ANALYSIS OF THE REQUIREMENTS FOR HUMAN TOLL-LIKE RECEPTOR 3 DOMINANT NEGATIVITY AND SIGNAL TRANSDUCTION

A Senior Scholars Thesis

by

MATTHEW HICKEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Biochemistry

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Analysis of the Requirements for Human Toll-like Receptor 3 Dominant Negativity and Signal Transduction (April 2009)

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Toll-like receptors are an important part of the innate immune system and mediate infection via the recognition of pathogen-associated molecular patterns (PAMPs). Toll-like receptor 3 (TLR3) recognizes foreign-derived double stranded RNA as its ligand, and is active as a homodimer. Previous research has indicated that specific residues in TLR3's extracellular domain (ECD) are responsible for dimer-dimer interactions between TLR3s, and the apparent specificity of this interaction has allowed for modulation of TLR3 signal through the use of dominant negative mutants. Here we present a class of mutants which lack the inter-disulfide cap region of the ECD (Δ123-635, hereafter called TLR3N-CT), yet still exhibit dominant negative properties. The degree of dominant negative inhibition of TLR3N-CT is comparable to that of TLR3 ΔToll interleukin-1 receptor (TIR), the previously established standard for dominant negativity. Tyrosine mutants, such as Y759F, have been shown to dramatically reduce TLR3 signal induction by interfering with cytoplasmic signaling adapters. Our mutant, TLR3N-CT Y759F, retained the ability to act as a dominant negative inhibitor of TLR3,

thus indicating that the observed reduction in induced/uninduced signal was not due to ligand-independent activation of the mutant. Furthermore, the mutant TLR3N-CTΔTIR was generated to investigate the role of the cytoplasmic TIR domain in dimer-dimer interactions. This mutant was not a dominant negative inhibitor of TLR3 activity, indicating a possible role of the TIR domain in the dominant negative interaction between TLR3N-CT and wild type TLR3. It is possible that this TIR-TIR interaction is either in the incorrect confirmation for signaling or, contrary to previous reports that ligand binding and dimerization are necessary only to bring the TIR domains together, that more than simple TIR-TIR interaction is required for TLR3 signaling. However, expression studies by western blot have been unable to prove expression by any of the mutants previously discussed. Several explanations are possible, but it is likely that expression levels are sufficient for cell-based activity assays but too low for western blot detection.

DEDICATION

I would like to dedicate this research to my parents, who have taught me so much about life, love, and finding happiness in all things.

ACKNOWLEDGMENTS

I would like to thank my research advisor, Dr. Cheng Kao, for his direction, support, and encouragement throughout this research. He is an excellent teacher and researcher and I have learned a great deal from his example. I would also like to thank the members of Dr. Kao's lab for their constant willingness to help and to answer my incessant questions. I would specifically like to thank Scott Hoose for his help with my expression studies and Ranjith Kumar for his constant willingness to offer suggestions and helpful discussion on my research. Finally, I would like to thank my family for their support and for giving me the opportunity to pursue this and other opportunities to quench my curiosity through inquiry in science.

NOMENCLATURE

ECD	Extracellular domain
HA	hemagglutinin
LLR	leucine rich repeat
TLR	Toll-like receptor
TLR3N	TLR3 with deletion of residues 123-590
TLR3N-CT	TLR3 with deletion of residues 123-635
PAMP	Pathogen associated molecular pattern

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CHAPTER I

INTRODUCTION: INNATE IMMUNITY AND TOLL-LIKE RECEPTORS

The human immune system is generally divided into two branches: innate and adaptive immunity. The distinction is made based on the specificity of the response and the time from the initial recognition of the pathogen to the immune response. Adaptive immunity consists of antigen-specific responses carried out by B and T lymphocytes, and while this response is highly pathogen-specific, it takes several weeks to react after the initial presence of a pathogen. Innate immunity, in the broadest sense, can encompass all barriers to infection, such as skin as a physical barrier and lysozyme as an antimicrobial barrier, however the focus generally rests on the more cell-based responses of innate immunity. These responses can take place within a few hours but are not pathogen-specific; the innate response is much more systemic and results in a number of unwanted side effects, such as inflammation (1).

The innate immune system is initiated by receptors that can recognize pathogen-associated molecular patterns (PAMPs) and will, in turn, induce signal transduction to lead to the production of defense molecules (2). The Toll-like receptors (TLRs) are

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This thesis follows the style of the *Journal of Biological Chemistry*.

among this family of receptors. TLR3, the subject of this research, recognizes pathogen derived double-stranded (ds) RNA, a product of the replication for many viruses (2).

TLR3 is a single pass transmembrane protein that contains a ligand binding extracellular domain (ECD), the transmembrane domain (TM), and the intracellular domain (ICD) which can recruit kinases required for signal transduction. The ECD consists of N-terminus and C-terminus disulfide bond-capped ends as well as the binding site for dsRNA (4, 5).

Previous research has revealed that ligand binding by TLR3 induces the oligomerization required for signaling (6). Ligand binding takes place between the N- and C-terminal portions of the TLR3 ECD and includes residue E543. However, oligomerization should require the interaction of two or more TM domains (7, 8). Following ligand binding and dimerization, activated TLR3 recruits signal adaptor molecules and leads to the activation of the transcription factor NF-κB and the production of type I interferon (IFN) and proinflammatory cytokines (2). TLR3 may also be involved in a number of human diseases that are not necessarily mediated by pathogens, such as macular degeneration (10).

The ability to regulate TLR3 and the ensuing inflammatory response could be applied toward mitigating diseases. For example, suppressing TLR signaling could decrease the cytokines and chemokines that cause many symptoms. However, there is also need to

maintain some level of TLR3, as patients without functional TLR3 are subject to severe symptoms in association to gamma-herpes virus infection (3). A human single nucleotide polymorphism that decreases signaling by TLR3 has been linked to decreased incidence of the dry form of macular degeneration (10). The various roles of TLR3 in viral infection and human disease outcomes point toward a clear usefulness in modulating its activity.

My research is to understand how TLR3 recognizes ligand and activates signal transduction. The original hypothesis was that elimination of the ECD dimerization domain should result in a molecule containing the TM and ICD that could still interact with wild type TLR3. This interaction would result in one of two outcomes. One is that this ECD mutant will act in a dominant negative manner when it is complexed with the wild-type TLR3. A second possibility is that this interaction will result in a constitutively active (ligand independent) complex due to the loss of the normal ligand-dependent regulatory mechanisms. This research explores this interaction between wild type TLR3 and mutations of TLR3 that contain in frame truncations of the ECD.

CHAPTER II

EXPERIMENTAL PROCEDURES

Plasmid Construction

Stocks of wild type human TLR3 (GenBank accession number U88879) were obtained from Ranjith-Kumar. This TLR3 cDNA was cloned into the pcDNA3.1 vector as described previously (11). Using this construct, several TLR3 mutants were constructed: TLR3N and TLR3N-CT which include large deletions, and various point mutations of the wild type and TLR3N/TLR3N-CT constructs. In addition, hemaglutinin epitope-tagged versions of all previous constructs were made to allow detection of expression through western blotting.

Preparation of TLR3N and TLR3N-CT mutants

With the goal of eliminating the entire region between the N- and C-terminus disulfide "caps" of the extracellular region of TLR3, oligonucleotides were designed to introduce restriction enzyme recognition sites on either side of this region. Oligonucleotides 1 and 2 were first used to amplify the N-terminus region of the TLR3 ECD (Table 1). This amplified region included a NheI restriction site on the 5' end, while oligonucleotide 2 was designed to add an additional EcoRI and ClaI restriction cut site to the 3' end. The PCR product was generated by use of the *Pfu* polymerase, and then inserted into a pGEMT-easy vector (Promega Inc.). The pGEMT vector approach was chosen due to

difficulty encountered in trying to directly digest and ligate the short fragment back into the TLR3 vector. Following amplification and plasmid purification of the pGEMT constructs, they were digested with the restriction enzymes NheI and ClaI (New England Biosystems). The digested DNAs were then loaded on a 1.5% agarose gel to separate the amplified region from the larger vector. Correct pGEM-T insertions yielded two bands running at 400 bp and 3 kb, while correct pcDNA-TLR3 digestions yielded two bands running at 1.8 kb and 6.5 kb. The 400 bp pGEM-T fragment and the 6.5 kb pcDNA-TLR3 fragment were each then purified using a gel extraction kit (Qiagen), and ligated using T4 DNA ligase, resulting in the completed TLR3N construct. DNA sequencing was employed to confirm the correct construction of this mutant.

The TLR3N mutant removed the majority of the TLR3 ECD, but left the N- and C-terminal caps and leucine rich repeats (LLRs) 21 to 23 (4). To remove all of the uncapped LRRs, oligonucleotides 3 and 4 were designed to insert an EcoRI site at the 5' end of the C-terminus ECD disulfide cap and to amplify through the naturally occurring XhoI site. The resulting PCR product and the TLR3N construct were both digested with EcoRI and XhoI and ligated together to form the TLR3N-CT construct. This was sequenced to ensure that no unintended mutations were made.

Table 1. Primers used for plasmid construction

Primer Name	Primer Sequence	Purpose
1	5'- GGCTAGCAGTCATCCAACAGAATC -3'	To add ClaI and EcoRI resctriction enzyme sites to the 3' end of the ECT N-terminal cap of TLR3 for use in ligating this region with the C-terminal cap of the TLR3 ECD
2	5'- TCATCGATGAATTCGCAGAAGGCAAAGGTTTTATCAGAAAG -3'	
3	5'- AAAGAATTCAACCTGACTGAGTTAG ATATGCGCTTTAATCC -3'	To add an EcoRI restriction site to the 5' end of the C-terminal TLR3 ECD cap in order to ligate this with the EcoRI site inserted on the 3' end of the N-terminal cap
4	5'- GATGCTGTTAACAATTGCTTCTAGTTCAAAAACACC -3'	
dTIR 1	5'- GACAGAACAGTTTGAATAGGCAGCAT ATATAATTCATG -3'	To change the Tyr residue at position 756 in wild type TLR3 to a stop codon
dTIR 2	5'- CATGAATTATATATGCTGCCTATTCAAACTGTTCTGTC -3'	
Y759F 1	5'- CAGAACAGTTTGAATATGCAGCATTTA TAATTCATGCC -3'	To change the Tyr residue at position 759 in wild type TLR3 to a Phenylananine. This Tyr is an important phosphorylation site for TLR3 signaling
Y759F 2	5'- GGCATGAATTATAAATGCTGCATATTCAAACTGTTCTG -3'	
HA 1	5'- GAATCATGTACCCGTACGACGTCCCGGACTACGCCAGACAGAC TTTGCCTTGTATCTACTTTTGGG -3'	Adds the HA epitope, YPYDVPDYA to the N-terminal side of TLR3
HA 2	5'- CTGTCTGGCGTAGTCCGGGACGTCGTACGGGTACATGATTCTGT TGGATGACTGCTAGCCTTTCC -3'	
* non-complimentary additions are highlighted, added restriction sites are underlined		

Single nucleotide polymorphisms

Point mutations in the TIR signaling domain were generated in both wild type TLR3 and TLR3N-CT, resulting in the mutant forms of Y759F and a missense mutation, known as ΔTIR, which prevents the transcription of the TIR domain coding region of the gene. Oligonucleotides were designed to make the appropriate single base pair substitutions (Table 1) and *Pfu* polymerase was used for the PCR mutagenesis reaction. All mutants were sequenced across mutation sites to ensure that no unintended alterations were made. Resulting mutants were TLR3ΔTIR, TLR3 Y759F, TLR3NΔTIR, TLR3N Y759F, TLR3N-CTΔTIR, and TLR3N-CT Y759F.

HA-tagged mutants

In order to aid in the detection of these, at times severely altered, mutants, an HA tag (sequence YPYDVPDYA) was attached to the N-terminal side of each mutant. Oligonucleotides containing the insertion were generated and extended around each plasmid using *Pfu* polymerase (Table 1). Each mutant was sequenced across the HA insertion to ensure that no unintended alterations were made.

Cell-based reporter assay for TLR3 signaling

Methods for this assay were adapted from those reported by Sun, et al. (11). Briefly, HEK 293T cells were plated on a CoStar White plates at 1.5 $x10^5$ cells/mL with a total of 200 μL per well. Following incubation at 37°C in 5% CO_2 until the cells were 65-90% confluent, the cells were transfected with a mixture of Lipofectamine 2000 reagent (Invitrogen) and plasmids pNF-κβ-Luc (Stratagene Inc., La Jolla, CA) or ISRE-Luc, wild type or mutant TLR3, and phRL-TK (Promega Corp., Madison, WI). pNF-κβ-Luc and ISRE-Luc are both downstream transcription factors for the TLR3 signal transduction pathway and are attached to a firefly luciferase reporter. phRL-TK is a constitutively active gene attached to a *Renilla* luciferase reporter and is used for transfection control. Following transfection, the cells were incubated for 24 h at 37°C in 5% CO_2. At this time, the supernatant was discarded and the cells are induced with poly(I:C) at a concentration of 2.5 μg/mL. After 6 hours of induction, the cells were treated with Dual Glo Luciferase Assay System reagents (Promega). The resulting

luminescence, derived from plasmid expression, was quantified using the FLU-Ostar OPTIMA Plate Reader (BMG Labtech, Inc).

Western blotting

HEK 293T cells were transfected with Lipofectamine 2000 reagent (Invitrogen) and the plasmid of interest. Following an incubation of 24 hours, cells were lysed using lysis buffer (Promega Inc.) and sonicated to decrease the viscosity of the solution. Cell lysates were then combined with a protein loading buffer and loaded on a NuPAGE 4-12% bis-tris gel (Invitrogen). The iBlot Dry Blotting System (Invitrogen**)** was used to transfer protein samples to an iBlot-supplied polyvinylidene difluoride membrane. This membrane was probed with either polyclonal anti-TLR3 or monoclonal anti-HA antibody, followed by a peroxidase-conjugated secondary antibody. Blots were developed using the ECL Plus Western blotting detection system (Amersham Biosciences).

CHAPTER III

RESULTS AND DISCUSSION

Results and Discussion

Inter-cap ECD deletion exhibits dominant negative inhibition of wild type TLR3

As would be expected, TLR3N-CT was unable to induce signaling when expressed in cells. However, when co-expressed with the wild type TLR3, it was able to reduce signaling by the WT activity. A control for this reaction is the construct TLR3-ΔTIR, a known dominant negative mutant (6) [Fig. 1]. In fact, TLR3N-CT had comparable dominant negative activity to TLR3-ΔTIR. The assay for TLR3 signal activation uses a firefly luciferase reporter expressed from either an NF-kB or ISRE promoter that is known to be responsive to TLR3. *Renilla* luciferase expressed from a constitutive promoter was used as a transfection control and all firefly luciferase signal was normalized over *Renilla* signal. HEK293T cells were used because they do not contain any endogenous TLR3 (12). Activity is reported as a fold induction of poly I:C induced/uninduced normalized signal.

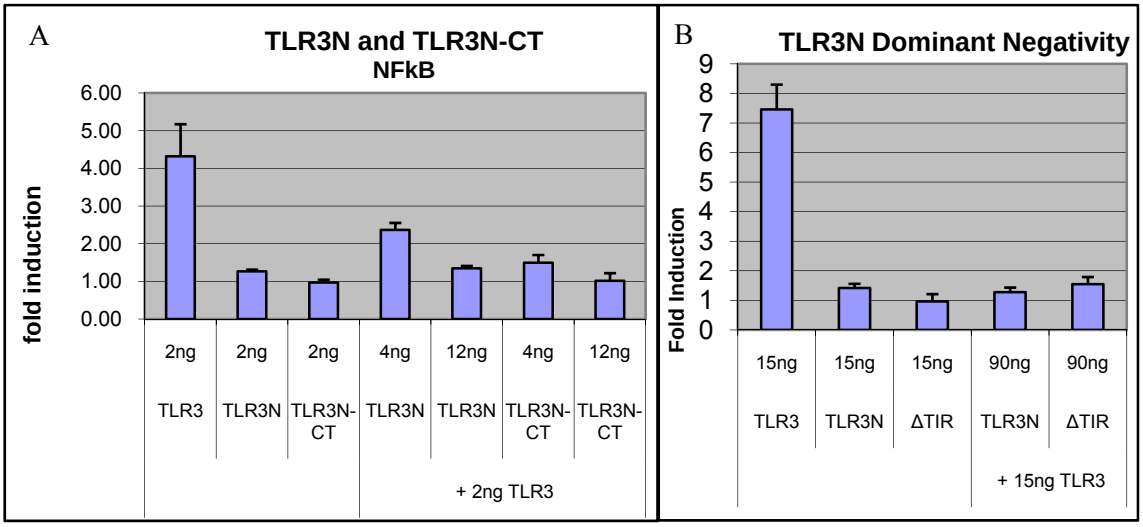

FIGURE 1. **TLR3N and TLR3N-CT act as dominant negatives to wt TLR3.** *(a)TLR3N-CT demonstrates dominant negativity similar to that of a previous mutant, TLR3N (deletion of residues 123-590). Results were obtained 6 hours after induction with poly I:C. (b) TLR3N demonstrates dominant negativity similar to that of TLR3ΔTIR. Results were obtained 24 hours after induction with poly I:C. All samples were normalized with pcDNA to ensure equal plasmid concentration amongst samples of the same trial. Different induction times are a result of a procedural change to shorter times in attempts to reduce background effects.*

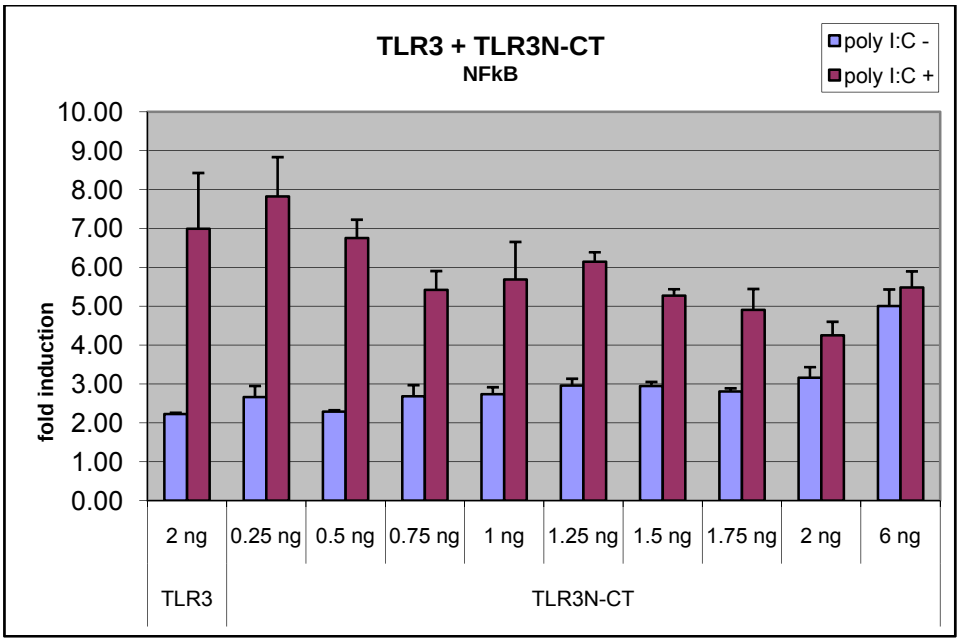

FIGURE 2. **Ligand independent activation of TLR3N-CT.** *Increase in ligand independent activation represents potential cause of decrease in fold induction for firefly/renilla luciferase ratio (fold induction) observed in Figure 1. All samples include 2ng wild-type TLR3. Results were obtained 6 hours after induction with poly I:C.*

In addition to the observed reduction in signal transduction when TLR3N-CT is cotransfected with WT TLR3, ligand independent activation is also observed [Fig. 2]. The decrease in ligand-induced signal and increase in ligand-independent activation points toward two potentially coincident explanations for TLR3N-CT activity.

TIR phosphorylation site mutant TLR3N-CT Y759F

It has been previously reported that the mutation Y759F in wild type TLR3 results in a sharp decline in TLR3 signaling (9). This is likely due to decreased ability to interact with signal adapter molecules. Here, the mutant TLR3N-CT Y759F was constructed to determine whether ligand-independent activation was responsible for the decrease in fold induction observed with the co-transfection of TLR3N-CT and WT TLR3. As shown in Figure 3, the removal of this phosphorylation site in the TLR3N-CT mutant had no effect on its dominant negative properties.

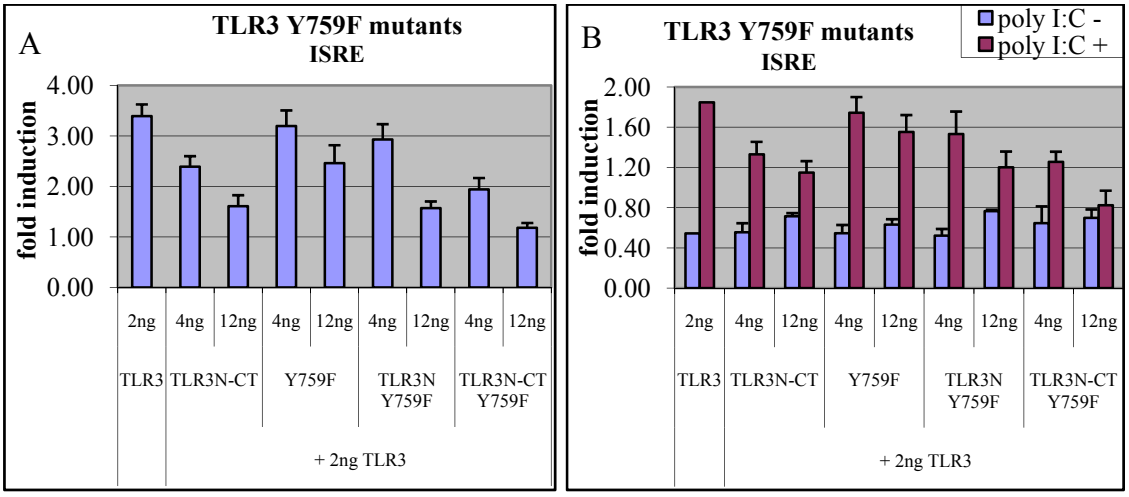

FIGURE 3. **TLR3N-CT Y759F dominant negativity and ligand-independent activity.** *Results were obtained 6 hours after induction with poly I:C. (A) TLR3N-CT Y759F exhibits similar dominant negativity to TLR3N-CT mutant. (B) While some ligand-independent activation is still evident, TLR3N-CT Y759F lacks the primary phosphorylation site for TIR activation, indicating that any ligand-independent activation present is due to causes aside from TLR3 manipulation.*

Because TLR3 signal induction is mediated through cytoplasmic tyrosine residues, it is thought that a Y759F mutant will silence any potential ligand-independent activation observed in the TLR3N-CT mutant. From Figure 3, it is apparent that TLR3N-CT Y759F retains dominant negative activity. This result indicates that the dominant negative properties of both TLR3N-CT and TLR3N-CT Y579F are not likely to be caused by ligand-independent activation.

TIR domain deletion – TLR3N-CT ΔTIR

Based on the observation that dominant negative interaction was occurring with the TLR3N-CT mutant despite the fact that the dimerization domain was missing, it became necessary to determine which domains were interacting. Figure 4 demonstrates that the removal of the TIR domain from the TLR3N-CT ECD mutant results in the loss of dominant negative properties. This observation leads to two possible conclusions. The first is that the TIR domain contains a secondary dimerization site which allows for dominant negative interaction when the sterically bulky ECD is not present. The loss of the putative dimer interaction explains the loss of dominant negativity reported in Figure 4. Alternatively, the loss of dominant negative activity could be due to an effect on expression.

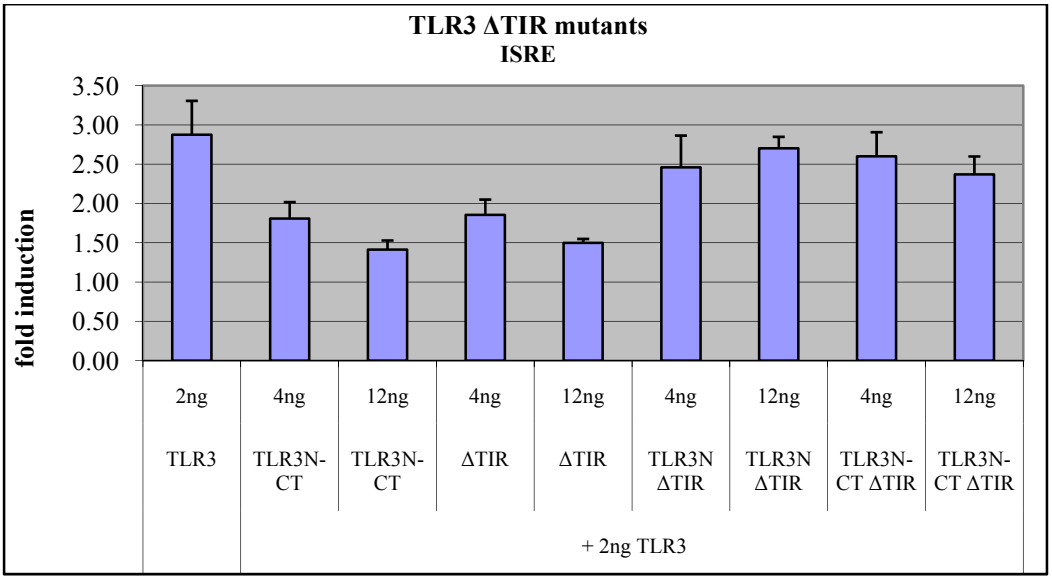

FIGURE 4. **Dominant negativity is lost in TLR3N-CT ΔTIR.** *The deletion of the TIR domain in TLR3N and TLR3N-CT results in a loss of dominant negative interaction with wt TLR3. Results were obtained 6 hours after induction with poly I:C.*

HA-tagging for expression studies

Due to the significant manipulation of wild-type TLR3 to obtain some of the mutants used in these experiments, an alternative to the use of a polyclonal αTLR3 antibody was necessary to ensure detection of protein expression by western blot. As a convenient solution to this problem, each mutant and wild-type protein described above was tagged with the hemagglutinin (HA) protein of sequence YPYDVPDYA. Figure 5 demonstrates the dominant negative properties of each of these HA-tagged mutants as compared with their untagged counterparts. The comparible dominant negativity of each of the HA mutants to their counterparts indicates that the HA tag had no significant disruptive effect on the activity and interactions of the mutants.

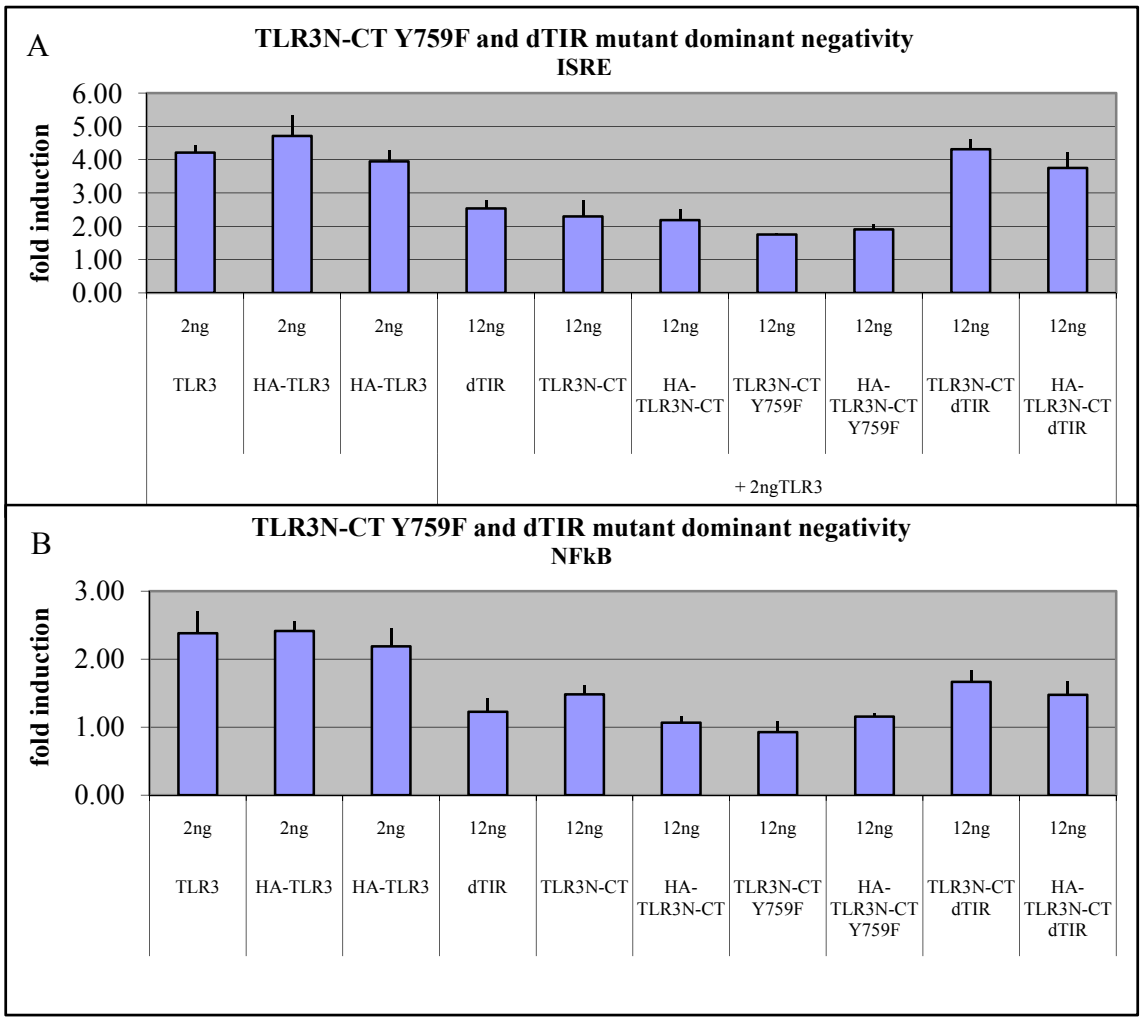

FIGURE 5. **Dominant negativity of HA-tagged mutants.** *Renillia/Luciferase assay comparing dominant negativity of HA-tagged mutants to their untagged counterparts. Reporter genes are ISRE (A) and NFκB (B). Results were obtained 6 hours after induction with poly I:C.*

TLR3 mutant activity

It was necessary to prove that none of the mutants used in these dominant negativity assays were themselves able to be activated. To examine this, each mutant was transfected individually into HEK293T cells and then induced according to the above protocol. Figure 6 reports the signal induction in response to poly I:C.

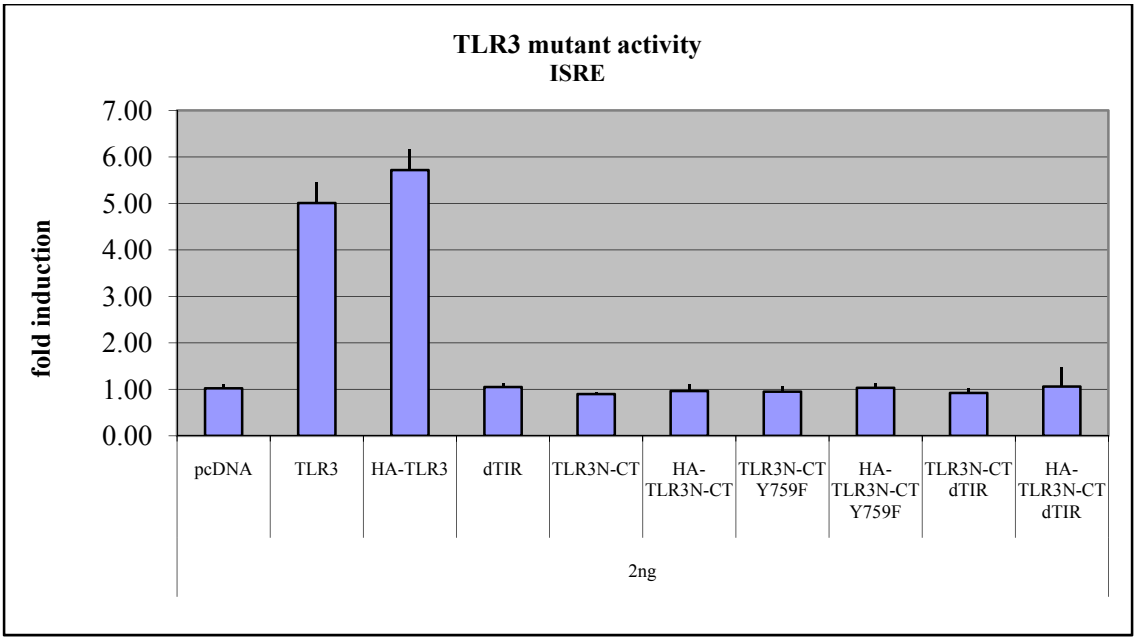

FIGURE 6. **Signal induction from various TLR3 mutants.** *Each mutant was individually transfected into HEK293T-cells, and following induction with poly I:C signal fold induction was measured. Results were obtained 6 hours after induction with poly I:C.*

Expression analysis by western blot

Western blots were conducted in order to establish the expression of the mutants studied in these experiments. In order to detect the wild type and HA-tagged ECD mutants, both a polyclonal αTLR3 antibody and an αHA antibody were used. After several trials with αTLR3, no expression could be found in any of the mutants or wild type strains (data not shown). Positive controls of pBeth HA-TLR3 and pcDNA TLR3 ECD were detected. The αHA was unable to detect expression for even the positive controls, despite several attempts and successful uses by other lab members. The lack of expression as reported by western blot leaves room for several possible explanations. It is, of course, possible that none of these mutants are being expressed and that all activity assay data is merely an artifact of some unknown alternative variable. However, this explanation is

unsatisfactory for several reasons. First, the ability of wild type TLR3 and reduced ability of the mutants to induce ISRE/NFκB lends credence to the argument that the TLR3 mutants used in these experiments are expressive. Secondly, since wild type TLR3 can ordinarily be detected by αTLR, the question shifts away from whether or not the particular genes are expressed to whether or not the western procedure is being conducted correctly and whether the plasmids are working correctly. Since the western was repeated several times with the same results, it is unlikely that procedural errors are to blame. Both of these observations do lead, however, to the possibility of a promoter mutation on all of the plasmids. The presence of such a mutation could explain a level of expression which is sufficient to detect ligand, but not sufficient to be detected by western blot.

CHAPTER IV

CONCLUSION

The ability to modulate TLR3 activity through dominant negative inhibition holds promise for better understanding and controlling of human inflammatory disease. This research specifically advances the study of TLR3 by identifying a potential new mutant which exhibits a new mechanism for dominant negativity. The problems of expression still need to be resolved in order to validate the promise of this new mutant class for modulation of TLR3 signaling.

Previous research has proposed that dominant negative interaction occurs at the dimerization site in the ECD (7). These conclusions were based on the observed loss of dominant negative inhibition in mutants containing a substitution in proposed dimerization residues, such as E442K (7), however this research provides evidence that this interaction may be more complex than originally thought. Since, the mutants studied here lack any contribution or opposition to dimerization at the originally proposed ECD sites, the role of the TIR and transmembrane domains in dimerization can be observed.

Beyond the unsolved expression issues, there are still several other questions left unanswered by this research. It remains unclear which portion of the TLR3N-CT mutant is contributing to its dominant negative interactions. If expression data can be obtained,

this data will help elucidate whether or not the lack of dominant negative inhibition by TLR3N-CTΔTIR is due to lack of protein expression or to the presence of a secondary dimerization domain in the TIR domain. If TLR3N-CT is expressed, further experimentation will then be necessary to determine whether these mutants are folding correctly and whether their dominant negative interactions are in the same orientation as wild type TLR3 dimer interactions. The orientation in which these mutants are interacting is of interest for a better understanding of both wild type TLR3 interactions and of domains which could be manipulated to modulate wild type signaling.

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