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INNATE IMMUNITY: TOLL-LIKE RECEPTORS, NF-κB ACTIVATION, AND COXSACKIEVIRUS

A Major Qualifying Project Report

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ABSTRACT

The purpose of this project was to determine whether the Coxsackievirus B (CVB) uses toll-like receptors (TLR) to enter cells. In the signal transduction pathway of Toll-like Receptors, phosphorylation of the inhibitor protein IκB (by either of the IκB kinases (IKK)1 or IKK2) leads to the degradation of IκB. IκB is always associated with NF-κB, which is a transcription signaling molecule. When IκB is degraded, NF-κB is translocated into the nucleus, and ultimately causes cytokines, especially interleukin-8 (IL-8) to be synthesized and released. This project involved the creation of a DNA reporter plasmid that in the presence of free cellular NF-κB expresses the reporter protein dsRed-Express-1. This NF-κB reporter plasmid was transiently transfected into several different Human Embryonic Kidney (HEK 293) cell lines which were each stably transfected with different TLRs. Known ligands for these TLRs were used to test the specificity of the expression of the fluorescent signal. Once the system was shown to work well with positive control ligands, Coxsackievirus was used as a ligand, and it was shown that Coxsackievirus does indeed activate NF-κB, but not by the classic pathway, no IL8 synthesis was detected. So CVB does not appear to interact with any of the TLRs used in this specific HEK cell line, but it does not fully rule out an interaction between TLRs and CVB in other cells.

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ACKNOWLEDGEMENTS

First of all I would like to thank Dr. Robert Finberg and Dr. Evelyn Kurt-Jones for being so kind and letting me work in their labs. I would like to thank Dr. Neeta Shenoy, whose tireless efforts to help me with my project were incredible. Huge thanks are owed to everyone in the Finberg lab who helped me along the way when I had questions and problems. Glennice Bowen, Melvin Chan, Damon Asher, Jennifer Wang, Leisa Mandell, and Anna Cerny have been extremely helpful and especially patient with me during my time working in the lab, and I am very thankful for their assistance. Also, thanks to Sarah Shin for helping me to learn sterile techniques in the cell culture hood. A special thanks for Dr. Jennifer Wang for providing the d2EGFP-NF-κB plasmid for the project. And, thanks to Dave Adams, for initiating this project and helping with the writing of the report.

BACKGROUND

Immunology is considered to be a relatively new science. Most scientists contribute its origin to Edward Jenner, who in 1796 discovered that cowpox, or vaccinia, induced protection against human smallpox, hence the term vaccination which we still use today. Robert Koch then proved, in the late 19th century, that infectious diseases are caused by microorganisms, and each one is responsible for a different pathology. Today we recognize four different classes of disease-causing microorganisms, or pathogens: viruses, bacteria, fungi, and parasites. In the 1880s, Louis Pasteur devised a vaccine against cholera in chickens, and a vaccine against rabies. These practical advances sparked searches into the mechanism of protection that these vaccines provided, and thus the development of the science of immunology (Janeway et al 2001).

Toll-like Receptors

The Toll receptor was originally found in *Drosophilia* and was determined to be essential in the determination of the dorso-ventral pattern in embryo development, it was also determined that Toll-mutant flies were highly susceptible to fungal infection (Hashimoto et al 1988; Lemaitre et al 1996). These studies showed how the innate immune system has the ability to specifically recognize invading microorganisms, and since then mammalian homologues have been found and subsequently names Toll-like receptors (TLR) (Takeda 2005). The TLR family contains at least 11 members (TLR1–TLR11) (Takeda 2005). The cytoplasmic portion of Toll-like receptors is very similar to

The cytoplasmic portion of the IL-1 receptor family, and is therefore referred to as the Toll/IL-1 receptor (TIR) domain (Takeda 2005). Despite this cytoplasmic similarity, the extracellular portions are not conserved between the two types of receptors. "IL-1 receptors possess an Ig-like domain, whereas Toll-like receptors bear leucine-rich repeats (LRRs) in the extracellular domain (Takeda 2005)." The physiological functions of TLRs have mostly been determined by the analysis of knockout mice. Each TLR recognizes specific portions of micro-organisms that are conserved (Takeda et al 2003). Thus, the mammalian innate immune system can detect invasion by pathogens via the recognition of microbial components by TLRs (Janeway and Medzhitov 2002).

TLR Signaling Pathway

The TLR signaling pathways begins in the TIR domains. This was first revealed in the C3H/HeJ mouse strain, which contains a point mutation of a proline residue in the TIR domain of TLR4 (Poltorak et al 1998; Hoshino et al 1999). "In the signaling pathways downstream of the TIR domains, an important role for a TIR domain-containing adaptor, MyD88, was first characterized. MyD88 possesses a TIR domain in its C-terminal portion, and a death domain in its Nterminal portion. Upon stimulation, MyD88 recruits a serine/ threonine kinase, IL-1 receptor-associated kinase (IRAK), to TLRs through interaction of the death domains of both molecules. IRAK becomes activated and then associates with TRAF6, leading to NF-κB activation (Takeda 2005)." Therefore, the signaling protein MyD88 is required for cytokine activation as shown in Figure 1 (Takeda 2005).

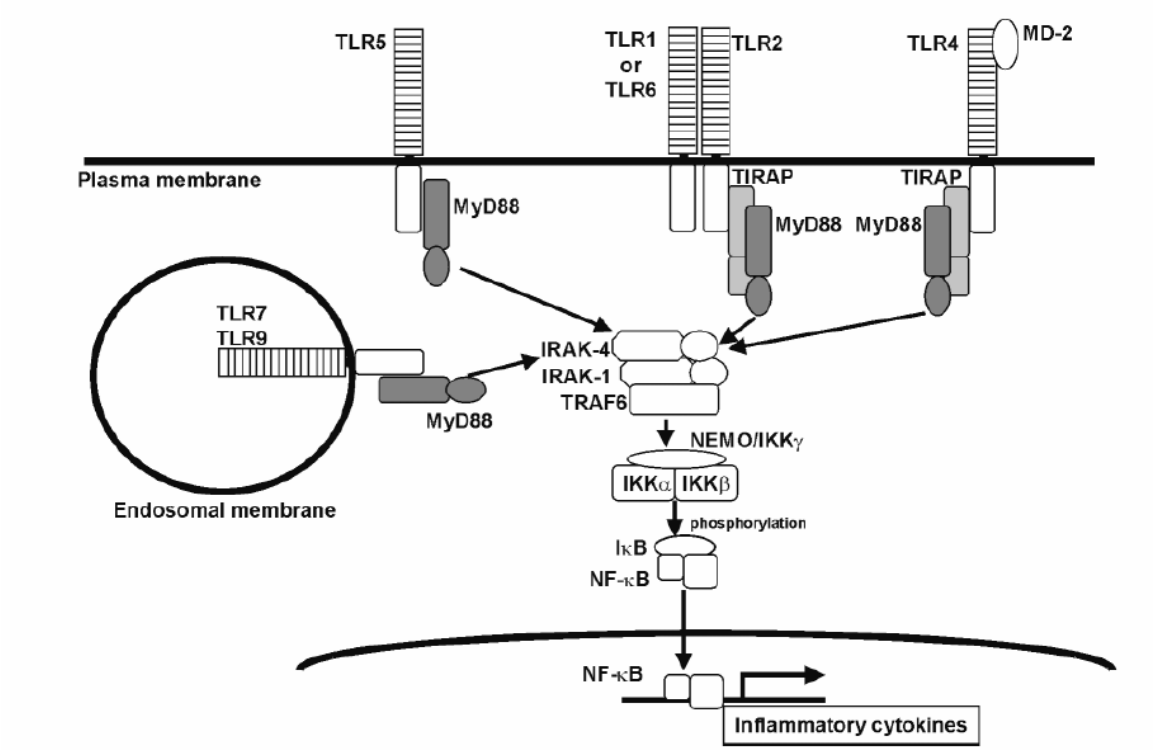


Fig. 1. MyD88-dependent pathway. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of a TLR, and recruits IRAKs to the receptor upon ligand binding. IRAKs then activate TRAF6, leading to activation of the IκB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. The IKK complex phosphorylates IκB, resulting in nuclear translocation of NF-κB which induces the expression of inflammatory cytokines. TIRAP is involved in the MyD88-dependent pathway via TLR2 and TLR4.

In TLR3 and TLR4 pathways, NF-κB activation can also occur via a TRIF pathway that is independent of MyD88 shown in figure 2 (Takeda 2005).

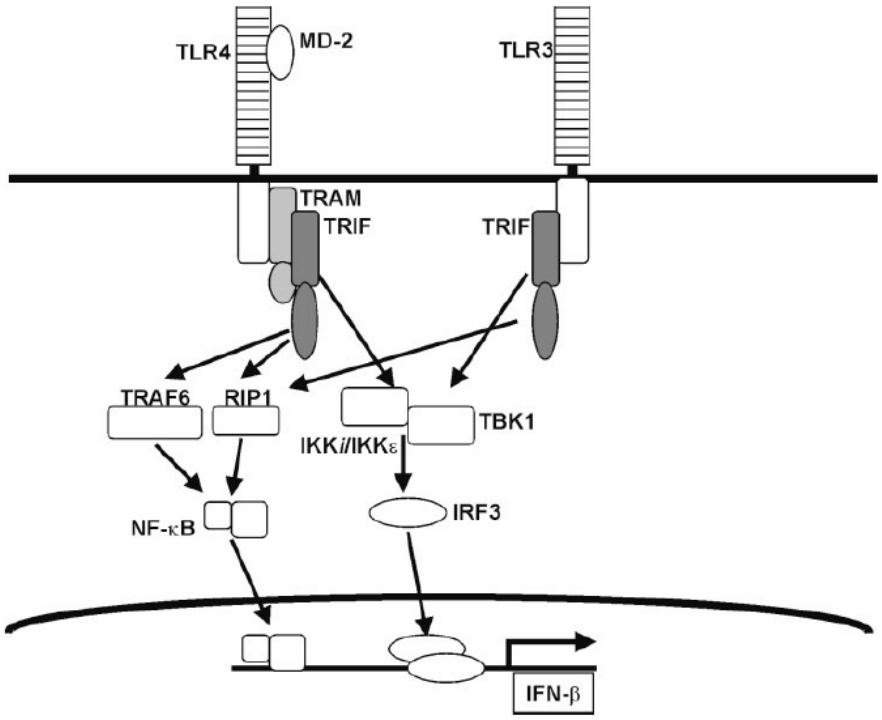


Fig. 2. MyD88-independent pathway/TRIF-dependent pathway. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. TRIF is essential for the MyD88-independent pathway. IKK-related kinases, IKKi/IKKε and TBK1, mediate IRF-3 activation downstream of TRIF. TRAM is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway. RIP1 and TRAF6 are indicated to mediate TRIF-dependent activation of NF-κB.

NF-κB Activation

There are two pathways by which NF-κB is activated. The classical pathway which is based on IKKb-dependent IkB degradation, is essential for innate immunity (Bonizzi and Karin 2004). "The activation and nuclear translocation of classical NF-kB dimers is associated with increased transcription of genes encoding chemokines, cytokines, adhesion molecules [intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial–leukocyte adhesion molecule 1 (ELAM)], enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis" (Ghosh et al 1998; Bonizzi and Karin 2004) as shown in Figure 3a. These

proteins recruit inflammatory and phagocytic cells to the site of the infection (Bonizzi and Karin 2004).

Recently, there has been a second pathway that has begun to be described. This pathway, dependant upon IKKα (Senftleben et al 2001) and independent upon IKKβ and IKKγ (Dejardin et al 2002) is shown in figure 3b (Bonizzi and Karin 2004). In the alternative pathway, the signal transduction pathway does not originate from the TLRs (Bonizzi and Karin 2004). Also shown in the figure, is the difference in the proteins produced based on the activation of NF-κB, the secondary pathway produces proteins that are necessary for adaptive immune responses, while the classical pathway produces proteins used in innate immune responses (Bonizzi and Karin 2004).

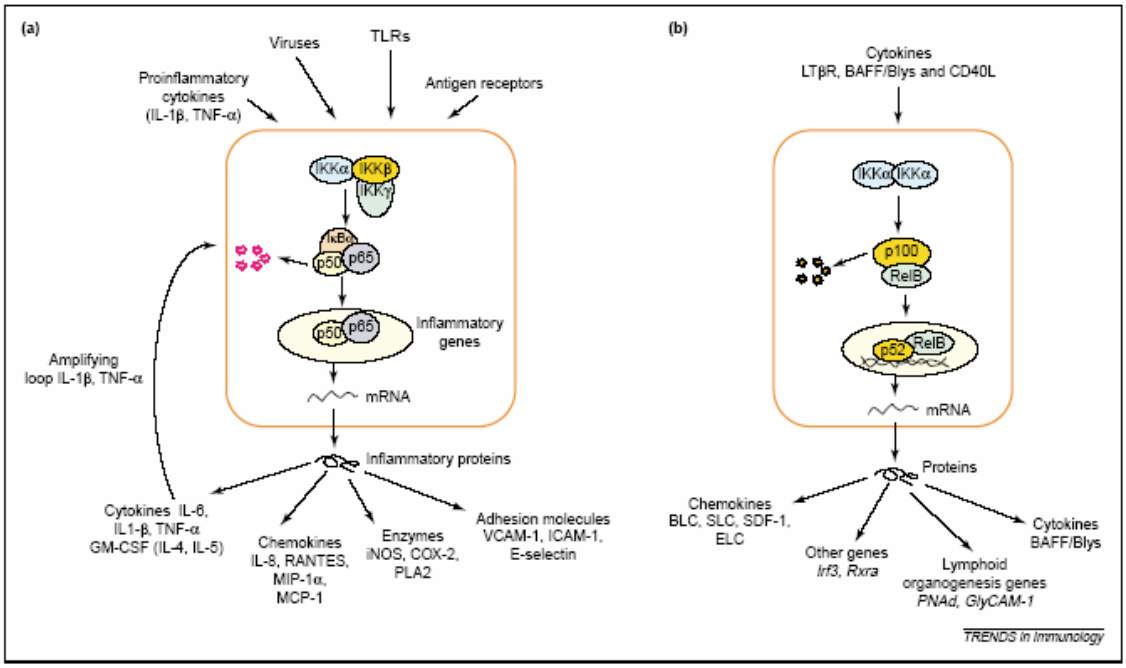


Figure 2. Classical and alternative NF-κB pathway. (a) The classical NF-κB pathway is activated by a variety of inflammatory signals, resulting in coordinate expression of multiple inflammatory and innate immune genes. The proinflammatory cytokines IL-1β and TNF-α activate NF-κB, and their expression is induced in response to NF-κB activation, thus forming an amplifying feed forward loop. (b) The alternative pathway for NF-κB results in nuclear translocation of p52–RelB dimers, is strictly dependent on IKKα homodimers and is activated by LTβR, BAFF and CD40L by NIK. Many data strongly suggest that the alternative pathway plays a central role in the expression of genes involved in development and maintenance of secondary lymphoid organs. Abbreviations: BAFF, B-cell-activating factor belonging to the TNF family; BLC, B-lymphocyte chemoattractant; CD40L, CD40 ligand; COX-2, cyclooxygenase 2; ELC, Epstein–Barr virus-induced molecule 1 ligand CC chemokine; GM-CSF, granulocyte–macrophage-colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; IKK, IκB kinase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LT, lymphotoxin; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; NIK, NF-κB-inducing kinase; PLA2, phospholipase 2; SDF-1, stromal cell-derived factor-1α; SLC, secondary lymphoid tissue chemokine; TLRs, Toll-like receptors; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule-1.

Figure 3: Classic and Alternative NF-κB Pathways.

An important thing to notice is the production of the cytokine IL-8 through the classic pathway, and the non-production of IL-8 through the alternative pathway.

Coxsakievirus B

Coxsakievirus B (CVB) is an enterovirus that is closely related to poliovirus. CVB is a much less of a health hazard then poliovirus is, however, it still remains a pathogen that is of concern (Asher and Finberg 2004). In humans, a range of acute and chronic diseases are caused by CVB (Brown and O'Connell 1996). The gastrointestinal (GI) tract is a major site of CVB replication, however, symptoms induced by CVB in the GI are not usually seen (Flint 2004). CVB is a picornavirus, a family of viruses that is the leading cause of aseptic meningitis in adults (Whitton 2002). In infants, CVB is of particular concern, as it can cause life-threatening aseptic meningitis, myocarditis, and

fulminant hepatitis (Goren 1989, Gorwishankar 1914, Kaplan et al 1983; Kawashima et al 2004; Wang et al 1998).

One of the most surprising characteristics of CVB is that most adults have been infected at one time. CVB is the leading cause of adult myocarditis (Kearny et al 2001). Studies show: 86% of the tested population had neutralizing antibodies against 2 serotypes of CVB (Eggers and Mertens 1987). Of those exposed, about half are expected to have an episode of acute viral mycocarditis (O'Connell 1987). In a given population and at any given time point, around 1% of the population may be experiencing a subclinical episode (Gravanis and Sternby 1991). Patients with myocarditis have mild symptoms such as rash, myalgia or upper respiratory or they are asymptomatic (Whitton 2002). In general, most infections caused by CVB are resolved naturally without any further complications; however, myocarditis can sometimes lead to death (Asher and Finberg 2004). It has been found that asymptomatic myocarditis is a major cause of sudden, unexpected death in young persons (Drory et al 1991; Ward 1978).

PROJECT PURPOSE

The purpose of this project is to determine whether or not Coxsackievirus B (CVB) interacts with any of the Toll-like receptors (TLR). Unpublished laboratory data suggests that mice infected with CVB show an up-regulation of TLR 4 in the heart and spleen compared to non-infected control mice. This led the laboratory to believe that there may be some interaction between CVB and the TLRs. To determine this, an *in vitro* reporter system was created that expresses a fluorescent signal upon NF-κB activation. NF-κB is activated by two different pathways, one of which originates with an external signal in the TLRs, and the other is caused by cellular signaling. To determine which pathway was used to activate NF-κB, an IL-8 ELISA was performed. Only the TLR pathway will cause the cytokine IL-8 to be released. Therefore, we could better determine whether or not CVB is recognized by the TLRs.

METHODS

Transformation of Competent E. coli Cells Using Heat Shock

Beginning with the two commercially available plasmids (pNF-κB-d2EGFP and pDsRed-Express-1) competent E. coli cells were transformed using a heat shock protocol. The E. coli cells transformed with pNF-κB-d2EGFP were grown up on agar trays containing ampicillin, and cells transformed with pDsRed-Express-1 were grown up on agar containing kanamycin.

Cell Culture Medium

Medium used was Dubelco's Modified Eagle's Media (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, 1% penicillin, and 1% streptomycin. Media were stored at 4°C.

Mini-prep

Mini-preps were performed using the mini-prep kit (Qiagen) followed by restriction enzyme digestion and southern blot to determine if the plasmids had been transformed correctly.

Maxi-prep

Once the plasmids had been determined to be correct, a maxi-prep done using the Maxi-prep kit (Qiagen) to isolate a large amount of plasmid DNA.

Ligation

To create the pNF-κB-DsRed-Express-1 plasmid, both of the commercially available plasmids were digested in SacI and HindIII. Then the kappa enhancer element from pNF-κB-d2EGFP (KB_4) and the TK promoter were ligated into the MCS of pDsRed-Express-1. The ligations were performed by Dr. Neeta Shenoy.

Sequencing

Plasmids were sent to the UMASS Medical School sequencing facility to verify the accuracy of the cloning.

Transient Transfection

HEK 293 cell lines were transiently transfected with plasmids using Genejuice and according to factory specifications from Novagen.

Stimulation

24 hours post-transfection, the cell culture media was changed and replaced with either: plain media; media containing TLR-specific ligands, media treated the same way as the media containing CVB; media containing UV-inactivated CVB; media containing the full concentration of CVB; and media containing a 1:100 dilution of media containing CVB. CVB was prepared and purified by Dr. Neeta Shenoy. Ligands used: TNF-α, PAM2 (TLR2 ligand), LPS (TLR4 ligand), Poly I:C (TLR3 ligand), CPG DNA (TLR9 ligand).

Fluorescence Microscopy

24 hours post-stimulation, cell cultures were placed under the microscope in a dark room. Fluorescent filters were used, and digital pictures were taken using the SPOT Advanced Program.

IL-8 ELISA

24 hours post-stimulation, supernatants from the cell cultures were removed and an ELISA specific for IL-8 was performed. ELISA's were performed using a 1:5 sample dilution, following the BD Biosciences factory protocol (Franklin Lakes, New Jersey). Plates were prepared, and then the antibodies were added by Dr. Neeta Shenoy.

RESULTS

The purpose of this project was to create an *in vitro* system to determine whether CVB interacts directly with TLRs. This project began with the goal of creating a plasmid which would express a fluorescent red signal upon NF-κB activation. The commercially available plasmid pNF-κB-d2EGFP was not used for this because the fluorescent protein DsRed-Express-1 used as a reporter in this MQP aggregates less.

In the lab, a plasmid had just been created by Dr. Alexsandr Repik that was supposed to contain kappa enhancer elements and express a DsRed fluorescent signal. The plasmid was created by starting with pNF-κB-d2EGFP (Fig. 4) and pDsRed-Express-1 (Fig 5). Then two primers were created: a forward primer containing 3 NF-κB enhancer elements, the MCS TATA box, and the beginning of the DsRed-Express-1 expression portion; and a reverse primer containing the end of the DsRed-Express-1 expression portion. Using PCR, pDsRed-Express-1 as the backbone, and the primers, a PCR product containing the MCS TATA box, 4 NF-κB enhancer elements, and the DsRed-Express-1 fluorescent protein was produced. Then, using BglII and XbaI, pNF-κB-d2EGFP was digested and the TK promoter and d2EGFP were removed, and then subsequently replaced with the PCR product to form pNF-κB-DsRed-Express-1 (Fig. 6) with an additional 3 enhancer elements than the original GFP plasmid.

Figure 4: pNF-κB-d2EGFP

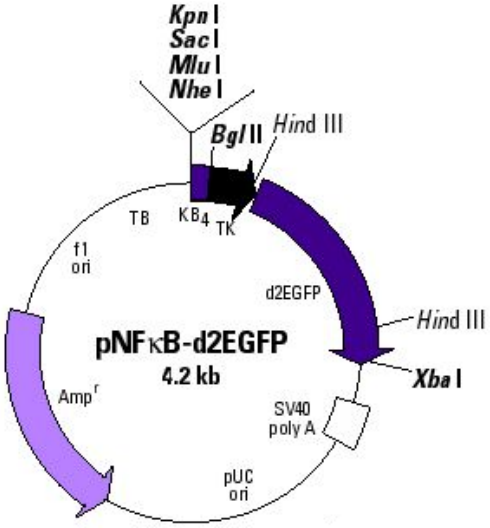
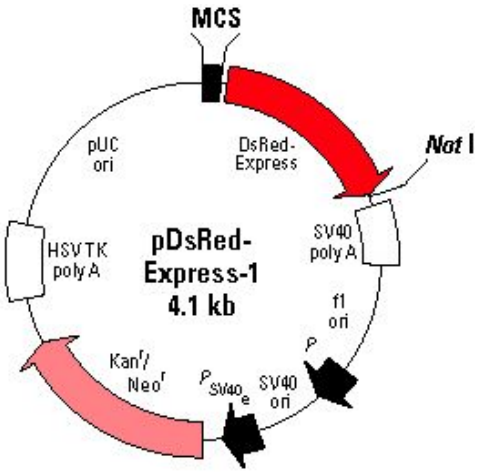


Figure 5: pDsRed-Express-1



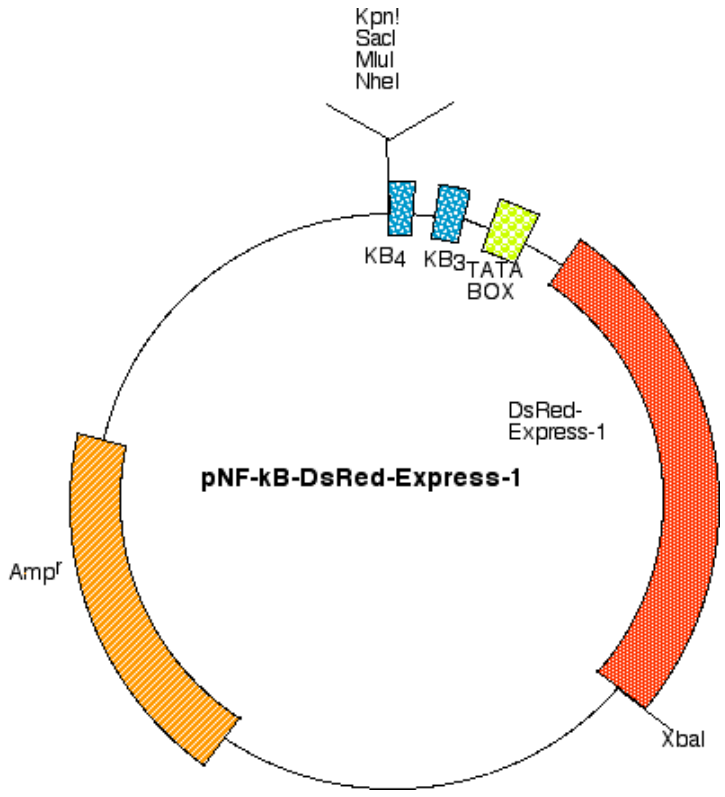


Figure 6: PCR pNF-κB-DsRed-Express-1.

As seen in figure 6, there is no promoter for this vector. However, the creator of this vector advocated that this vector should work, and the TATA BOX would serve as the promoter. Therefore, the project moved forward with transient transfections in HEK 293 cell lines that had previously been stably transfected with different TLRs. Six weeks of transfections went by without a positive result (Table 1).

It was determined that there may be a problem with this vector, so it was sent to the UMASS Medical School sequencing facility, and it was then determined that the vector did not have a promoter.

Table 1: Transfection Results with the PCR pNF-κB-DsRed-Express-1

Experiment	Result	Notes
1	No	H2.14 DsRed Espressed, No expression in YFP TLR4/MD2 GFP Positive Contrlol Expressed in H2.14
2	No	Transfection Failed
3	No	NF-kB GFP expressed, DsRed Only expressed in H4.14
4	No	GFP expressed in all cell

		lines, no DsRed expression
5	No	Used lipofectamine and genejuice in parallel, GFP expressed, no DsRed expressed
6	No	Freshly Prepped DsRed expressed in H2.14 and H4.14, GFP expressed in all cell lines

It was therefore decided to start over again with the two commercially available plasmids. A new approach was devised, and it was determined that this problem could be solved by a simple ligation. Both plasmids were digested with HindIII and SacI. For pNF-κB-d2EGFP this causes two cuts to be made, one just downstream of the TK promoter, and one just upstream of the kappa enhancer elements. These two cuts essentially remove the enhancer region and the promoter from pNF-κB-d2EGFP. For pDsRed-Express-1, this also causes two cuts to be made, each inside of the MCS (Figure 7). The enhancer elements, and promoter were ligated into the MCS, and pNF-κB-DsRed-Express-1 was created by Dr. Neeta Shenoy (Figure 8).

Figure 7: pDsRed-Express-1 MCS

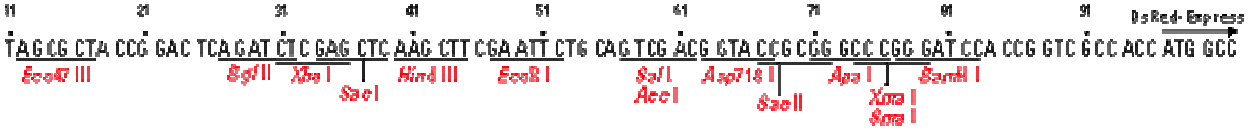
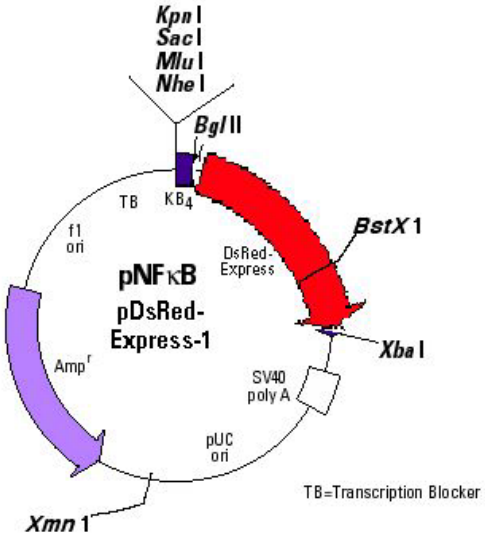


Figure 8: pNF-κB-DsRed-Express-1



Several different clones were grown and then tested through transfection and stimulation with known ligands. This was repeated many different times to determine which clone performed with the best results. Figures 9, 10, and 11 show the first trial with the first clone, and they shows a positive result, as there is an up-regulation of fluorescent signal where TLR signaling should be occurring.

Figure 9: Transfection with DsRed N=1 in HEK 293 Cells

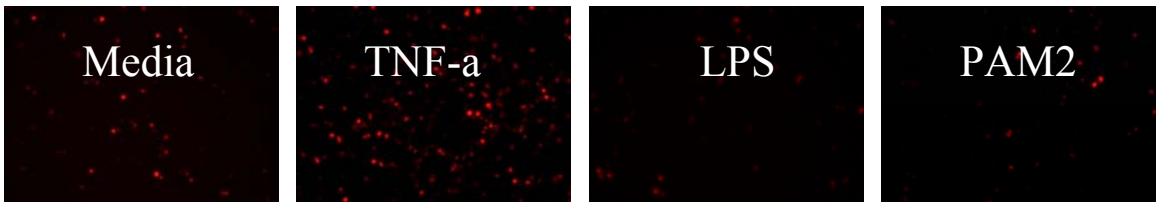


Figure 10: Transfection with DsRed N=1 in H2.14.12 Cells

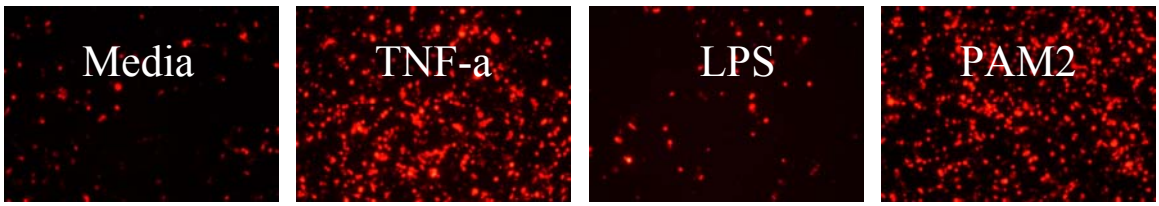
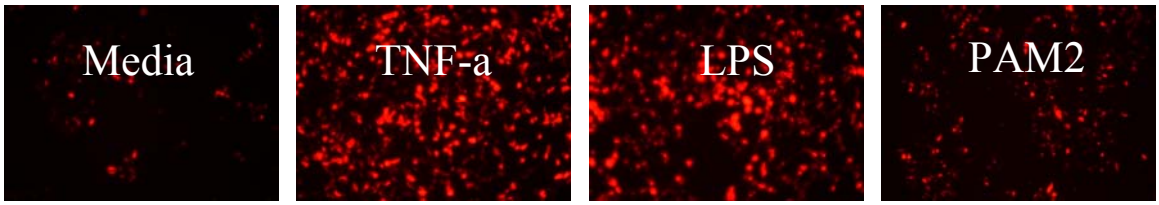
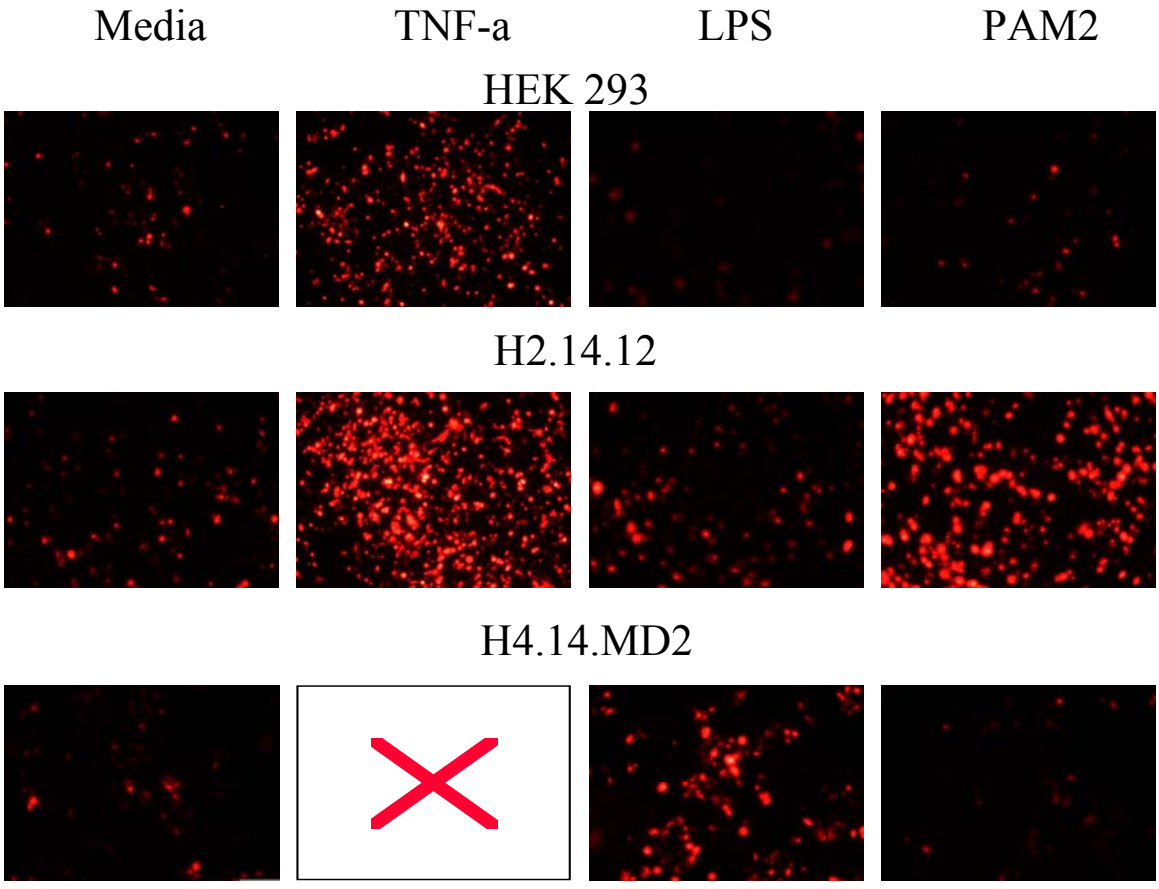


Figure 11: Transfecton with DsRed N=1 in H4.14.MD2 Cells



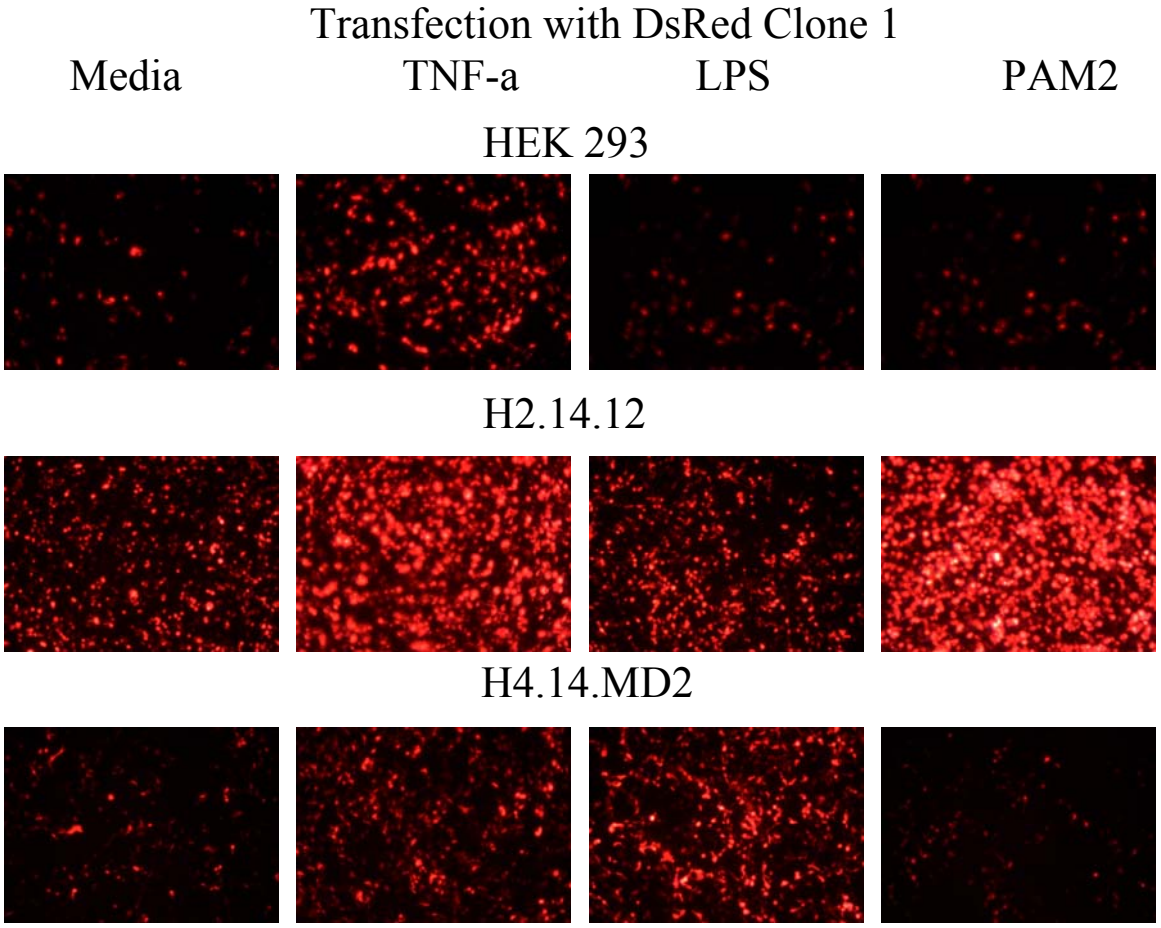
The transfection was then performed again, with the same results, however, this time there was a failure in the transfection in one of the wells (Figure 12).

Figure 12: Transfection with DsRed N=2



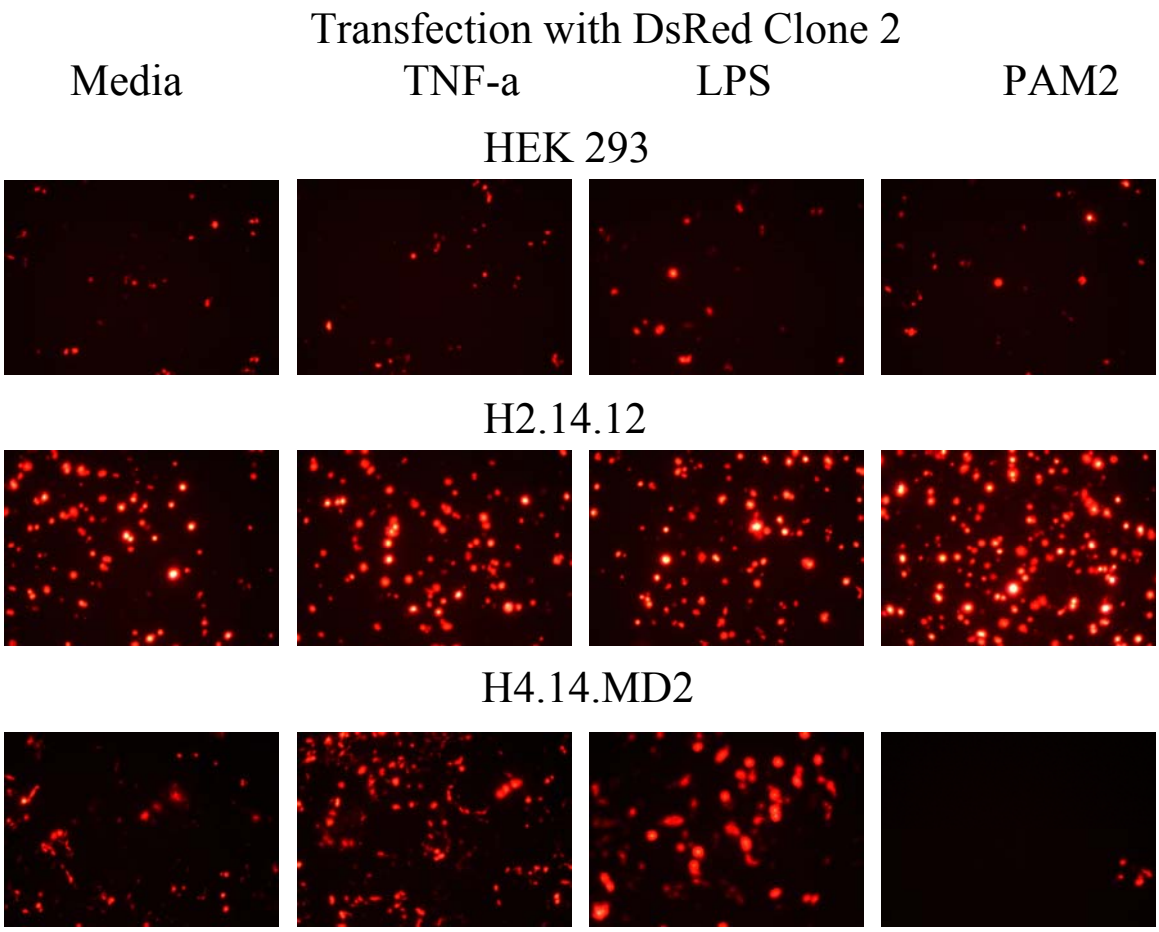
Then, the same transfection was performed with a clone of DsRed, that was from a separate ligation (Figure 13). This clone showed similar results, however there seemed to be a high amount of background expression of DsRed.

Figure 13: Transfection with DsRed Clone 1



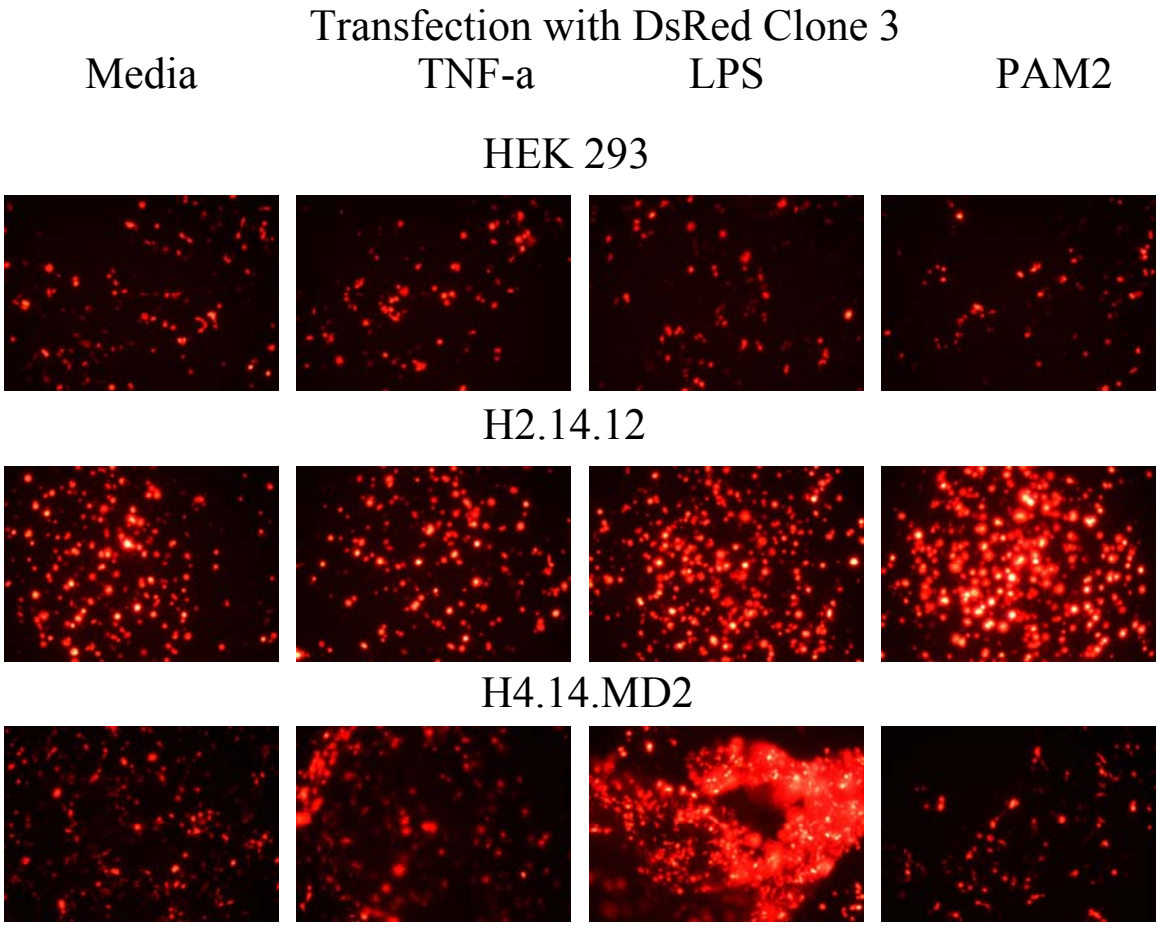
The same transfection protocol was then used to transfect these cells with clone 2 (Figure 14). The results with clone 2 produced a high amount of background expression of DsRed, however there was no specific up-regulation of signal where TLR activation should be occurring.

Figure 14: Transfection with DsRed clone 2



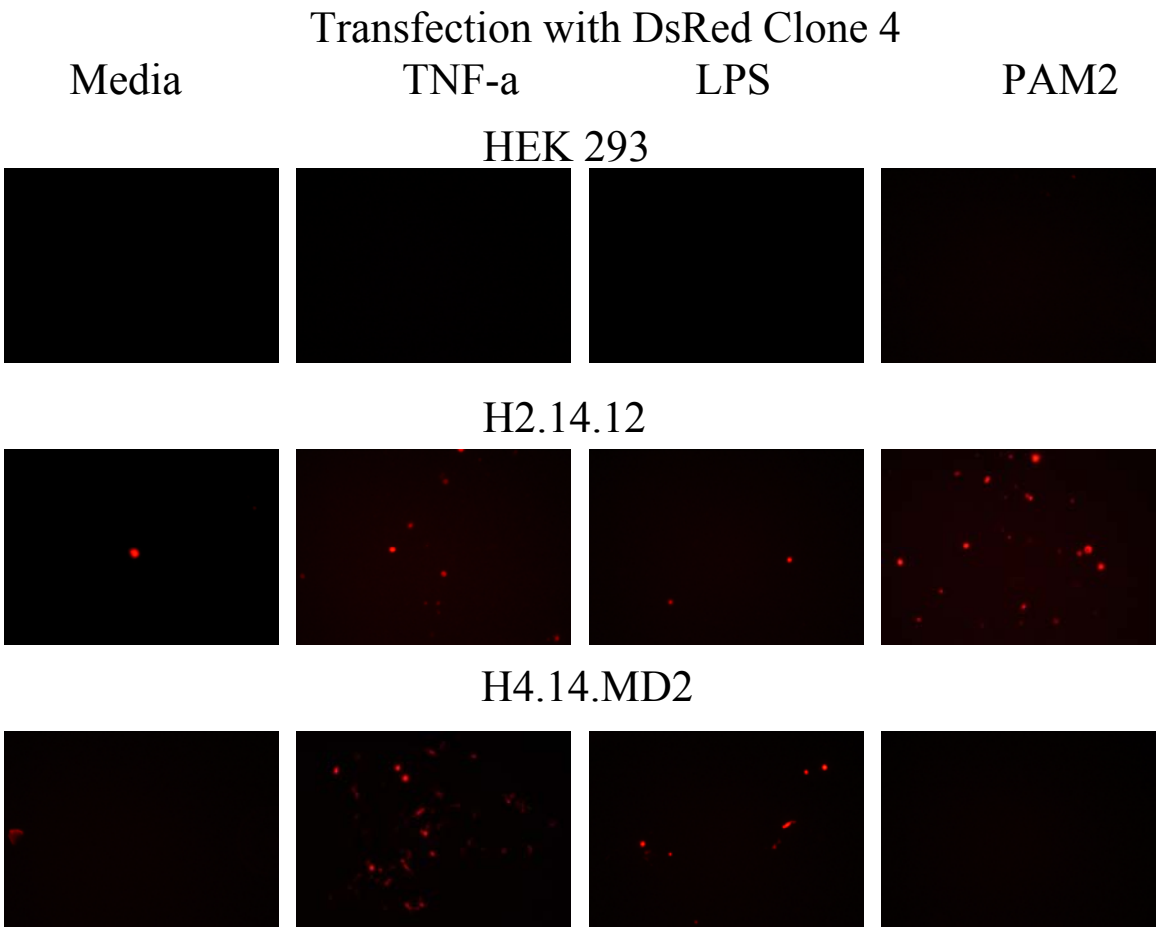
Then the same transfection protocol was performed using clone 3. Similar results were obtained (Figure 15).

Figure 15: Transfection of HEK cells using Clone 3



Then the transfection was performed with the DsRed Clone 4 (Figure 16). This transfection looks as if there was something wrong with the ligation as little fluorescent signal was observed, even with the positive ligands.

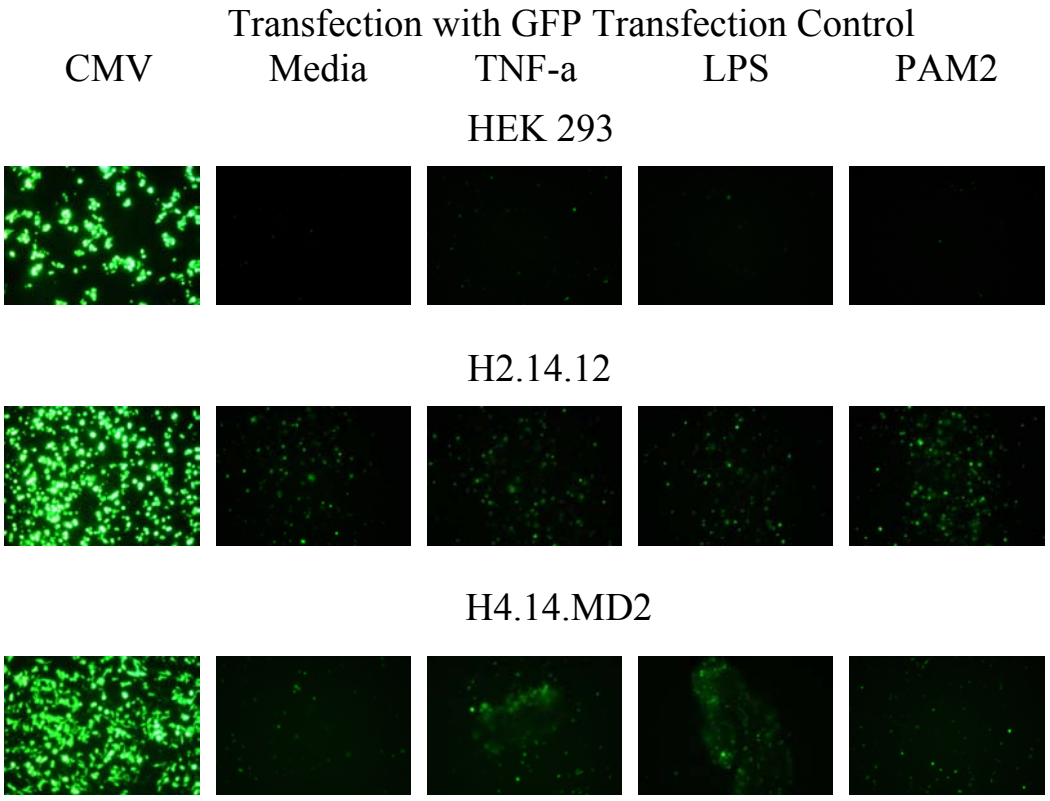
Figure 16: Transfection with DsRed Clone 4



Next, the same transfection was performed with the pNF-κB-d2EGFP plasmid, and a plasmid obtained from a Dr. Jennifer Wang called pCMV-GFP (Figure 17). pCMV-GFP is a green fluorescent protein-expressing plasmid with a cytalomegalovirus promoter. The pCMV-GFP was used as a control to show the transfection protocol was working correctly. The pNF-κB-d2EGFP plasmid was used as another control to

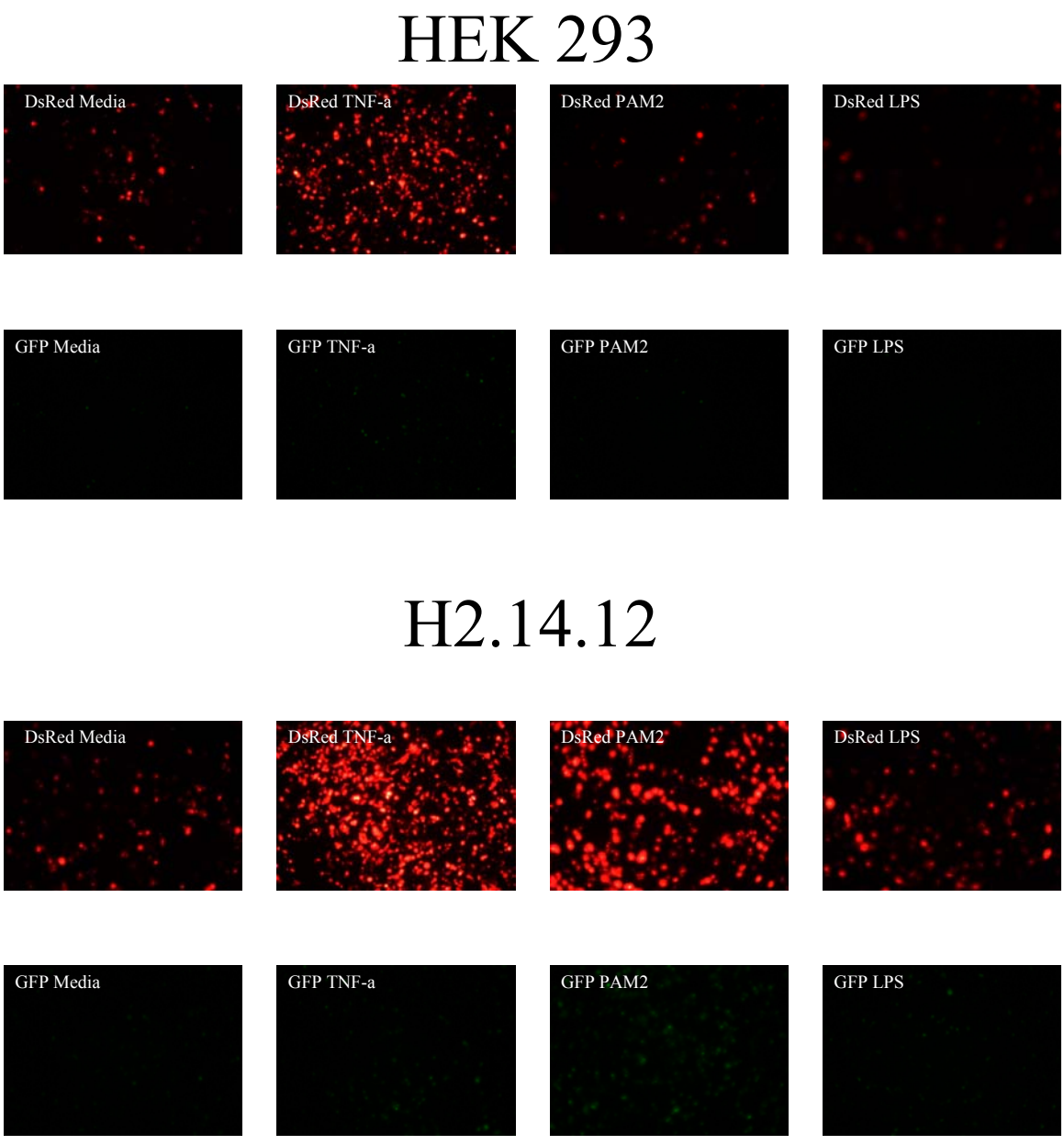
determine if the stimulation protocol was working correctly. This data shows that the transfection protocol and NF-κB stimulation were working well.

Figure 17: Transfection with GFP



Two more transfections were completed with DsRed (Figures 18 and 19). All of these results indicate that this system expresses a fluorescent red signal upon NF-κB activation dependant upon TLR activation.

Figure 18: Transfection with DsRed N=3



H4.14.MD2

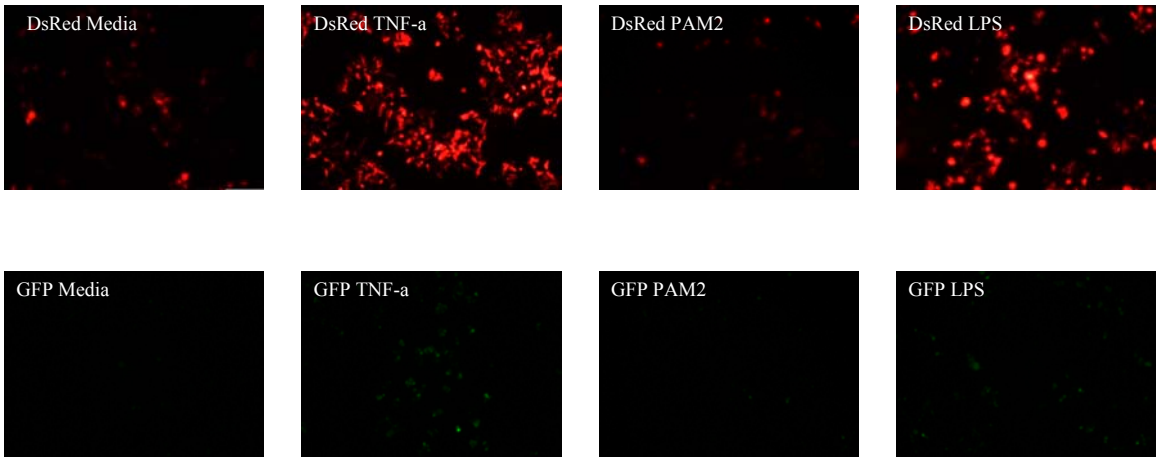
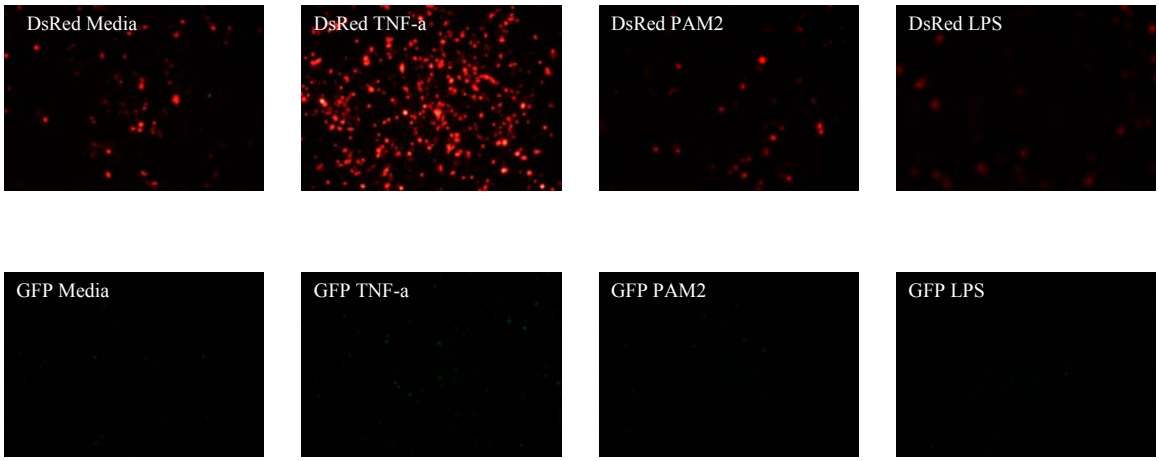
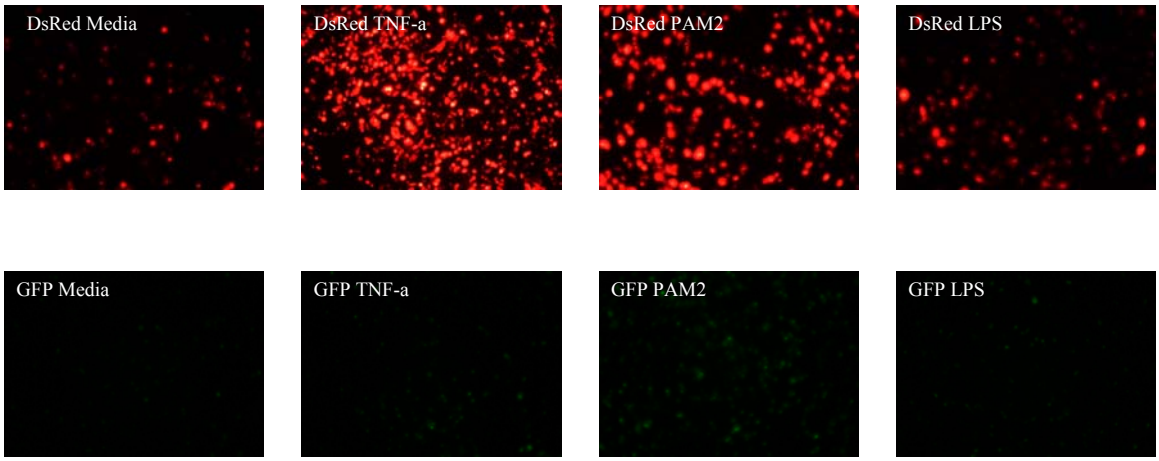


Figure 19: Transfection with DsRed N=4

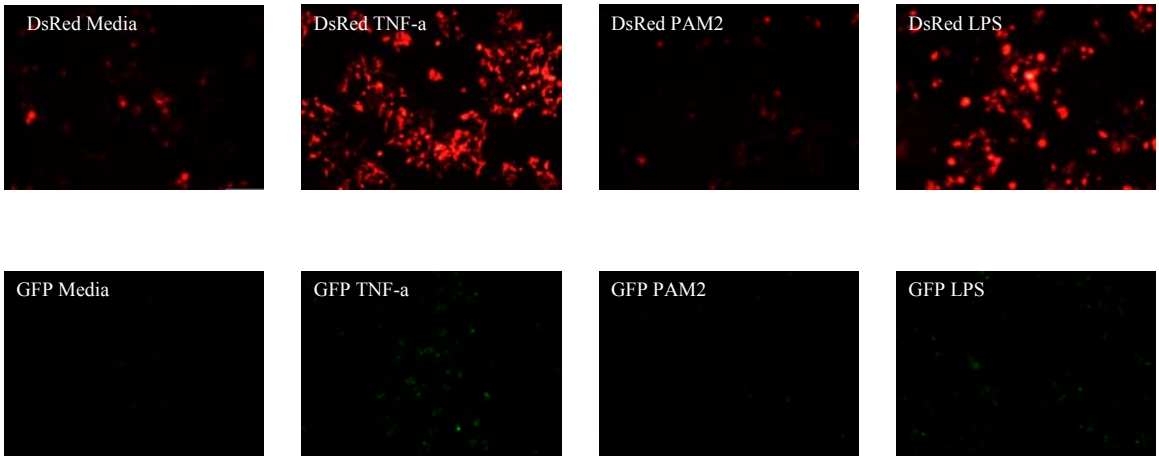
HEK 293



H2.14.12



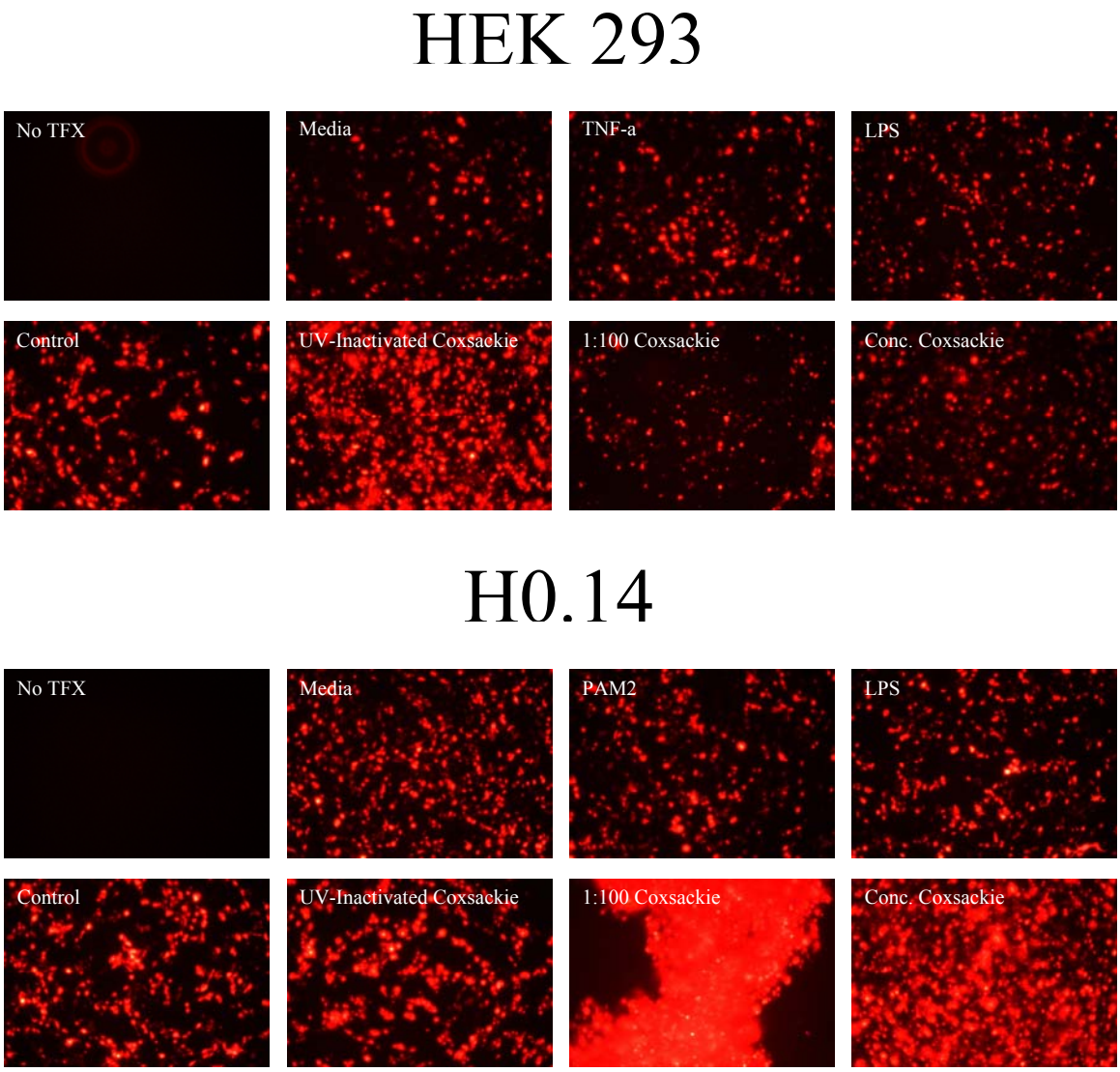
H4.14.MD2



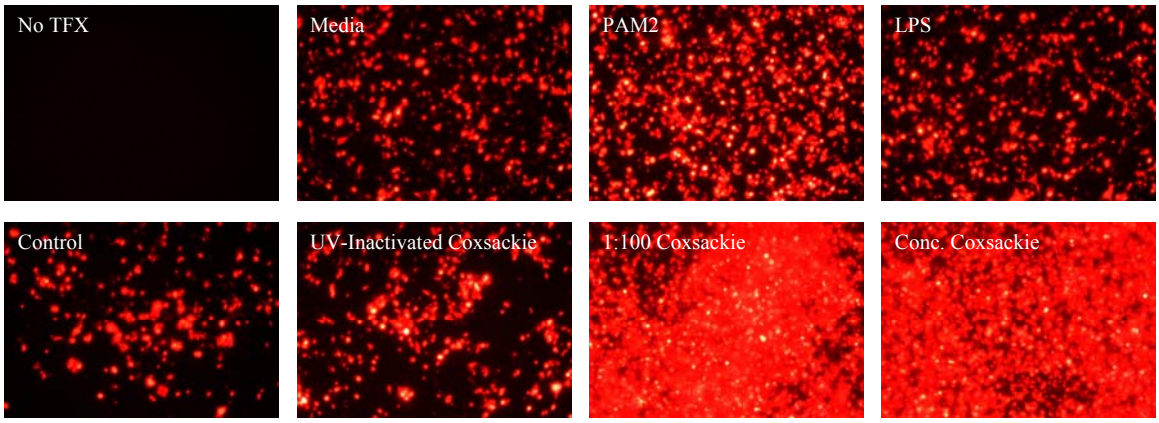
Once it was determined that the cell line stably transfectd with TLR genes produced the most reliable system for detecting NF-κB activation, media containing CVB were added into the cell flasks to determine if CVB interacts with any of the TLRs in the system (Figure 20) (Figure 21). The cells stimulated with the UV-inactivated,

concentrated CVB, and 1:100 dilution of CVB, showed high levels of NF-κB activation, and had also lifted off the bottom of the wells. Also, in Figure 21, there was a slight problem with the fluorescence photography, as the DsRed expression photos were recorded in black and white.

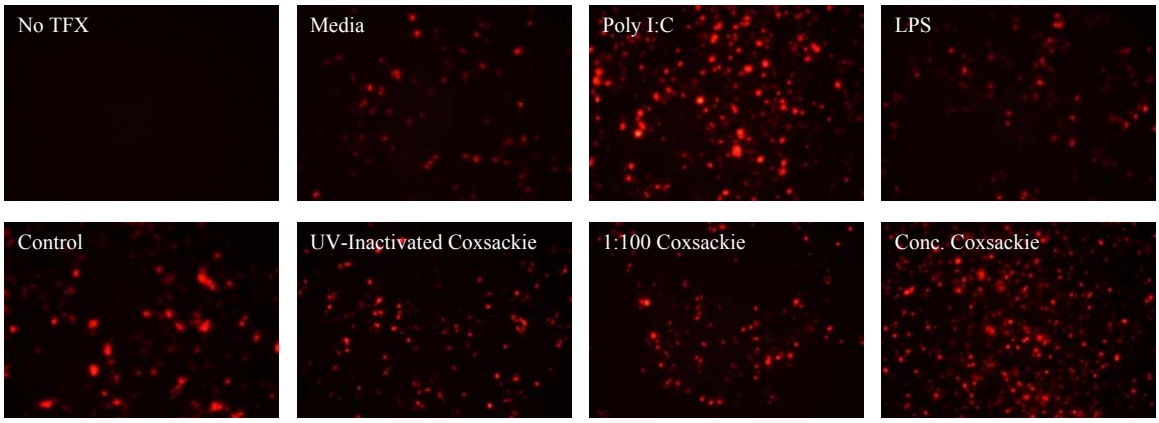
Figure 20: Stimulation with Coxsackievirus N=1



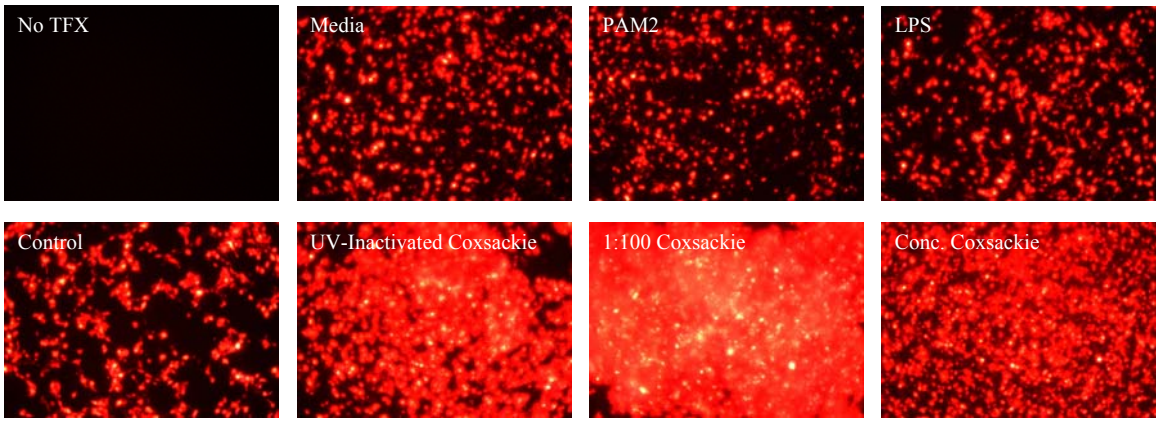
H2.14



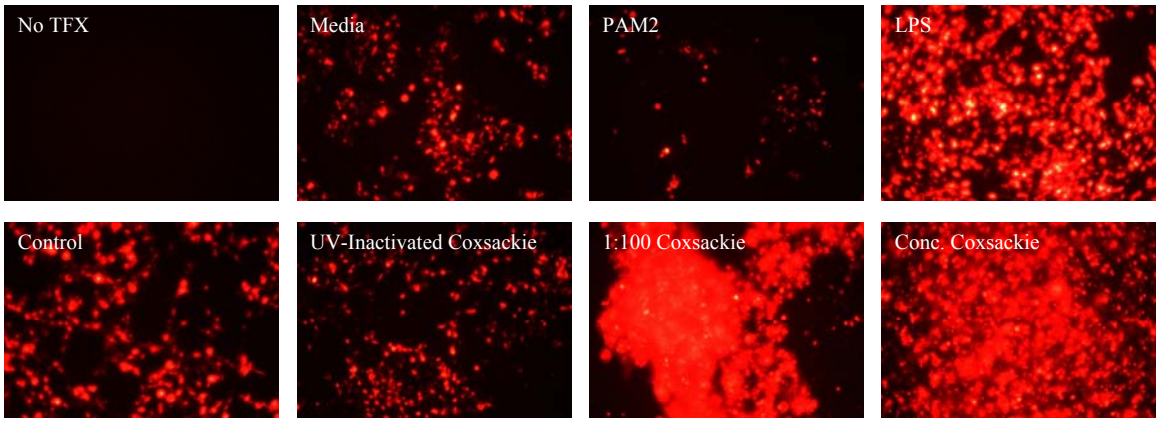
HEK TLR3



H4.14



H4.MD2



HEK YFP TLR9

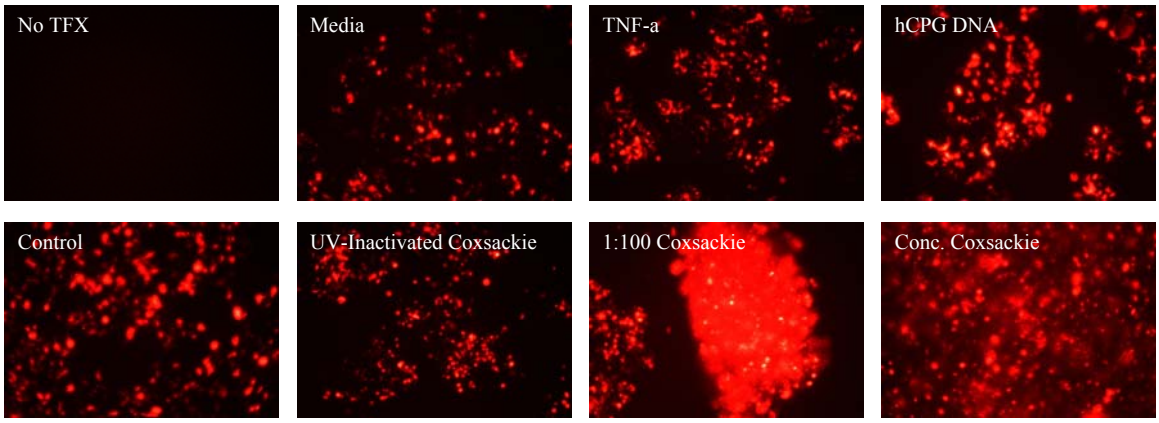
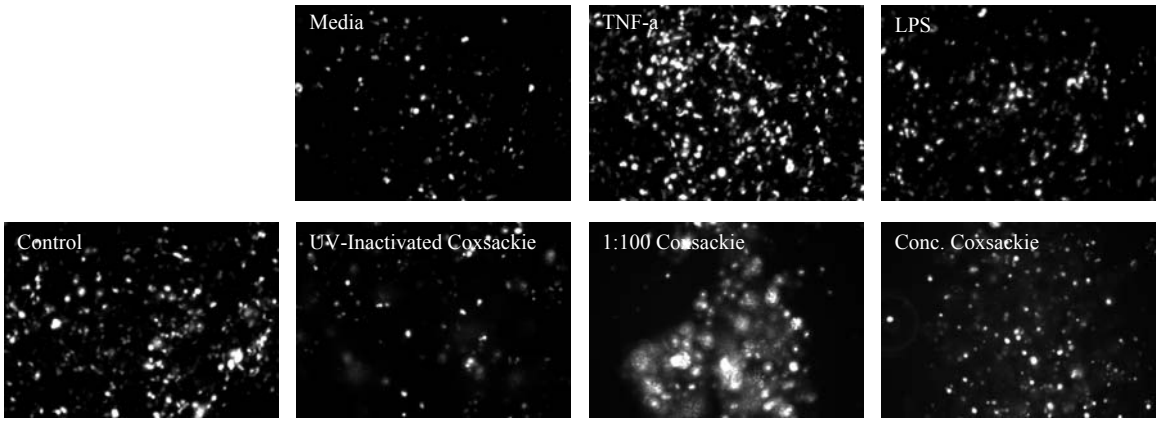
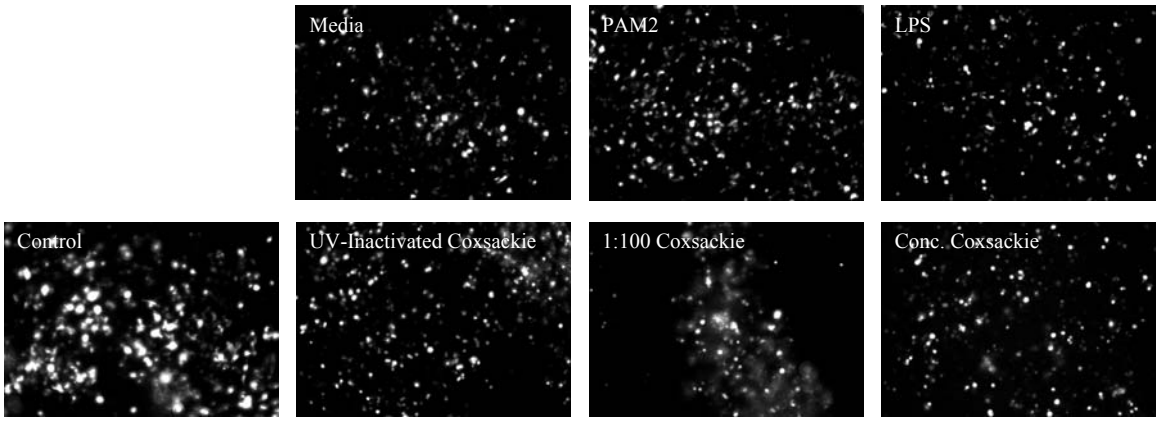


Figure 21: Stimulation with Coxsackievirus N=2

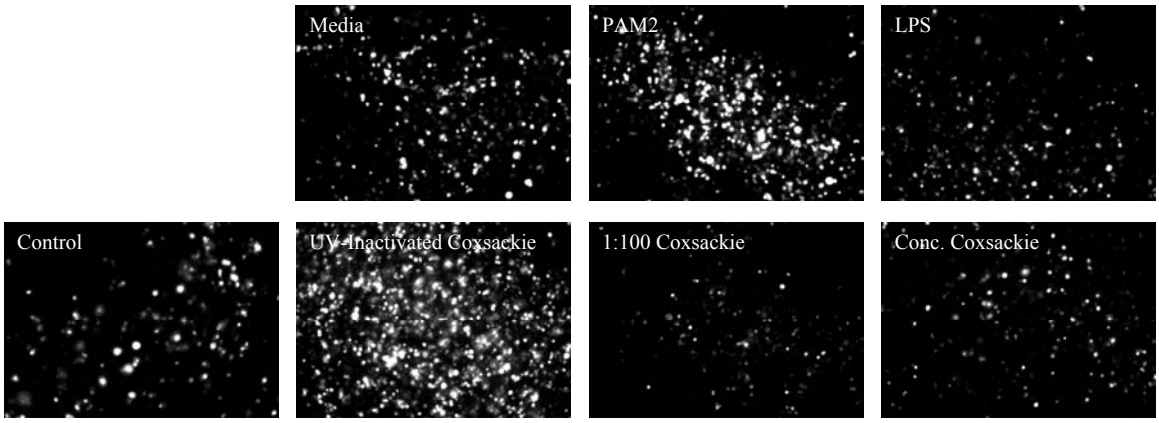
HEK 293



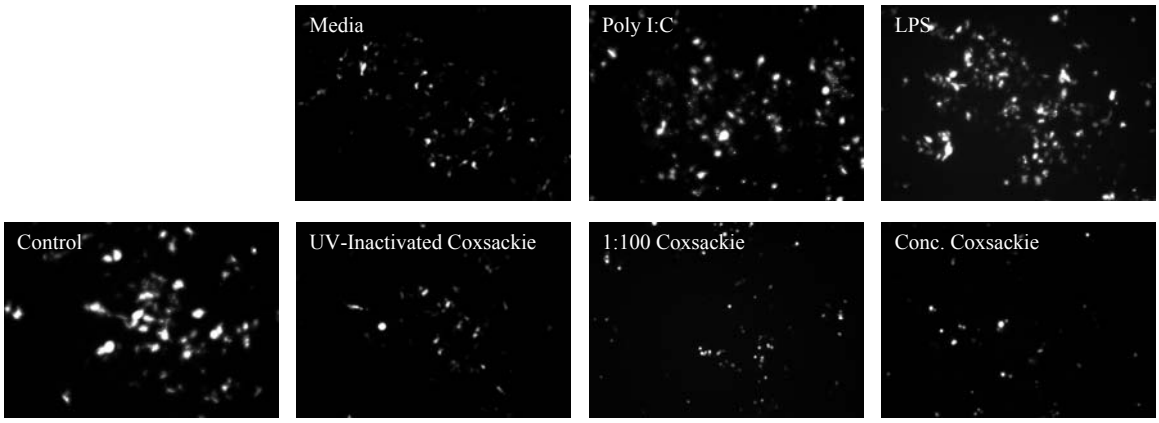
H0.14



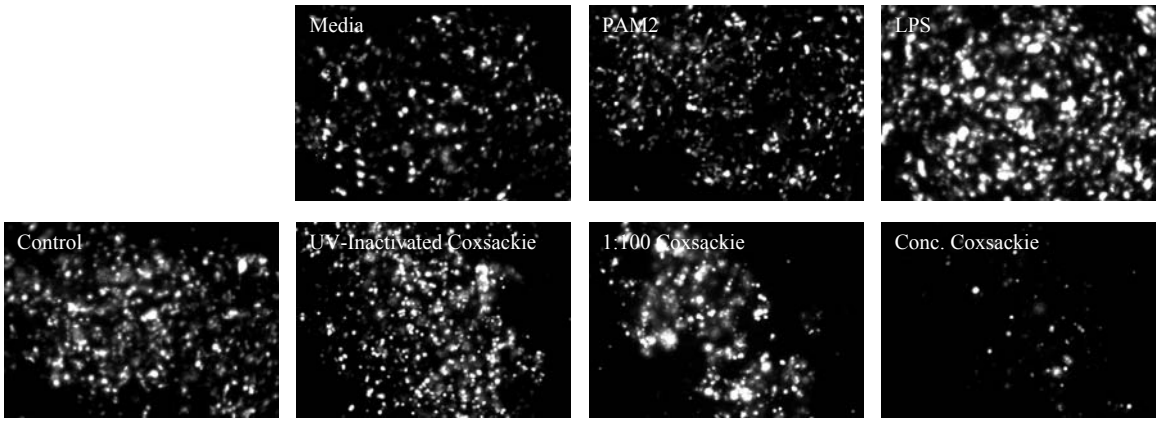
H2.14



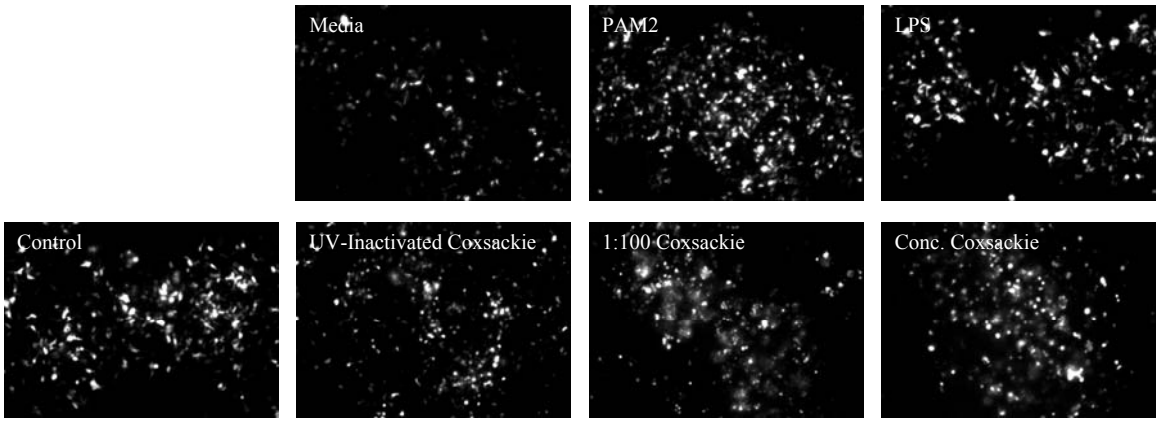
HEK TLR3



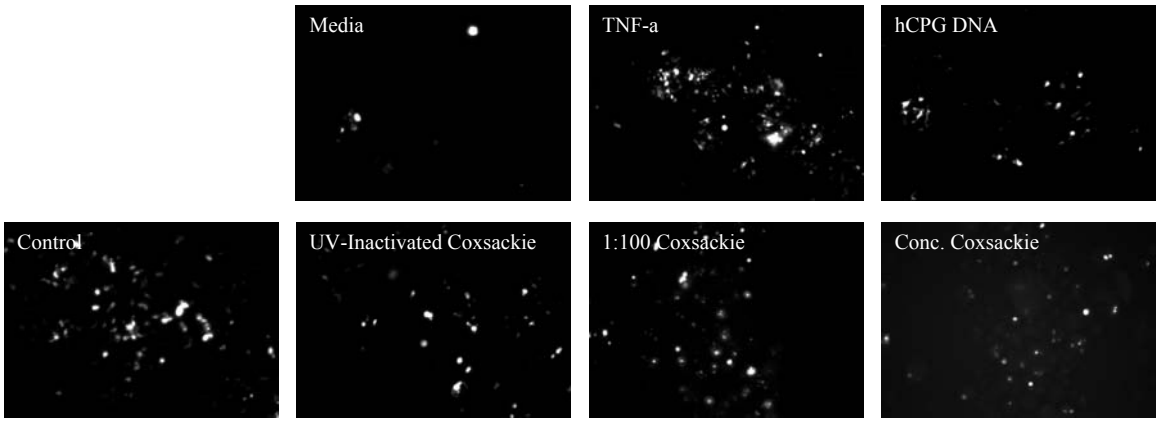
H4.14



H4.MD2



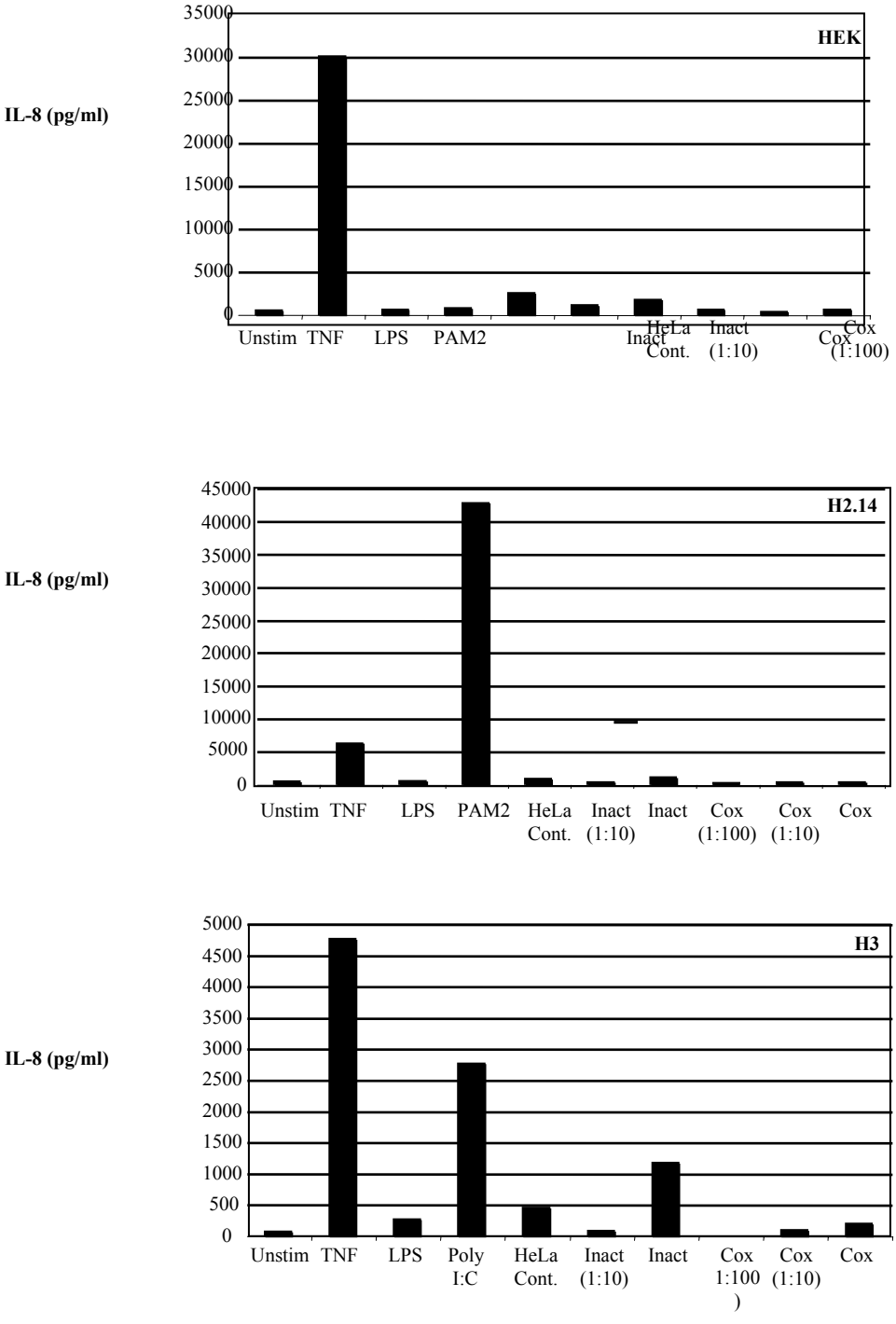
HEK YFP TLR9

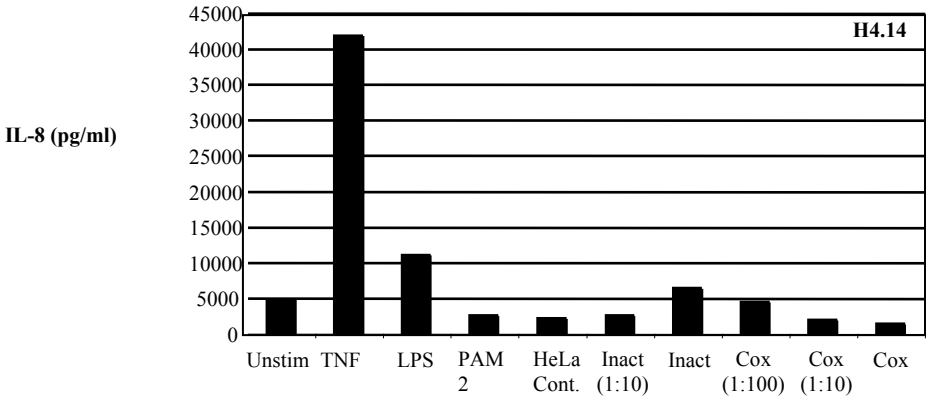


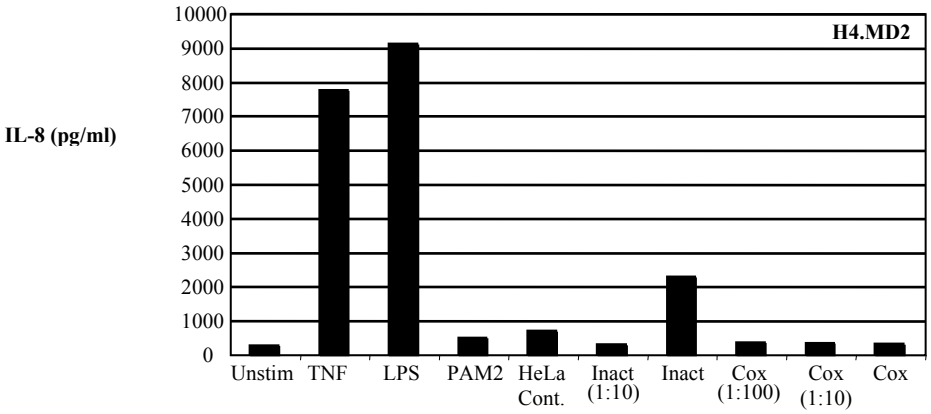
These data indicated that treatment of the reporter cells with Coxsackievirus activated NF-κB, so we next performed an experiment to determine which pathway activated NF-κB. We performed an IL-8 ELISA to determine whether IL-8 was produced by the cells stimulated by Coxsackievirus (Figure 22). These results show normal IL-8 production by the cells stimulated with TLR ligands, and in the cells stimulated by Coxsackievirus there is IL-8 production similar to non-stimulated cell lines.

This adds to the evidence that in this HEK *in vitro* system, CVB is not activating NF-κB through the TLR signal transduction pathway, but through an alternative pathway.

Figure 22: IL-8 ELISA's Performed by Dr. Neeta Shenoy




IL-8 (pg/ml)
H4.14
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Unstim
TNF
LPS
PAM 2
HeLa Cont.
Inact (1:10)
Inact
Cox (1:100)
Cox (1:10)
Cox


IL-8 (pg/ml)
H4.MD2
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Unstim
TNF
LPS
PAM2
HeLa Cont.
Inact (1:10)
Inact
Cox (1:100)
Cox (1:10)
Cox

DISCUSSION

This project began with a few complications regarding the construct created by Dr. Alex Repik, but that is all part of the scientific process. After steps were taken to determine that the construct was missing a promoter, a much simpler approach to making a construct was taken. A new construct made by Dr. Neeta Shenoy was shown to have all of the necessary parts through restriction digest and southern blot, as well as sequencing, so an initiative was made with the transfection of the HEK cell line. One of the important factors in choosing a clone to move forward was sufficient up-regulation of the DsRed signals to be obvious to the naked eye when NF-κB activation was occuring. This was because the original purpose of the project was to create a construct that was to be used to create transgenic zebra fish, and therefore the signal would have to be very strong and defined.

After moving forward with the construct determined to have the best signal specificity and strength, transfections in HEK cell lines were completed several times to make sure that the signals were significant following activation by known TLR ligands. Once the *in vitro* system had shown it could express a strong DsRed signal upon NF-κB activation, it was time to move forward with Coxsackievirus stimulation. However, by revisiting the Bonizzi and Karin (2004) paper, one must keep in mind that there are two pathways to NF-κB activation. The pathway to NF-κB activation that follows the classical pathway, which originates with a receptor like the TLRs, and results in the production of specific cytokines and chemokines. This is the one we would observe if

CVB interacts with the TLRs used in this *in vitro* system. This *in vitro* system has the ability to determine what cytokines and chemokines are being produced by the cells by removing the supernatant media and performing an ELISA. One of the specific chemokines that can be analyzed is IL-8. This chemokine is usually indicative of NF-κB activation through the classical pathway.

When Coxsackievirus was used to stimulate the NF-κB activation system, there was a high amount of NF-κB signaling in the cells where UV-inactivated virus, 1:100 dilution of the virus, and the concentrated form of the virus had been added to the cells. This would normally lead to the belief that this had occurred via the classically pathway, however, it happened in every cell line with different TLRs stably transfected into each respective cell line. This raised a few questions. Another strange thing that happened is that the cells stimulated with CVB titers lifted off the bottom of the wells. To determine whether this NF-κB signaling occurs from the classical pathway, or the alternative pathway, an IL-8 ELISA was performed to see if this chemokine was produced. The data indicated no IL-8 was produced in this *in vitro* system by the cells stimulated with CVB titres. This leads to the belief that the NF-κB signaling is occurring through an alternative pathway, which does not originate in the TLRs.

The results of this MQP are a definitive conclusion about the *in vivo* effect of CVB. This only shows what happens in this specific HEK cell line. Also, there are many cytokines and chemokines that can be analyzed, not just IL-8. While IL-8 is usually produced, there are also other cytokines produced. Also, other cell lines should be analyzed other then HEK cells. This virus may interact with TLRs in other types of cells,

and using a similar *in vitro* system it can be determined which TLR the virus may use to enter those cells. Eventually the data will shed light on what may be happening *in vivo*.

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