TLR Signaling Pathways in Immunity & Beyond



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TLR Signaling in Immunity & Beyond

The Big Question:

How to tease apart complex TLR pathways?

Key Consideration:

What signaling mechanisms are you studying?

Research Tools: TLR detection, activation & inhibition

Presented by Lisa Heiden, PhD June, 2014



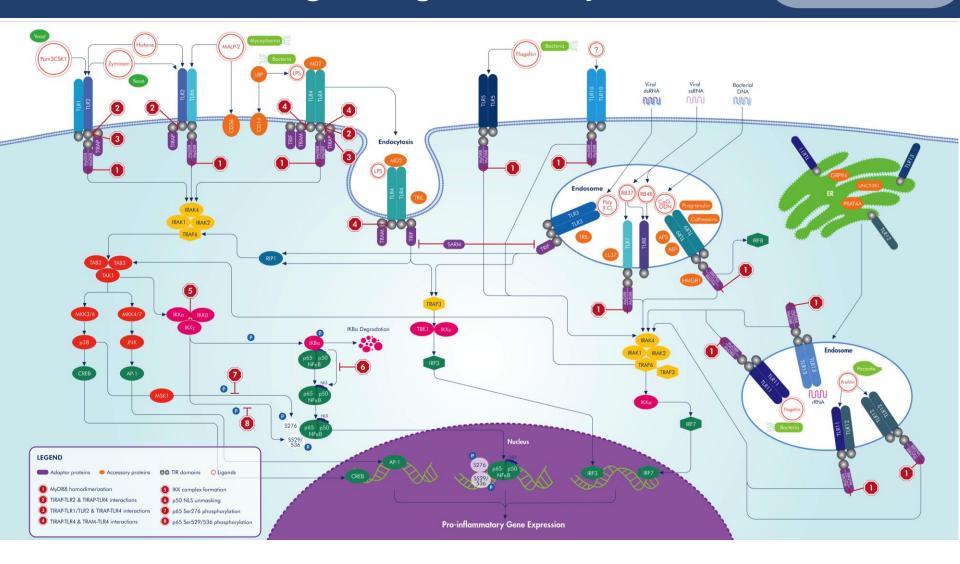
TLR Signaling Tools: Overview

Toll-like Receptors (TLRs): first defined as key regulators of innate immunity, later also found to have broad roles

- TLR signaling overview
- II. Activation & inhibition assays
- III. Emerging awareness of TLR posttranslational modifications



I. TLR Signaling Pathways







TLR Signaling Roles

TLR activation



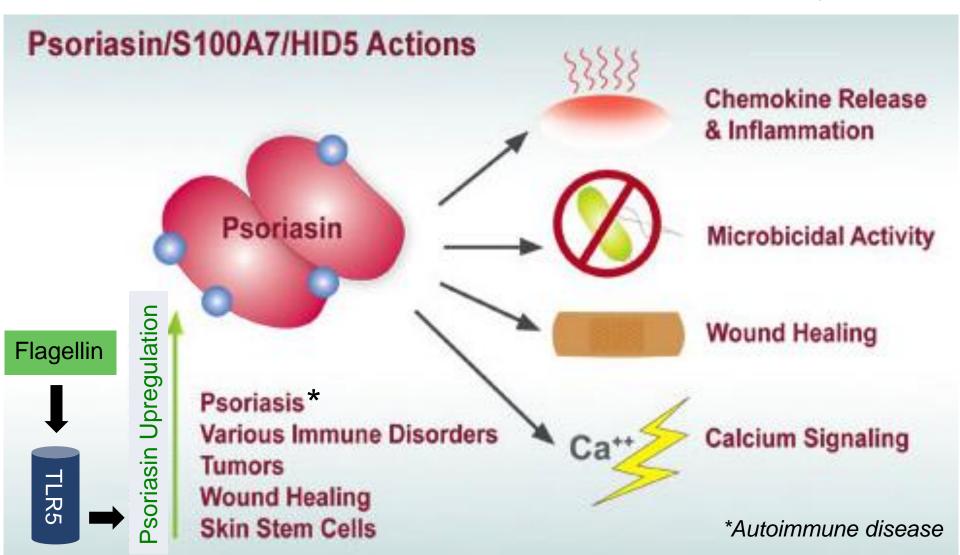
Immune responses

- Innate immunity
- Bridge to adaptive immunity
- Diseases
 - Autoimmunity
 - Cancer



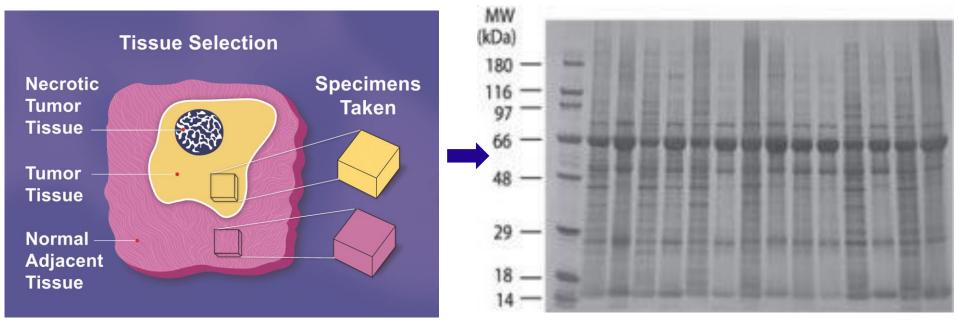
TLRs in Immunity and Disease

First line of defense: Cells recognize and respond to pathogens through TLR receptors, leads to production of factors that kill pathogens



Clinical Tissue Lysate Model System

Study expression of proteins in tumors and normal adjacent tissue



Tissue Lysate Manufacture

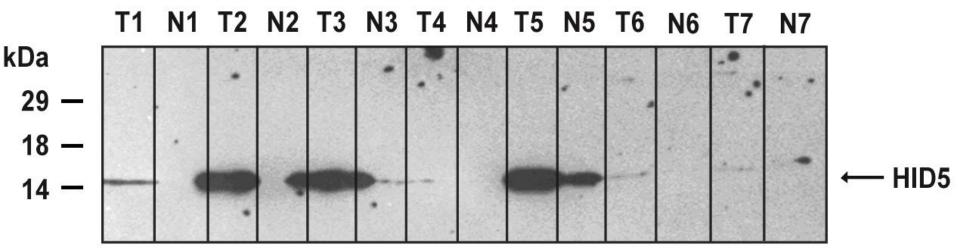
INSTA-Blots: 7 Donor Patients

Breast ductal carcinoma is a model system for studying psoriasin regulation



Psoriasin Upregulation & Disease

Cross-talk between inflammation and disease pathways Breast ductal carcinoma: an inflammatory disease?



INSTA-Blot Breast Tissue OncoPair: 7 Donor Patients, NBP2-29911
Breast ductal carcinoma (T)/normal adjacent (N) clinical tissue lysates

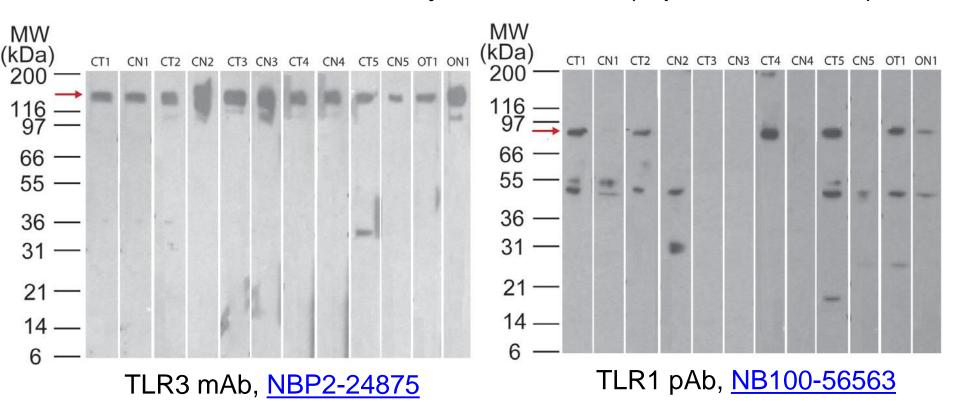
Psoriasin/HID5/S100A7 mAb, NB100-56559

Note: HID5 stands for High in Ductal Carcinoma



TLR Dysregulation in Cancer?

Tumor versus Normal Adjacent Tissue (7 patient donors)



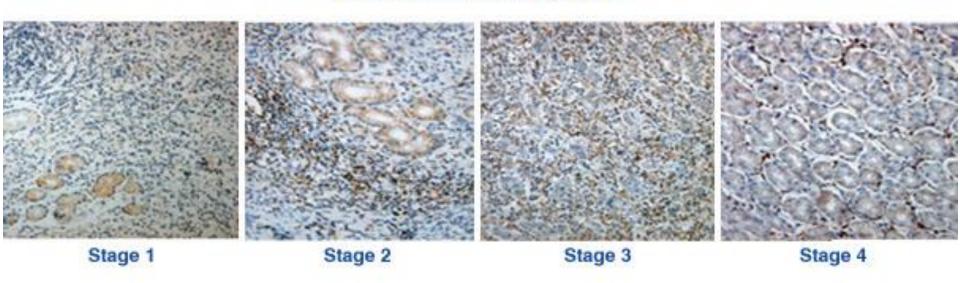
T = Tumor tissue, N = Normal adjacent tissue C = Colon, O = Ovary(all lysates available, please inquire for catalog numbers)



TLRs in Cancer

Tumor cells and the microenvironment

TLR4: clone 76B357.1



Gastric carcinoma, 4 patients: TLR4 (<u>NB100-56566</u>) expression in gastric epithelial tumor cells and tumor-infiltrating lymphocytes. Expression is primarily cytoplasmic, occasionally nuclear and surface.



Cancer: Microenvironment

Tumor environment: immune and other cells, blood vessels, extracellular matrix, signaling molecules, tumor cells, normal adjacent cells.

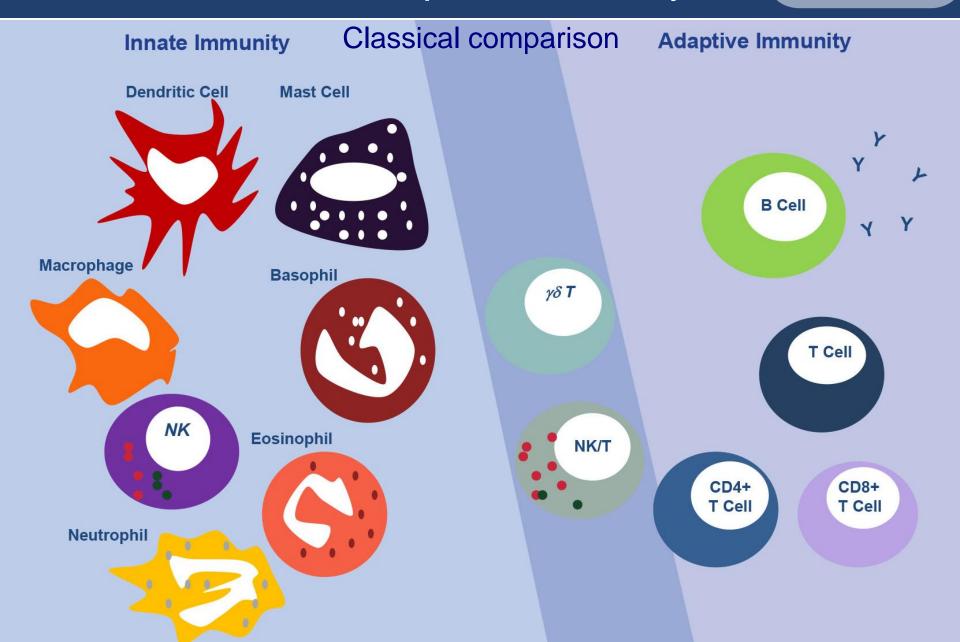
TLR expression and immunity in the tumor and microenvironment interplay and influence tumor growth.

Goal: Harness immune system is being harnessed to manage cancer

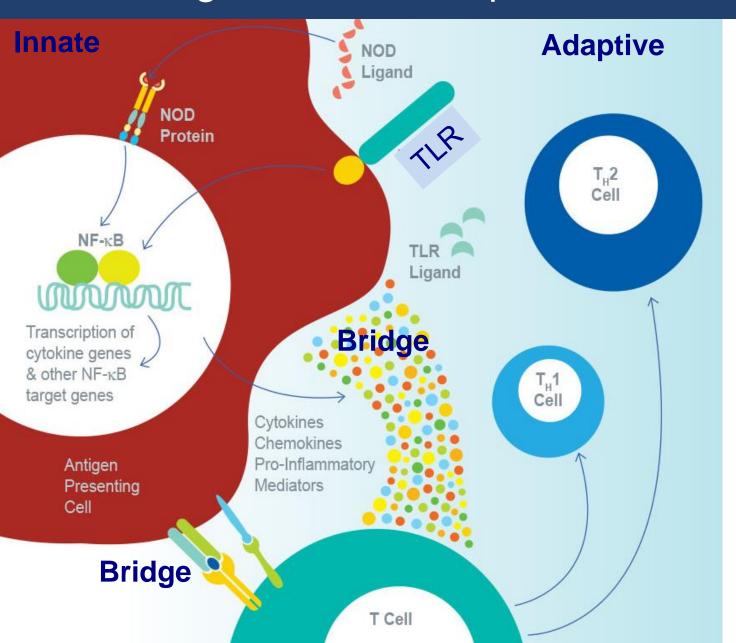
However: "an increasingly sophisticated understanding of the immune system has highlighted how much we still have to learn"

Angela Colmone, American Association for the Advancement of Science/
2014 Annual Meeting

Innate versus Adaptive Immunity



TLRs Bridge Innate & Adaptive Immunity



Example:

TLR signals activate T cells

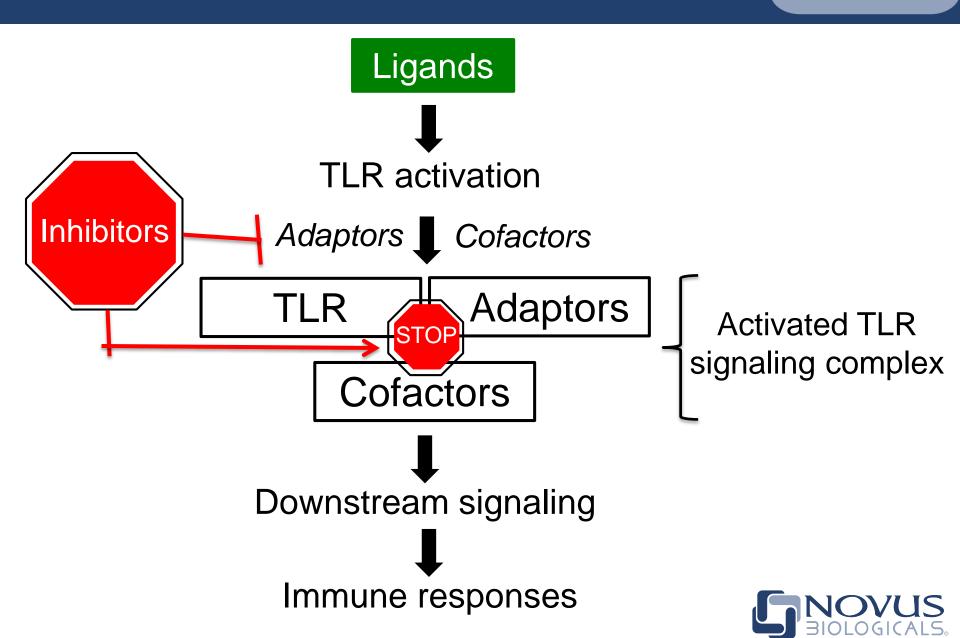


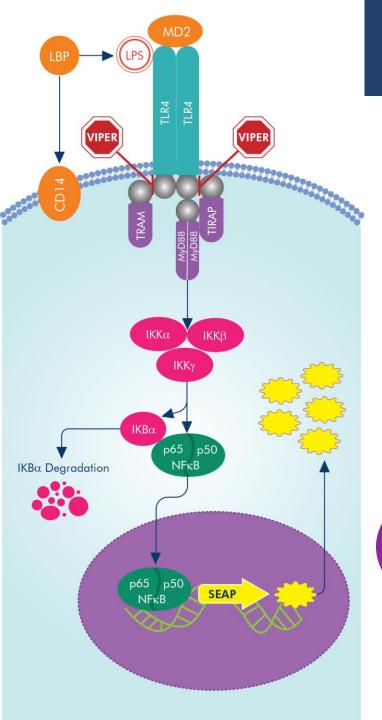
TLR Signaling I

- I. TLR signaling overview: activation, immunity, disease
 - Signals activate pathways
 - Normal immune response & disease roles
 - May be dysregulated in diseased cells & disease microenvironment
- II. Activation & inhibition assays
- III. Emerging roles of TLR posttranslational modifications



II.TLR Signaling Perturbation Strategies





TLR Signaling Tools

Model System

Ligands

: LPS (<u>NBP2-25295</u>)



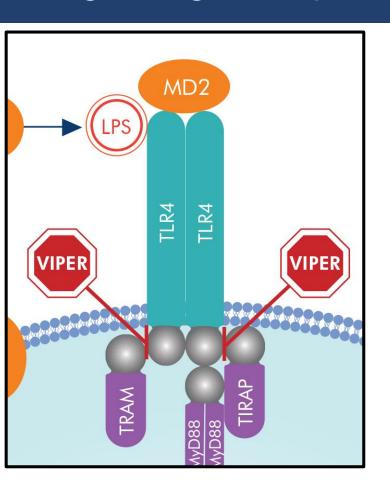
: VIPER/TLR4 (<u>NBP2-26244</u>)

Reporter Cell Lines

: TLR4/MD2/CD14 NF-kB/SEAP (<u>NBP2-26503</u>)



Signaling Disruption Mechanism: VIPER



Viral inhibitor peptide of TLR4 A46 vaccinia sequence

VIPER: KYSFKLILAEYRRRRRRRRR

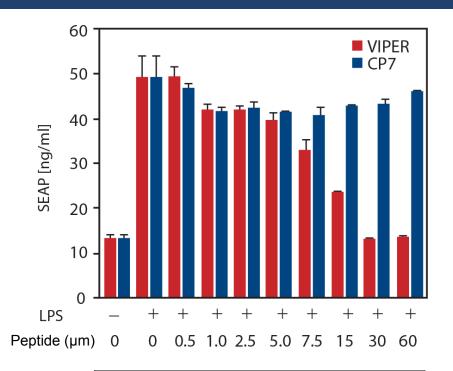
Control: RNTISGNIYSARRRRRRRR

R = cell permeable sequence

VIPER (NBP2-26244) binds to TLR4, TIRAP, TRAM TIR domains Blocks TLR4/TRAM & TLR4/TIRAP TIR-TIR interactions



VIPER Inhibits LPS Activation of TLR4



Reporter Cell Line

TLR4/MD2/CD14/ NF-kB SEAP (<u>NBP2-26503</u>)

Pretreatment

Peptides, 1 h (NBP2-26244)

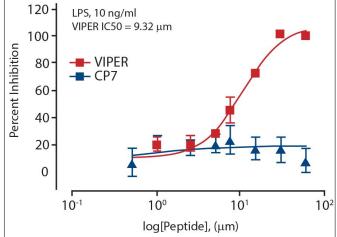
Ligand Activation

LPS, 24 h (NBP2-25295)

Readout

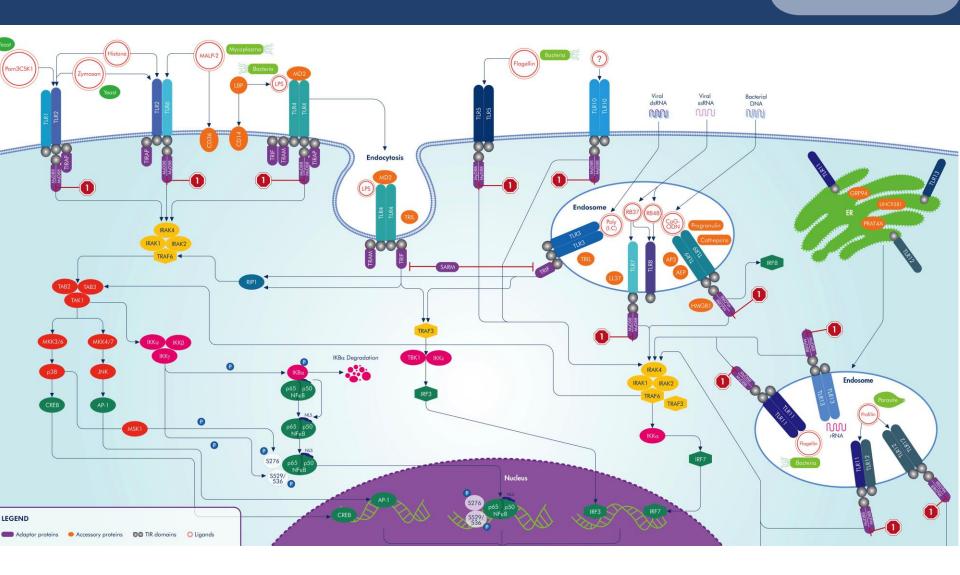
SEAP Assay Kit (NBP2-25285)

IC50 = inhibitor concentration where response is reduced by half





Inhibiting MyD88: TLR Common Adaptor



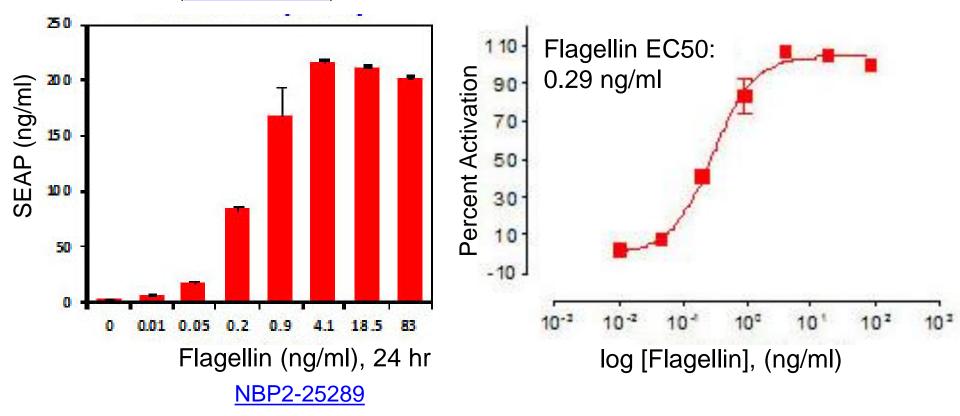




Flagellin Activation Model System

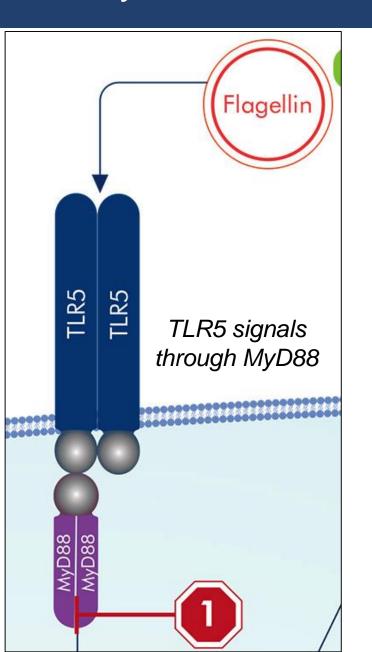
TLR5/NF-kB SEAP Reporter Cell Line

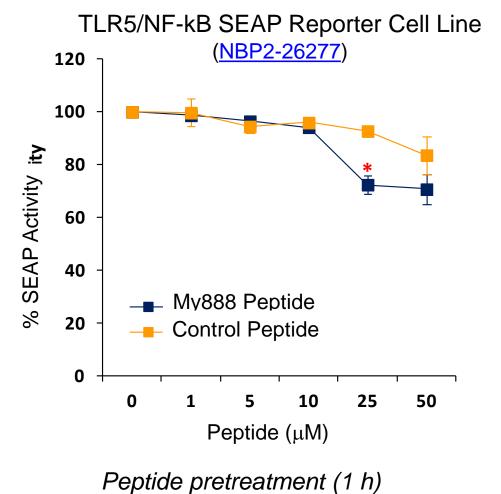
(NBP2-26277)





MyD88 Inhibition of TLR Activation



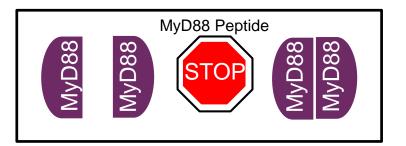


Flagellin stimulation (24 h, 1 ng/ml)

(NBP2-29328)

(NBP2-25289)

MyD88 Inhibitory Peptide Citations



MyD88 Inhibitory Peptide Set (NBP2-29323)

- 1. DRQIKIWFQNRRMKWKKRDVLPGT
- 2. DRQIKIWFQNRRMKWKK*
- *Cell permeable sequence

*Protein transduction (PTD) sequence from Drosophila antennapedia. PTD technology widely used, first described in 1994.

Highly published: in vitro & in vivo*

Essential consideration: Readout system

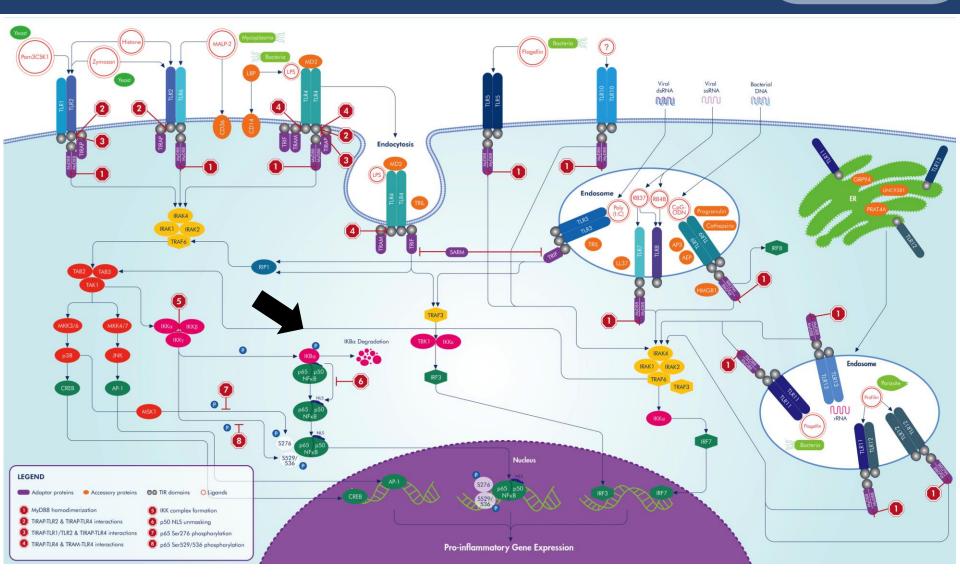
Various readout assays

- SEAP reporter
- CAT reporter
- Phosphorylation levels
- Nitrate: nitric oxide levels
- RT-PCR of a target
- WB of a target
- Cytokine ELISA

*In vivo mouse models: various injection schemes



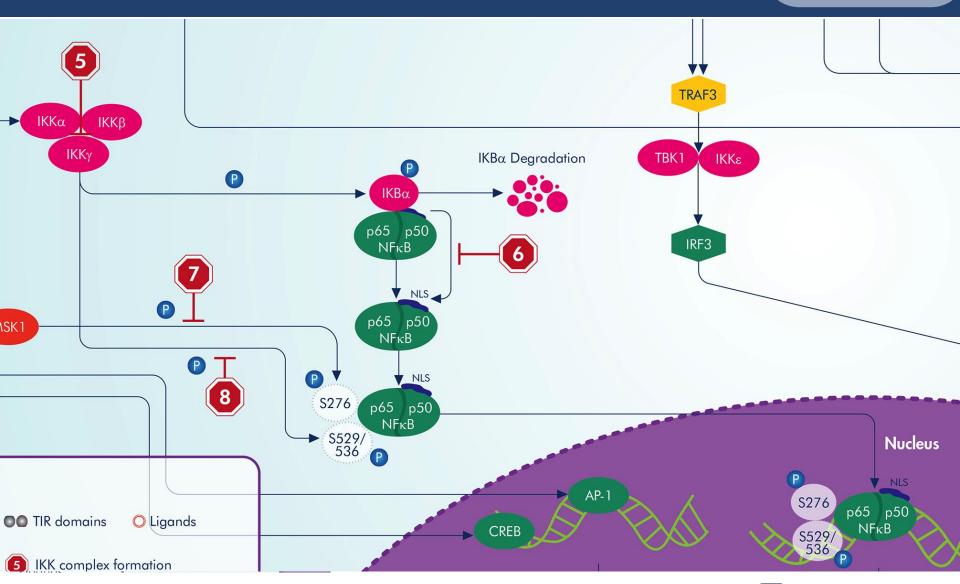
Inhibiting TLR Downstream: NF-kB



TLR signals propagating through NF-kB activation

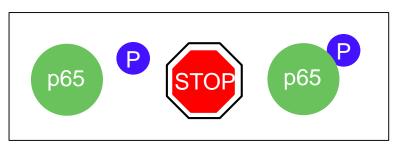


NF-KB Signaling Peptide Inhibitors



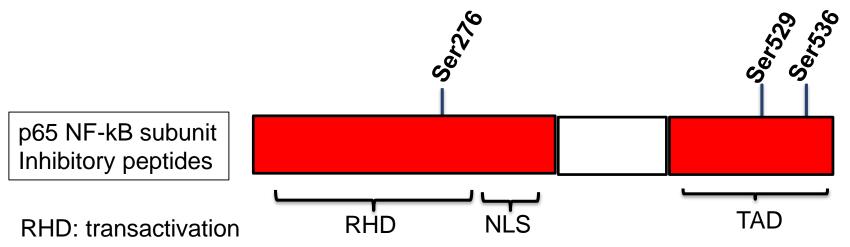


p65 pSer Peptide Inhibitor Mechanisms



Ser276 (<u>NBP2-26505</u>) & Ser529/536 (<u>NBP2-29321</u>) p65 inhibitors:

Decoy phosphorylation sites block endogenous NF-kB activation



NLS: nuclear localization

RHD: DNA binding and dimerization

p65Ser276: DRQIKIWFQNRRMKWKKQLRRPSDRELSE

p65 Ser529/536: DRQIKIWFQNRRMKWKKNGLLSGDEDFSS

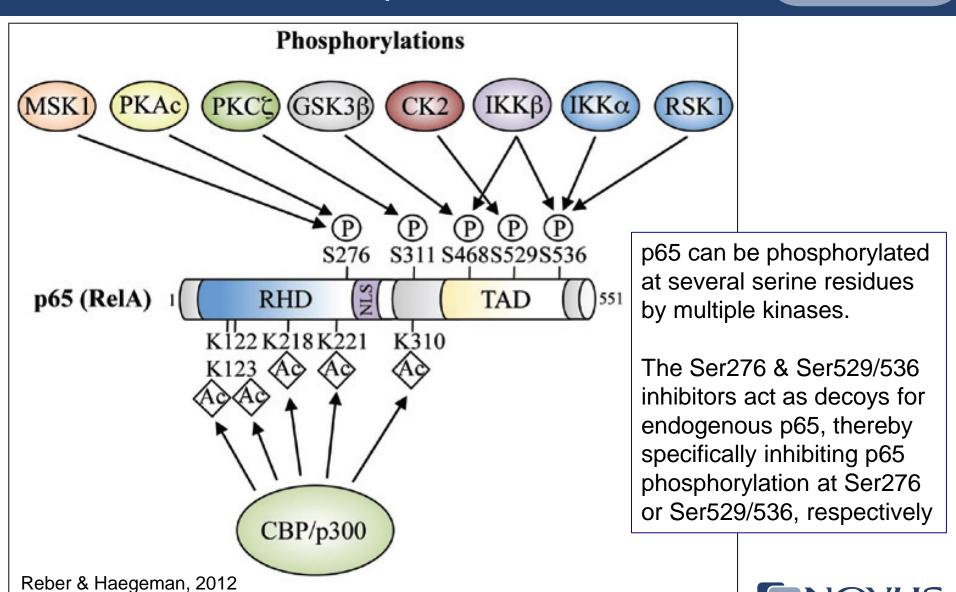
Control peptide: DRQIKIWFQNRRMKWKK



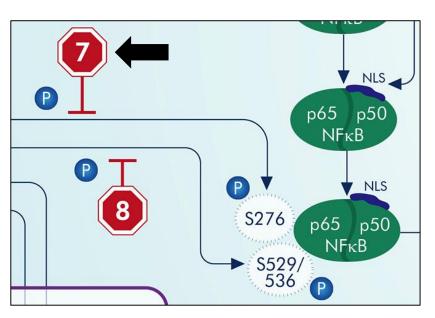
Posttranslational p65 Modifications

Acetylations

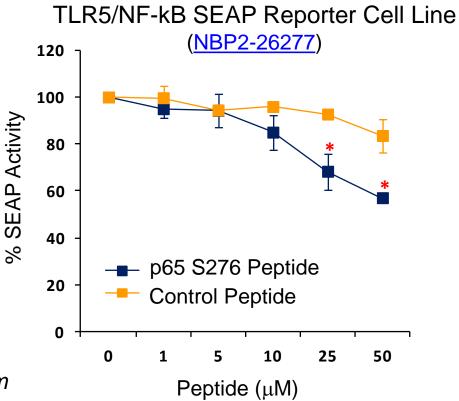
Landes Bioscience



p65 Ser276 P Inhibition of NF-kB



p65 S276 phosphorylation of p65 is an NF-kB activation step. Active NF-kB translocates from the cytoplasm to the nucleus



Peptide pretreatment (1 h) (NBP2-26505)

Flagellin stimulation (24 h, 1 ng/ml)

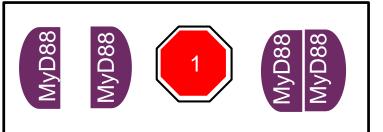
(NBP2-25289)

TLR & NF-kB Peptide Inhibitors

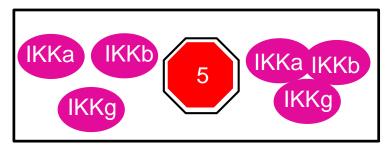


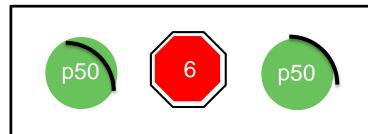
- MyD88 homodimerization
- TIRAP-TLR2 & TIRAP-TLR4 interactions
- 3 TIRAP-TLR1/TLR2 & TIRAP-TLR4 interactions
- TIRAP-TLR4 & TRAM-TLR4 interactions

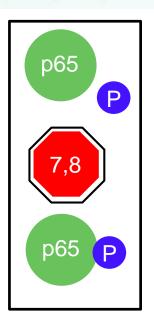
- 5 IKK complex formation
- 6 p50 NLS unmasking
- 7 p65 Ser276 phosphorylation
- 8 p65 Ser529/536 phosphorylation







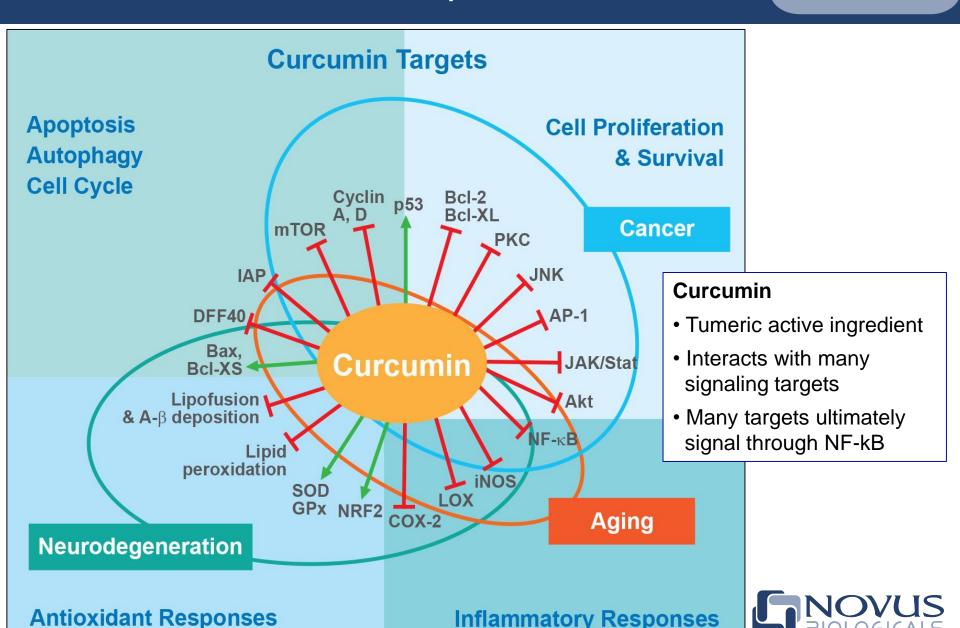




Inhibit at specific, critical nodes in signaling pathways

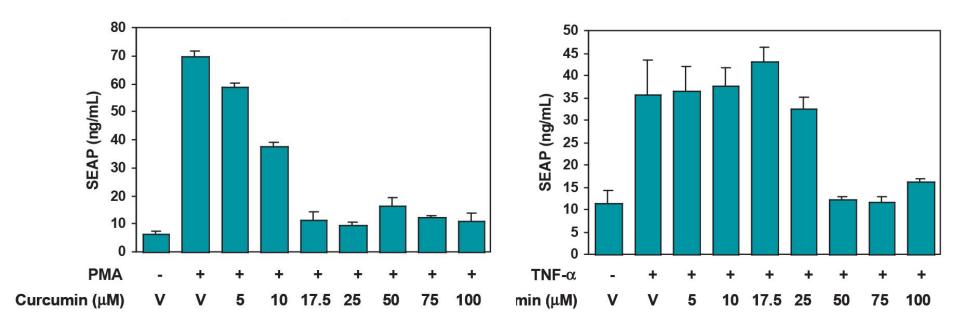


Curcumin: Pleotrophic Inhibitor



Curcumin Inhibition of NF-kB Signaling

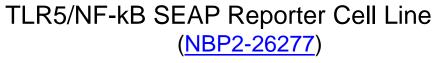
NF-kB SEAP Reporter Cell Line (NBP2-26260)

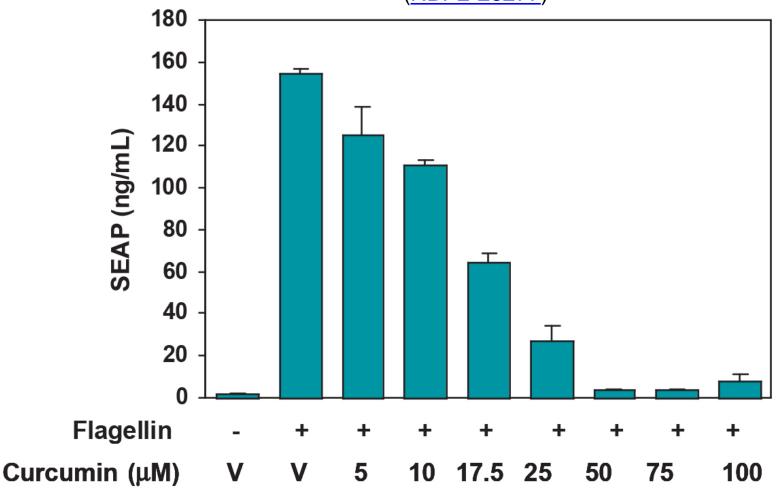


Curcumin pretreatment (2 h) (NBP2-26243)
PMA or TNF stimulation (24 h, 10 ng/ml)



Curcumin Inhibition of TLR5/NF-kB





Curcumin pretreatment (2 h), Flagellin stimulation (24 h, 10 ng/ml) (NBP2-26243) (NBP2-25289)



TLR Signaling II

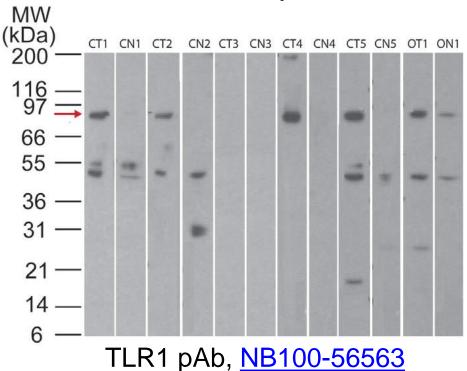
- TLR signaling overview
- II. Activation & inhibition assays
 - Ligands, inhibitors, responses
 - Specific and pleotropic inhibitors
 - Inhibit signaling nodes between TLR and adaptor proteins or downstream targets
 - TLR/NF-kB SEAP Reporter cell lines
- III. Emerging awareness of TLR posttranslational modifications



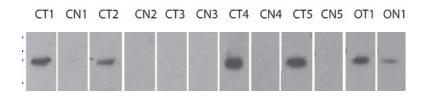
III. TLR Modifications & WB Patterns

Myth of the Single Band Western Blot





Often publish in this format



Thereby perpetuating the myth..... and Potentially overlooking important information

Tumor versus Normal Adjacent Colon & Ovary Tissue (7 donors)



TLR Posttranslational Modifications Era

Rethinking the single WB band dogma: Elucidating new TLR mechanisms

Toll-like receptor (TLR4) shedding and depletion: acute proximal tubular cell responses to hypoxic and toxic injury. Zagar et al *Am J Physiol Renal Physiol* 292:F304-F312 (2006). **TLR4 pAb** (NB100-56580)

WB (mouse renal cortex and urinary samples)

TLR4 was observed at the following molecular weights:

- 1. Two ~90 kDa bands reflecting different degrees of TLR4 glycosylation
- 2. 30 kDa in post ischemic urine samples, reflecting a presumptive **TLR4 cleavage** product
- 3. 60 kDa and 30 kDa bands, reflecting presumptive **TLR4 cleavage** products in urine samples at 18 hours post cisplantin treatment. At 48 hr only the 30 kDa band was present

Scouring the TLR WB Literature

Nucleic acids exert a sequence-independent cooperative effect on sequence-dependent activation of Toll-like receptor 9. Kindrachuk et al. *JBC* 282: 13944-13953 (2007). **TLR9 mAb** (NBP2-24729)

- 1. WB: TLR9 stably transfected bovine kidney epithelial cells, these **cells secrete recombinant TLR9 (ligand binding domain)** into the media. The data shows the **molecular weight of TLR9 was reduced following deglycosylation.**
- 2. EMSA: **TLR9 formed monomeric and dimeric nucleoprotein complexes** with the plasmid CMV. Protein component of shifted band was excised from the gel then subjected to WB.



Many Interesting TLR WB Citations

Short DNA sequences and bacterial DNA induce esophageal, gastric, and colorectal cancer cell invasion. Kauppila et al. Acta Pathol Microbiol Immunol Scand. DOI 10.1111/apm.12016 (2012). **TLR9 mAb** (NBP2-24729)

1. WB and ICC (MDA-MG-231, OE33, AGS, CaCo-2 cells).

Full length TLR9 120 kDa and 72 kDa cleaved bands were observed in WB.



Banding Patterns & Underlying Biology

The role of UNC93B1 protein in surface localization of TLR3 receptor and in cell priming to nucleic acid agonists. Pohar et al. *JBC* 288: 442-454 (2013). **TLR3 mAb** (NBP2-24875):

- 1. WB: HUVEC cells, TLR3 was detected at ~130 kDa. IFN-Beta treatment upregulated TLR3 expression and induced TLR3 glycosylation (TLR3 form higher than 130 kDa).
- 2. WB: Transfected HEK293T. TLR3 was detected at ~130 kDa. When UNC93B1 was over-expressed with TLR3, TLR3 glycosylation was increased and a TLR3 form higher than 130 kDa was observed. UNC93B1 expression also led to upregulation of TLR3 cell surface expression. PNGase treatment resulted in a TLR3 form with a molecular weight less than 100 kDa.



WB Correlations with Other Applications

Toll-like receptor 3 and RIG-1-like receptor activation induces innate antiviral responses in mouse ovarian granulosa cells. Yan et al. *Mol Cell Endocrinol* 372:73-85 (2013). **TLR3 mAb** (NBP2-24875):

- 1. WB: (primary mouse granulosa cells, primary mouse peritoneal macrophages), F(WT and TLR3-/- granulosa cells). TLR3 was detected at ~108 kDa in both granulosa cells and macrophages. The specificity of the TLR3 mAb was validated in the WT and TLR3-/- mouse model system by WB whereby TLR3 was recognized in granulosa cells from the WT but not the TLR3-/- mice.
- 2. **IF** (mouse granulosa cells) and **IHC-P** (mouse ovary)

Recall: Pohar et al. *JBC* 288: 442-454 (2013). **TLR3 mAb** (NBP2-24875):

WB: HUVEC cells, TLR3 was detected at ~130 kDa.



TLR Signaling III

- TLR signaling overview
- II. Activation & Inhibition assays
- III. Emerging awareness of TLR posttranslational modifications
 - WB patterns may vary
 - Cleavage
 - Glycosylation
 - Varying molecular weights
 - Highly validated & highly published
 - Consider published entire banding pattern
 - Identify more citations on Scholar.google.com

TLR Signaling



- I. TLR signaling overview
- II. Activation & Inhibition assays
- III. Emerging awareness of TLR posttranslational modifications



Thank You for Attending!



Better tools. Better research!

Please contact Dr. Lisa Heiden for more information lisa.heiden@novusbio.com