

DEVELOPMENT AND STANDARDIZATION OF A FUNCTIONAL TOLL LIKE RECEPTOR PATHWAY TEST

M L Altrich PhD, C T Thompson, S L Stoops, and J F Halsey PhD
IBT Laboratories, Lenexa, KS 66219

Abstract

Toll-like Receptors (TLRs) make up part of the innate immune system. They recognize pathogen-associated molecular patterns (PAMPs) found on viral and bacterial pathogens. The binding of TLR-ligands initiates a signal transduction cascade that results in the production of cytokines including TNF- α , and IL-6. Recently human diseases, both infectious and autoimmune, have been linked to inappropriate TLR function. Therefore, a clinical assay evaluating an individual's functional TLR status would be extremely useful. Diluted or whole blood was incubated at 37°C for 24 hours with various concentrations of TLR ligands: Lipoteichoic acid (TLR2), Poly I:C (TLR3), LPS (TLR4), Flagellin (TLR5), Zymosan (TLR2/6), Loxoribine (TLR7), ssRNA (TLR8), and bacterial DNA (TLR9). Following the incubation, the plasma was removed and the concentrations of TNF- α and IL-6 were measured with a multiplex assay. Whole blood stimulated immediately after draw stimulated the highest level of cytokine production and the stimulation with LPS and bacterial DNA were the most time sensitive. The optimal concentration of TLR ligand and blood was optimized on a small number of donors. These conditions were used to determine 95% reference ranges in healthy controls. Due to the requirements for testing shipped specimens the validation and the reference ranges were determined on blood challenged 24 hours following blood draw. The mean level and reference ranges for TNF- α and IL-6 varied for each TLR and is given in Table 1.

Table 1. Reference ranges for TLR function assay.

Reference Ranges	LTA TLR 2	Poly I:C TLR 3	LPS TLR 4	Flagellin TLR 5	Zymosan TLR 2/6	Loxoribine TLR 7	ssRNA TLR 8	Bacterial DNA TLR 9
IL-6								
mean ng/mL	23.59	0.32	14.67	17.17	23.91	5.45	10.99	4.80
range ng/mL	13.38-36	0.011-2.48	7.81-26.09	9.21-36	10.53-36	1.42-10.78	3.3-17.9	0.68-17.58
TNF- α								
mean ng/mL	1.37	0.0082	0.37	0.87	1.85	0.030	5.60	0.20
range ng/mL	0.28-3.09	0.0002-0.029	0.02-0.93	0.094-3.11	0.109-6.09	0.004-0.066	3.03-8.88	0.03-0.55

The role of TLR signaling in multiple disease states is becoming increasingly clear. A functional TLR assay may be useful in evaluating susceptibility to both infectious and autoimmune diseases.

Introduction

- Toll like Receptors (TLR) are part of the innate immune system
- Recognize pathogen-associated molecular patterns (PAMPs)
- Binding of TLR-ligands initiates a signal transduction cascade that results in the production of cytokines including TNF- α , IL-6
- Goal: Establish methodology to determine if an individual has a functional TLR system**

References

- Beutler, B., Inferences, Questions and Possibilities in Toll-like Receptor Signaling. *Nature* 2004; 430: 257-263.
- Deering, et al., Development of a Clinical Assay to Evaluate Toll-like Receptor Function. *Clinical and Vaccine Immunology* 2006; 13 (1): 68-76.

For more information contact:
Michelle Altrich: michellea@ibtreflab.com
(913) 492-2224

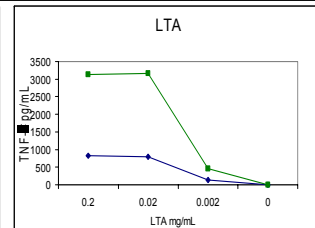
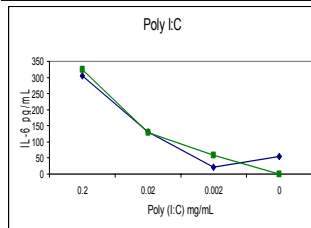
Materials and Methods

- Control Population**
 - Healthy Controls** - (n=16) Whole blood was collected into sodium heparin vacutainers
- TLR Ligands**
 - All TLR ligands were purchased from InvivoGen
- TLR stimulation**
 - TLR ligand concentration**
 - Whole blood (2 donors) was incubated with 3 different concentrations of TLR ligands (over a 3 log scale) and the plasma was evaluated for IL-6 and TNF- α production.
 - Blood concentration**
 - Whole blood or blood diluted 1:2 or 1:4 (two donors) was incubated with the optimal concentration of TLR ligands and the plasma was evaluated for IL-6 and TNF- α production.
 - Time to Setup**
 - Whole blood (2 donors) was incubated with the optimal concentration of TLR ligands and the plasma was evaluated for TNF- α production.
 - Samples were set up immediately after blood draw and 4, 8, 24 and 48 hours after blood draw.
 - Determining Reference Ranges**
 - Whole blood was incubated with the optimal concentration of TLR ligands and the plasma was evaluated for IL-6 and TNF- α production (n=16).
- Cytokine measurement**
 - Levels of cytokines were measured using R&D Systems multiplex kits for IL-6 and TNF- α

TLR Ligand concentration

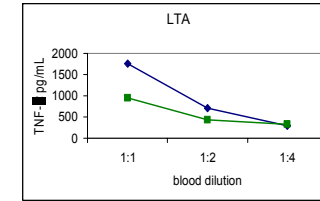
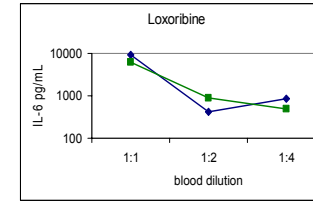
Three different concentrations of TLR ligands were tested. The optimal concentration is shown in **bold**. Representative data from these experiments is shown for Poly I:C and LTA.

TLR Ligand (mg/mL)	High	Med	Low
LTA	0.2	0.02	0.002
Poly I:C	0.2	0.02	0.002
LPS	0.1	0.01	0.001
Flagellin	0.01	0.001	0.0001
Zymosan	0.2	0.02	0.002
Loxoribine	1.0	0.1	0.01
ssRNA	0.01	0.001	0.0001
Bacterial DNA	0.1	0.01	0.001



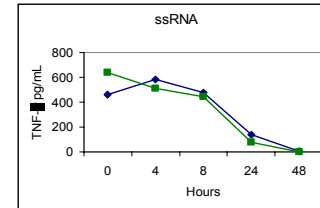
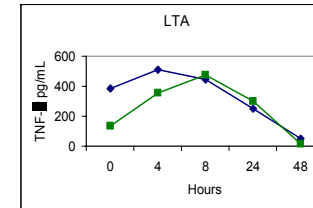
Blood Concentration

Three different concentrations of blood were tested (undiluted, 1:2 and 1:4 diluted). The optimal concentration was determined to be undiluted. Representative data from these experiments is shown for loxoribine and LTA.



Time to Setup

Samples were setup immediately after draw and 4, 8, 24 and 48 hours after blood draw. The optimal setup time was between 0 and 8 hours. Representative data from these experiments are shown for LTA and ssRNA.



Reference Ranges

Due to the requirements for testing shipped specimens the validation and reference ranges were determined on blood challenged 24 hours after draw. Sixteen normal donors were used to determine reference ranges. Graphs with TNF- α data are shown. Complete reference ranges are given in Table 1.

