5-2023

Implications of Toll-Like Receptor 10 Mutants in Cell Signaling

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Spring 5-15-2023

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This Biology Honors Thesis

Implications of Toll-Like Receptor 10 Mutants in Cell Signaling

by

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is submitted in partial fulfillment of the requirements for the

Biology Honors Program

at the

University of San Francisco

Submission date: 05/10/2023

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Abstract

Our innate immune system serves the important purpose of quickly recognizing microbial pathogens and providing the first line of defense against infection. Toll-Like Receptors (TLRs) are one class of receptors responsible for enacting our bodies' innate immune responses. Specifically, TLR10 is an extremely under-researched receptor. Some naturally occurring mutations in TLR10 have been linked with a worsening prognosis of rheumatoid arthritis, with previous research uncovering a mutant form of TLR10 that impairs innate immune system-controlled inflammatory responses. However, other research has found that wild-type forms of TLR10 also contribute to pro-inflammatory pathways in healthy cells. To better understand the wild-type form and the naturally occurring mutants, we wish to explore the impacts on cell localization and signaling in different forms of TLR10 in healthy cells. This was done by generating the mutant plasmid, followed by transfection of different forms into HEK cells. Flow cytometry revealed that the mutant forms do not localize to the cell surface at the same level as the wild-type. Similarly, they do not activate the NFkB signaling pathway to the same extent as the wild-type. This helps us to understand how mutations in TLR10 can affect different cell types and further study will elucidate more information.

Introduction

The innate immune system is a crucial component of any immune response. It is the first line of defense, responding quickly to any and all threats to the organism. Pathogen recognition receptors (PRRs) which are present on cell surfaces and intracellular compartments and respond to components of pathogens known as pathogen-associated molecular patterns (PAMPs) are a part of the innate immune system and can be found in all cells. A specific type of PRR is toll-like receptors (TLRs). Humans possess ten toll-like receptors, numbered accordingly from 1-10. [1] Each TLR recognizes one or multiple PAMPs and activates different signaling pathways, which generally results in inflammation and the activation of the adaptive immune system.

Toll-like receptor 10 is one TLR that we know very little about. Mice are used as a model organism in the study of TLRs and TLR10 is not present in mice. Research centered around this TLR has increased in recent years, with more knowledge arising about it. Recently, a few bacterial ligands for TLR10 have been discovered, which include *L. monocytogenes*, *B. burgdorferi* which is the pathogen behind Lyme disease, as well as the lipopolysaccharides of *H. pylori.* This information is crucial to determining the functions of TLR10 as well as directing future research.

In researching TLR10, one approach is to focus on its single nucleotide polymorphisms (SNPs) to understand the differences between the mutant form and the wild-type form, to provide more information about how the wild-type form may function. One specific study focused on the SNP *rs11466657* and it was found that those with this SNP had a worsened disease prognosis for a number of diseases [3].

One of the diseases that this SNP is implicated in is rheumatoid arthritis [5]. Rheumatoid arthritis is an autoimmune inflammatory disease, causing chronic inflammation in joints [4]. The inflammatory response is where TLR10 is relevant. The researchers studying the mutant form of TLR10 examined its role in inflammatory pathways. These experiments found that the mutant TLR10 had significantly higher levels of components of the NFkB pathway, an inflammatory pathway. This suggested that wild-type TLR10 plays a role in inhibiting NF-kB and inflammation.

However, other research examining the role of TLR10 in intestinal epithelial cells indicates that wild-type TLR10 has a pro-inflammatory role, with increased NF-kB expression. [2] This supports the idea that TLR10 may have different functions depending on cell type and disease state of the individual it

is found in. In this specific study, wild-type TLR10 showed activation of the NFkB pathway in response to L. Monocytogenes in HEK293T cells, confirming this bacterium as a potential ligand and indicating the pro-inflammatory role. Other SNPs involved with TLR10 include *rs4129009*, which is I775V.

However, with the conflicting past research, to understand more about wild-type TLR10 and the effects that the mutant forms can have, studies can be performed examining factors such as cell signaling and more. I set out to generate a mutant plasmid containing rs11466657 that can be transfected into human cells. Following this, I aimed to compare cell localization and NFkB signaling differences between wild-type and mutant forms of TLR10. Based on the current information, I expect that mutant forms of TLR10 will have changed cell localization and decreased expression of the NFkB pathway.

Methods

Mutant Plasmid Generation - Gibson Assembly

The first step in studying TLR10 and its mutant forms required the generation of the mutant plasmid. To do this, I first performed a Gibson assembly consisting of three separate fragments. The first fragment was generated by a restriction digest followed by gel extraction and purification to generate a DNA fragment of ~6000bp. The wild-type plasmid, pcDNA3-TLR10-YFP, from AddGene, was cut with the restriction enzymes, BamH1 and Xho1. I performed gel electrophoresis on the DNA generated from the restriction digest, followed by gel extraction. The other two fragments were generated by performing polymerase chain reactions (PCRs). The first fragment was generated using the primers: 5' gcttggtaccgagctcggatcc 3' and 5' gcatttaattttctaactgatctccctggatgcagtcatttcagtagactttcagttctg 3'.
The second fragment was generated using the primers: 5' ctcgaggttctacaatctctctgat 3' and 5' catctgatggccttacgagaactaaatACTgcatttaattttctaactgatctccc 3'. After all three fragments were made, I performed Gibson Assembly using a master mix containing exonuclease, DNA polymerase, and DNA ligase. This DNA was then transformed into competent *E. coli* cells and plated onto plates containing LB and Ampicillin to screen for the plasmid. It was then mini-prepped for sequencing. Sequencing was performed through MCLabs. Ultimately, the successful generation of a mutant plasmid did not occur, leading to an alternative approach.

Mutant Plasmid Generation - Gene Fragment and Assembly

Since the Gibson Assembly method of generating the mutant plasmid did not work, I chose to design a gene fragment containing the mutation. This was ordered from Twist Bioscience, with the sequence:

5'-tatcttgaccacaattcatttgactactcaaatactgtaatgagaactataaaattggagcatgtacatttcagagtgttttacattcaacaggat
aaaatctatttgcttttgaccaaaatggacatagaaaacctgacaatatcaaatgcacaaatgccacacatgcttttcccgaattatcctacgaaa
ttccaatatttaaattttgccaataatatcttaacagacgagttgtttaaaagaactatccaactgcctcacttgaaaactctcattttgaatggcaat
aaactggagacactttctttagtaagttgctttgctaacaacacacccttggaacacttggatctgagtcaaaatctattacaacataaaaatgatg
aaaattgctcatggccagaaactgtggtcaatatgaatctgtcatacaataaattgtctgattctgtcttcaggtgcttgcccaaaagtattcaaat
acttgacctaaataataaccaaatccaaactgtacctaaagagactattcatctgatggccttacgagaactaaatACTgcatttaattttctaa
ctgatctccctggatgcagtcatttcagtagactttcagttctgaacattgaaatgaacttcattctcagcccatctctggattttgttcagagctgc
caggaagttaaaactctaaatgcgggaagaaatccattccggtgtacctgtgaattaaaaaatttcattcagcttgaaacatattcagaggtcat
gatggttggatggtcagattcatacacctgtgaataccctttaaacctaaggggaactaggttaaaagacgttcatctccacgaattatcttgcaa
cacagctctgttgatt-3'

I used a restriction digest with enzymes Bsu36I and BsrGI to get the second fragment. These were assembled using the NEB HiFi DNA Assembly Master Mix. After this, the DNA was transformed following the same steps as previously mentioned, and mini-prepped for sequencing as well.

Cell Culture

Two cell lines were used, HEK293T cells and HEK-Blue-hTLR2. These were maintained in DMEM supplemented with FBS, penicillin, streptomycin, and glutamine. The cells were kept in a 37C incubator at 5% CO2.

Transfection and Stimulation

Cells were transfected in 12 well plates with both TLR2 and TLR10, with a mass of 0.5ug for both plasmids, with a total of 1ug. After incubation for 30 hours, cells were stimulated with heat-killed *Listeria monocytogenes* at a low value (10^7 cells) and a high value (10^8 cells). A set of unstimulated cells were also included. This was performed with the HEK293T cells as well as the HEKBlue cells.

Flow Cytometry

Transfected cells were run through a flow cytometer (Cytek Northern Lights) to determine the percentage of cells that were expressing yellow fluorescent protein (YFP) on the cell surface. All TLR10 constructs have YFP at the N-terminal end so YFP+ is indicative of TLR10 surface expression.

HEKBlue Detection

The HEK-Blue-hTLR2 cells were seeded into 12 well plates at a density of 400,000 cells/mL. and transfected and stimulated according to the conditions above. After this, I plated the supernatant from each condition in triplicate into a 96-well plate, alongside a negative control. I placed the Quantiblue solution into each well and incubated it for 6 hours. After this, activity was measured by absorbance on a spectrophotometer at 630nm.

Results

Mutant Plasmid Generation - Gibson Assembly

The first attempt at plasmid generation, which was performed through Gibson Assembly was not successful. After multiple attempts at PCR and restriction digests, a Gibson Assembly was performed, and the DNA was transformed onto a selection plate. There was bacterial cell growth, leading to performing sequencing. Though sequencing results confirmed successful stitching of the whole plasmid, the mutation containing the base pair change was not inserted:

Mutant Plasmid Generation - Gene Fragment and Assembly

The second attempt at mutant plasmid generation was through the assembly of a gene fragment and another fragment made by restriction digest. Successful generation of an ampicillin-resistant plasmid was confirmed by transformation screening (Figure 1). The correct base pair change was confirmed by sequencing (Figure 2). Through these steps, I ultimately generated the correct mutant plasmid for *rs11466657*.

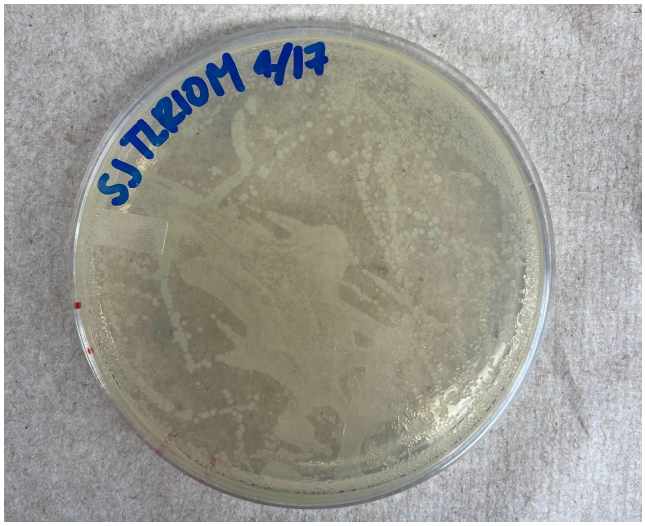

Figure 1. The results of a transformation for screening on an LB+Amp plate for the TLR10 plasmid.

Figure 2. Sequencing data demonstrating the mismatch between wild-type (bottom) and mutant (top) sequences

Flow Cytometry

To examine cell localization, flow cytometry was performed. Each plasmid was tagged with yellow fluorescence protein and one untransfected group of cells was used. Results showed that wild-type TLR10 had the highest expression of YFP at 66.52%, followed by the I775 mutant at 55.62%, and the I473T mutant at only 7.06%. (Figures 3 and 4).

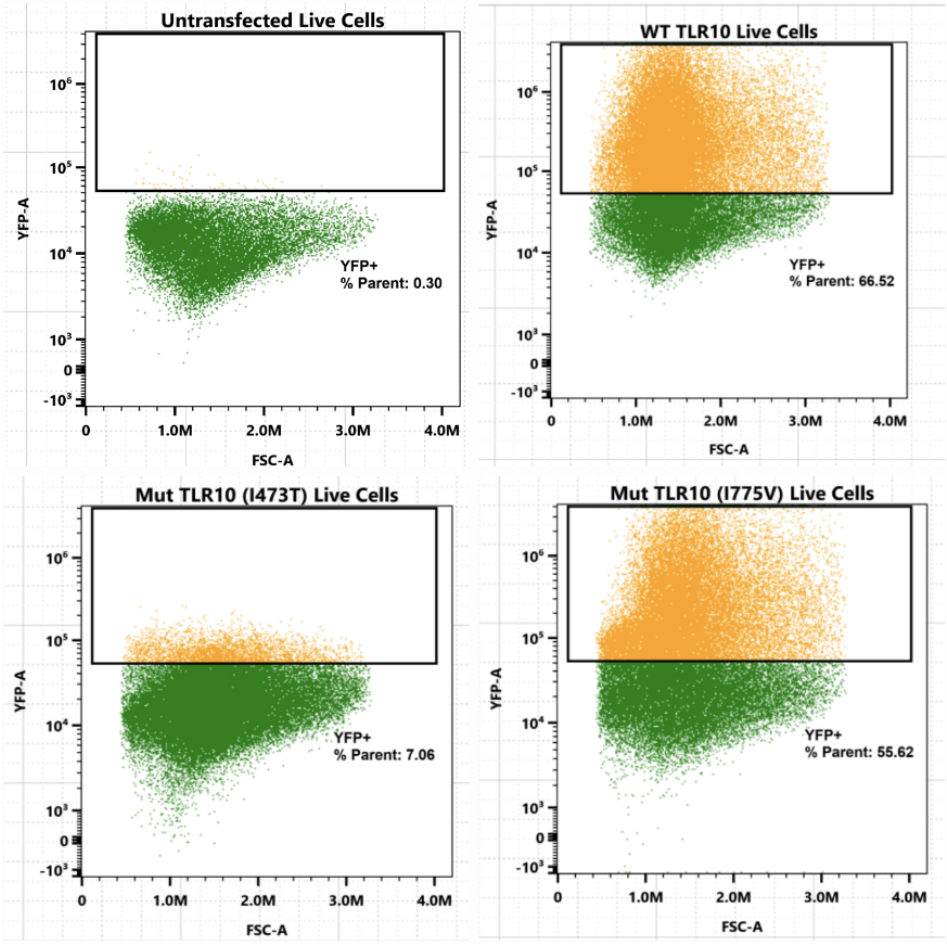

Figure 3. Results of the flow cytometry data, depicting the YFP expression in the different transfected live cells.

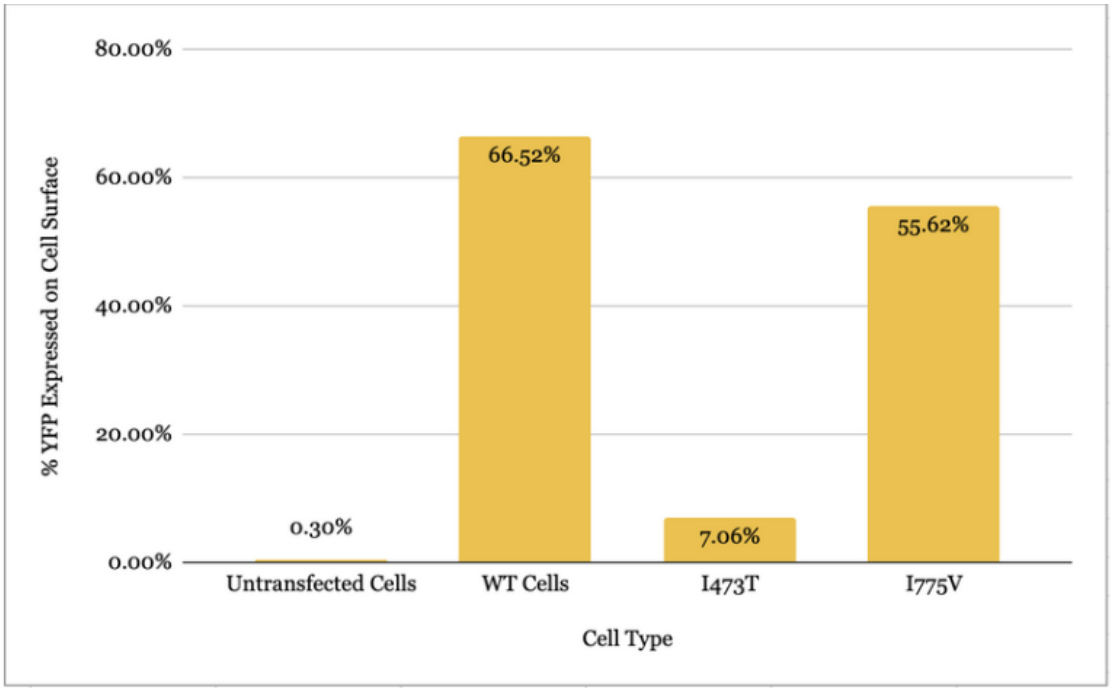

Figure 4. A graphical representation of the flow cytometry data.

HEK-Blue Detection

To examine NF-kB activity, a detection assay was performed on specialized HEK-Blue cells. After incubation, the Quanti-blue detection medium had sufficiently interacted with the expression proteins, leading to a color change, allowing us to see the differences in signaling between each transfection and stimulation type (Figure 5). This data was then quantified using absorbance measurements. The wild-type form had the highest amount of NF-kB, with the mutants being much lower (Figure 6).

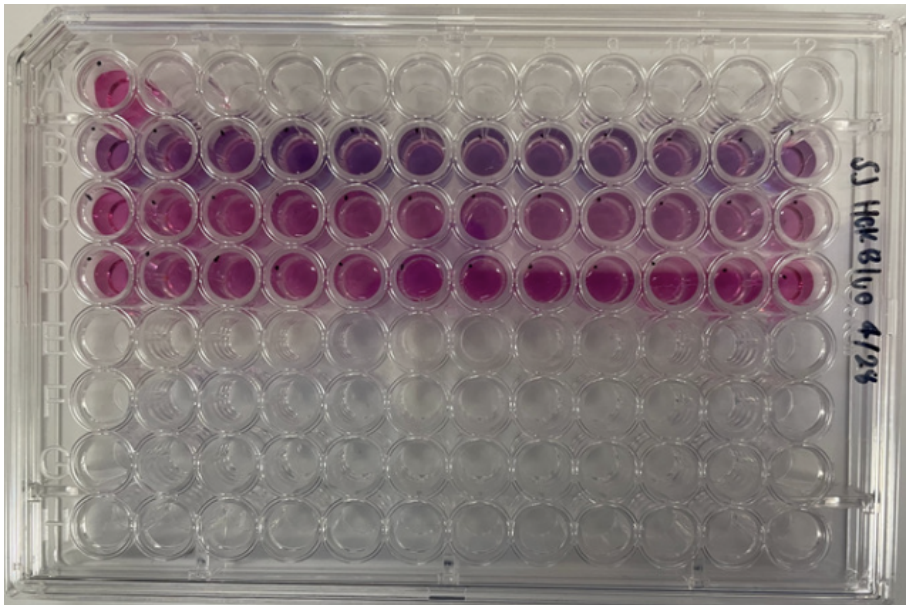

Figure 5. The plate containing the Hek-Blue cells after the addition of QuantiBlue and incubation.

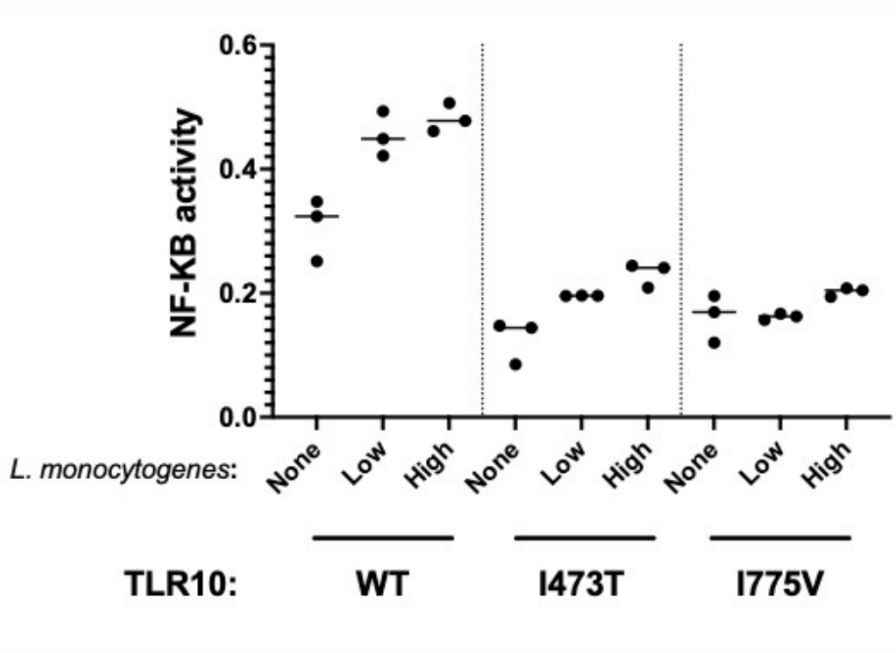

Figure 6. A graphical representation of the absorbance data collected through HEK-Blue detection, demonstrating NF-KB activity.

Discussion

Understanding the activity of Toll-like Receptor 10 and its mutant in different contexts is extremely important. Given that we know so little about TLR10, any information contributes to a greater knowledge surrounding it and will eventually lead to conclusions about its role.

The current research performed on TLR10 has led to indications that it has both pro- and anti-inflammatory properties. In intestinal epithelial cells and macrophages that were stimulated with *L. monocytogenes*, the wild-type form of TLR10 was found to have pro-inflammatory effects, leading to increased NF-kB activity. However, a study on wild-type TLR10 and its functional mutant, *rs11466657*, showed that the wild-type form inhibits NF-kB activity in the context of rheumatoid arthritis, an inflammatory disorder. These studies are just a few examples of the limited and conflicting information available on TLR10.

To elucidate the activity of wild-type TLR10 and its mutant forms further, I set out to study two mutant forms, *rs11466657* (I473T) and *rs4129009* (I775V). The first step in doing so was the generation of the mutant plasmid for the I473T variant, as the I775V had been previously generated by our lab. Since TLR10 is not naturally present in mice, creating these mutant plasmids is crucial. As previously mentioned, I took two approaches to this. The first approach, which was the generation of the mutant plasmid through Gibson Assembly was ultimately unsuccessful. This could be due to the difficulty in obtaining a high yield of the various DNA fragments, leaving only the wild-type form when being sent for sequencing. This led me to pivot and apply a second approach using a gene fragment with the mutation and a second fragment from the restriction digest. This was successful, leaving us with multiple mutant plasmids alongside the wild-type available for use in study.

After generating the necessary plasmids, I began analysis of the differences between wild-type and mutant forms. First, I analyzed flow cytometry results comparing the wild-type, I473T, and I775V. The use of untransfected cells serves as a negative control and a method to confirm that the transfection

did take place. As seen in the flow cytometry results, there are differences present between the expression levels of the wild-type and both mutants. The drastic change between the wild-type and the I473T variant indicates that this mutant form does not localize to the cell surface in the same way that the wild-type does and rather works intracellularly. If it localized at a similar level, the YFP expression in both would be similar. The slight change between the wild-type and I775V mutant indicates that I775V might still localize extracellularly but not at the same amount as the wild-type. These differences may be caused by the locations of both mutants. The I473T mutant is located in the LRR domain of the receptor while the I775V mutant is in the TIR domain (Figure 7). [5] Since the LRR domain is involved in pathogen recognition, a mutation in this could potentially impact this process.

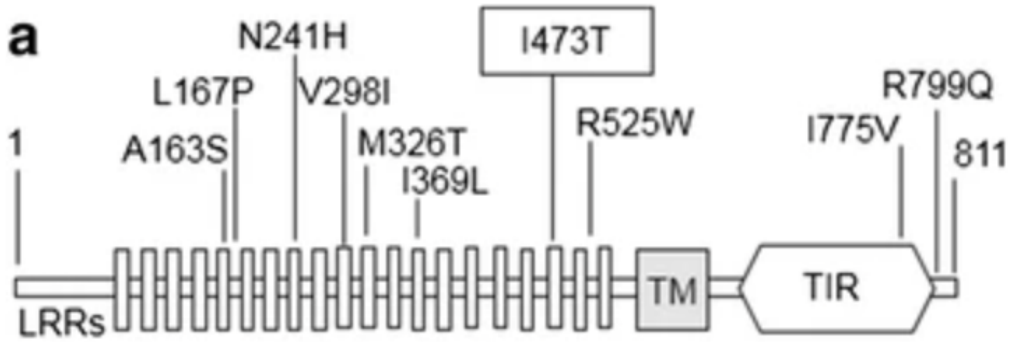

Figure 7. The TLR10 protein showing the location of both mutants of interest

These expression changes also inform the differences in NF-kB activity. These results showed that WT TLR10 has a higher baseline NFkB and a greater dose-dependent response to ligands than both mutant forms. I473T has the lowest baseline value which is in accordance with its expression changes but it does have more dose-dependent effects than I775V. Through these results, we have demonstrated that both mutant forms of TLR10 have decreased NF-kB expression, while the wild-type has increased expression in response to *L. monocytogenes.*

Determining the effects of these mutant forms helps to elucidate more information about the function of TLR10 and why certain mutant forms might have greater disease susceptibility. As we look ahead, further investigation on the activity of TLR10 is necessary to form conclusions on its definitive action, especially in the effects of this mutant form.

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