Better tools. Better research!

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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

I. TLR signaling overview
II. Activation & inhibition assays
III. Emerging awareness of TLR posttranslational modifications
TLR activity occurs through signaling pathways
TLR Signaling Roles

- Innate immunity
- Bridge to adaptive immunity
- Diseases
  - Autoimmunity
  - Cancer
First line of defense: Cells recognize and respond to pathogens through TLR receptors, leading to the production of factors that kill pathogens.

Psoriasisin/S100A7/HID5 Actions

- Chemokine release & inflammation
- Microbicidal activity
- Wound healing
- Calcium signaling

*Autoimmune disease
Clinical Tissue Lysate Model System

Study expression of proteins in tumors and normal adjacent tissue

Breast ductal carcinoma is a model system for studying psoriasin regulation

*Autoimmune disease*
Psoriasin Upregulation & Disease

Cross-talk between inflammation and disease pathways

*Breast ductal carcinoma: an inflammatory disease?*

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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)

TLR3 mAb, NBP2-24875

TLR1 pAb, NB100-56563
TLRs in Cancer

Tumor cells and the microenvironment

Gastric carcinoma, 4 patients: TLR4 (NB100-56566) 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
TLRs Bridge Innate & Adaptive Immunity

Example:

TLR signals activate T cells
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

- Ligands
  - TLR activation
    - Adaptors
    - Cofactors
  - Inhibitors

- TLR
  - Adaptors
  - Cofactors
  - Activated TLR signaling complex

- Downstream signaling
  - Immune responses
TLR Signaling Tools

Model System

Ligands: LPS (NBP2-25295)

Inhibitors: VIPER/TLR4 (NBP2-26244)

Reporter Cell Lines: TLR4/MD2/CD14 NF-kB/SEAP (NBP2-26503)
Signaling Disruption Mechanism: VIPER

**Viral inhibitor peptide of TLR4**
A46 vaccinia sequence

VIPER: \textcolor{red}{KYSFKLILAEY}RRRRRRRRRRR
Control: \textcolor{red}{RNTISGNIYSARRRRRRRRRR}

\textcolor{red}{R} = cell permeable sequence

VIPER \textbf{(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
(NBP2-26503)

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
TLRs except TLR3 signal through MyD88
Flagellin Activation Model System

TLR5/NF-κB SEAP Reporter Cell Line
(NBP2-26277)

Flagellin (ng/ml), 24 hr

SEAP (ng/ml)

Flagellin (ng/ml), 24 hr

Percent Activation

Flagellin EC50: 0.29 ng/ml

IC50 = inhibitor concentration where response is reduced by half
MyD88 Inhibition of TLR Activation

TLR5 signals through MyD88

Peptide pretreatment (1 h)  
(NBP2-29328)  
Flagellin stimulation (24 h, 1 ng/ml)  
(NBP2-25289)

TLR5/NF-κB SEAP Reporter Cell Line  
(NBP2-26277)
MyD88 Inhibitory Peptide Citations

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

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.

*In vivo mouse models: various injection schemes
Inhibiting TLR Downstream: NF-κB

TLR signals propagating through NF-κB activation
NF-KB Signaling Peptide Inhibitors
p65 pSer Peptide Inhibitor Mechanisms

Ser276 (NBP2-26505) & Ser529/536 (NBP2-29321) p65 inhibitors: Decoy phosphorylation sites block endogenous NF-κB activation

p65 NF-kB subunit
Inhibitory peptides

RHD: transactivation
NLS: nuclear localization
RHD: DNA binding and dimerization

p65 Ser276: DRQIKIWFQNRRMKWKKQLRRPSDRELSE
p65 Ser529/536: DRQIKIWFQNRRMKWKKNGLLSGDEDFSS
Control peptide: DRQIKIWFQNRRMKWKK
p65 can be phosphorylated at several serine residues by multiple kinases.

The Ser276 & Ser529/536 inhibitors act as decoys for endogenous p65, thereby specifically inhibiting p65 phosphorylation at Ser276 or Ser529/536, respectively.

Reber & Haegeman, 2012
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-κB Peptide Inhibitors

1. MyD88 homodimerization
2. TIRAP-TLR2 & TIRAP-TLR4 interactions
3. TIRAP-TLR1/TLR2 & TIRAP-TLR4 interactions
4. 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: Pleiotrophic Inhibitor

- Turmeric active ingredient
- Interacts with many signaling targets
- Many targets ultimately signal through NF-κB
Curcumin Inhibition of NF-κB Signaling

NF-κB 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-κB

TLR5/NF-κB SEAP Reporter Cell Line
(NBP2-26277)

Curcumin pretreatment (2 h), Flagellin stimulation (24 h, 10 ng/ml)
(NBP2-26243)
(NBP2-25289)
I. TLR signaling overview

II. Activation & inhibition assays
   - Ligands, inhibitors, responses
   - Specific and pleotrophic inhibitors
   - Inhibit signaling nodes between TLR and adaptor proteins or downstream targets
   - TLR/NF-κB SEAP Reporter cell lines

III. Emerging awareness of TLR posttranslational modifications
III. TLR Modifications & WB Patterns

Myth of the Single Band Western Blot

Researchers may observe this

Often publish in this format

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

Tumor versus Normal Adjacent Colon & Ovary Tissue (7 donors)
Rethinking the single WB band dogma: Elucidating new TLR mechanisms


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 cisplatin treatment. At 48 hr only the 30 kDa band was present

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.

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.
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.**

**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):**

1. **WB:** HUVEC cells, TLR3 was detected at ~130 kDa.
TLR Signaling III

I. 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
I. TLR signaling overview
II. Activation & Inhibition assays
III. Emerging awareness of TLR posttranslational modifications
Thank You for Attending!

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