutes using PicoTect™ Western Blot Chemiluminescent Substrate (Cat. No.NBP2-29912).
7. X-ray films are exposed to the blots for appropriate time periods. We normally use Hyperfilm™ –ECL films (Amersham Life Science Inc.) and expose to the blots for 10 sec, 1 min, 5 min, and 20 min to visualize the chemiluminescence signal corresponding to the specific antibody-antigen reaction.

Absorption of Antibody Activity with Synthetic Peptide:
To determine the specificity of the primary antibody, the antibody can be pre-absorbed with the specific synthetic peptide which was used as antigen for the development of the antibody. Mix the peptide and antibody at 2:1 to 5:1 (peptide:antibody) and incubate on a shaker at RT for 4 hours. Centrifuge at 10,000 RPM for 2 min. Use supernatant for further western blotting detection, as it is described in Steps 4-7 in the western blotting protocol.

Cell & Tissue Lysate Preparation Protocols

Researchers often have their own protocols for cell and tissue lysate preparation for western blot analysis. The following protocols are those routinely used at Novus. Specialized protocols are used for preparing the clinical tissue lysates, please see www.novusbio.com for additional information.

Cell Lysate Preparation Protocol

Buffers

- 1X Lysis buffer: 10 mM Tris, pH8.0, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi
- 100X PIC: 1.6 mg/mL Benzamidine HCl, 1.0 mg/mL Phenanthroline, 1.0 mg/mL Aprotonin, 1.0 mg/mL Leupeptin, 1.0 mg/mL Pepstatin A, dissolve in 100% ETOH and store at -20°C.
- 2X SDS sample buffer: 120 mM Tris-Hcl (pH 6.8), 20 mM EDTA, 4% SDS, 0.06% Bromophenol Blue, 20% glycerol, 0.4% beta-mercaptoethanol

Add to the 1X Lysis buffer just prior to use:
- 0.02 mg/mL Rnase
- 0.2 mg/mL Dnase
- 1 mM PMSF
- 1X Protease Inhibitor Cocktail (PIC)

For adherent cells

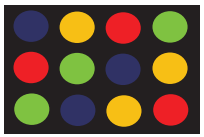
1. Rinse monolayer cells 3-4 times with PBS. On the final rinse, aspirate as much PBS as possible and add 5 ml of ice-cold PBS containing 0.5 mM EDTA and use a cell lifter or cell scraper to bring cells into suspension. Transfer suspension into a 50 ml centrifuge tube and add 5 ml ice-cold PBS to flask. Centrifuge cells at 1,500 RPM for 10 min at 4°C and aspirate supernatant. Repeat the PBS wash two more times. Continue with Step 2 below.

For suspension cells

1. Centrifuge suspension at 1,500 RPM for 10 min at 4°C and aspirate supernatant. Resuspend pellet in 15 ml PBS and centrifuge at 1,500 RPM for 10 min at 4°C and aspirate supernatant. Repeat 2 more times.
2. For every 1 x 10^6 cells add approximately 100 µL of ice-cold Lysis Buffer with 1X PIC and resuspend pellet, ensuring no clumps remain.
3. Incubate on ice for 60 min.
4. During incubation time, transfer contents of each tube to a microcentrifuge tube.
5. Centrifuge at 13,000 RPM for 30 min at 4°C.
6. Collect supernatant into an appropriately labeled tube and determine protein concentration using the BioRad modification of the Bradford assay.
7. Dilute the cell lysate with 2X SDS sample buffer (see recipe above) by combining equal volumes of 2X SDS sample buffer and cell lysate. Heat at 95-100°C for 3-5 min. Use immediately or aliquot and store at -20°C or -80°C. If storing lysates, warm prior to loading on SDS-PAGE gel.

Tissue Lysate Preparation Protocol

1. Weigh tissue sample in a 50 mL tube.
2. While keeping sample on ice, wash with cold 1X PBS and aspirate off PBS.
3. Repeat until wash buffer appears clear.
4. Add sufficient volume of cold lysis buffer to cover sample (about 3 times the weight of sample in volume; i.e. 500 mg sample will receive 1.5 mL lysis buffer)
5. Grind/homogenize tissue in tube and incubate on ice for at least one hour.
6. Transfer mixture to microcentrifuge tubes and spin at 13,000 RPM for 30 min at 4°C.
7. Poke through lipid layer and remove supernatant (this is the lysate). Discard cellular debris and lipids.
8. If necessary, recentrifuge the supernatant at 13,000 RPM and repeat Step 7 to obtain clean lysate free of lipid and debris.
9. Determine protein concentration using Bradford Protein Assay.
10. Dilute the tissue lysate with 2X SDS sample buffer (see recipe above) by combining equal volumes of 2X SDS sample buffer and cell lysate. Heat at 95-100°C for 3-5 min. Use immediately or aliquot and store at -20°C or -80°C. If storing lysates, warm prior to loading on an SDS-PAGE gel.



Novus Screening Services

Inflammation & Immune Signaling Pathways

Novus is a recognized leader in the provision of tools for studying cellular responses. We have experience in the manufacturing of reagents and development of applications and assay systems for studying pathways of cellular response, particularly pathways related to Immune Receptors with a focus on Toll-like Receptors. Over the past several years Novus has developed a broad product portfolio in the area of Toll-like Receptors (TLRs), and related areas of Inflammation and Immunology providing advanced research tools to accelerate the increasing volume of applied research in these areas of biology.

In parallel with their critical role in shaping and regulating cellular and immune responses, is the recognition of Toll-like Receptors as tunable targets to control these responses. Identifying new, more specific and more potent TLR agonists or antagonists is an area of focus for companies with intent for commercial development of diagnostics for monitoring response, or interested in therapeutics as prophylactic or curative drugs.

To help meet these objectives we now offer our new Novus Screening Services as a partner with expertise in cell signaling areas, as evidenced by the portfolio of products already commercialized. We are recognized as authorities in cell signaling pathways especially in the area of NF-κB related signaling pathways which are directly related to Inflammation and Immune Signaling Pathways.

Through Novus Screening Services our objective is to become your partner in accelerating your research and product development efforts and meeting your specific objectives efficiently and economically in accord with the scope, resources, and timetable structure desired.

Partnering Information & Initiation of Novus Screening Services Project

1 Contact Novus Screening Services

Tel: 1-888-506-6887
Fax: 303-730-1966
novus@novusbio.com

2 Assurance of Confidentiality

Exchange NDA assures confidentiality

3 Discussion of Overall Project

Project Scope, Timetable, Objectives

4 Novus provides SOW-Statement of Work

Systems, Assays, Reagents Planned

Data Analysis Formats and Requirements

Timetable & Milestones
Quotation Presentation

5 In Depth Discussion and Project Review

Review and revise SOW as agreed upon

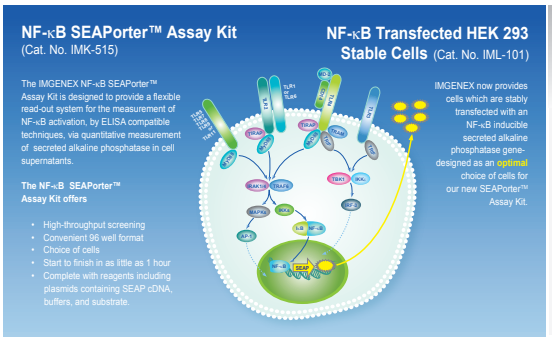
6 Agreement and Go Ahead

Start partnering with Novus

Toll-like Receptor	Key Ligands	Anti-Microbial			Adapter Molecules				Cell Location		Signaling Pathway					
		Bacteria	Virus	Fungi	MyD88	TRIF	TRAM	TIRAP	Cell Surface	Intra-Cellular	NF-κB	IRF3	IRF7	ROS	CRE-AP-1
TLR1	Lipids, Diacyl Lipopeptides Peptidoglycan	✓			✓			✓	✓		✓				✓
TLR2	Lipids, Peptidoglycan	✓			✓			✓	✓		✓			✓	✓
TLR3	dsRNA Poly(I):Poly (C)		✓			✓				✓	✓	✓		✓	
TLR4	LPS	✓	✓		✓	✓	✓	✓	✓		✓	✓			✓
TLR5	Flagellin	✓			✓				✓		✓				✓
TLR6	Lipids, Diacyl Lipopeptides Peptidoglycan	✓		✓	✓			✓	✓		✓				✓
TLR7	ssRNA		✓		✓					✓	✓		✓		✓
TLR8	ssRNA		✓		✓					✓	✓		✓		✓
TLR9	CpG-ODN		✓		✓					✓	✓		✓		✓
TLR10	Orphan				✓						✓				✓
TLR11	Uropathogenic Bacteria	✓			✓						✓				✓
TLR12	?				✓						✓				
TLR13	?				✓						✓				

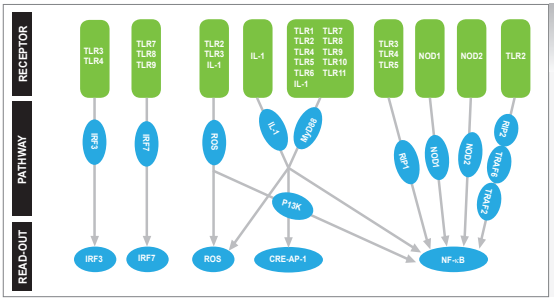
TLR & Inflammatory Ligand Discovery Screen

- Identification of TLR ligands in cell culture
- Identification of TLR ligands in tissue fluids
- Identification of TLR specific ligands



TLR & Inflammatory Ligand Activation Screen

- Pathway Analysis
- Activation Potential



TLR & Inflammatory Pathway Analysis

- Mediator Cascade Analysis
- Cytokines
- Chemokines
- Prostaglandins
- Interferons

Notes

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