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**The Effect of Inflammatory Stimuli on Cryptic Peptide Presentation for Immune Surveillance**

by

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University of California, Berkeley

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Professor Nilabh Shastri, Chair
Professor Russell Vance
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Professor Eva Harris

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## Abstract

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Cytolytic T cells eliminate infected cells by recognizing intracellular peptides presented by MHC class I molecules. The antigenic peptides are derived primarily from newly synthesized proteins including those produced by cryptic translation. Previous studies have shown that in addition to the canonical AUG codon, translation can be initiated at non-AUG codons. Furthermore, translation initiation at non-AUG codons such as CUG is mechanistically distinct from canonical translation initiation as it is resistant to protein synthesis inhibitors that cause global translation shutdown. Here, we show that Toll-like receptor (TLR) signaling pathways involved in pathogen recognition enhance presentation of the cryptically translated peptides. Moreover, infection of bone-marrow derived macrophages with different viruses and bacteria or treatment with pro-inflammatory cytokines also enhances presentation of cryptically translated peptides. Thus, translation and presentation of cryptic peptides may allow the immune system to detect intracellular pathogens that inhibit host translation and presentation of peptides from conventional sources.

Dedicated to my grandparents,
Mrs. Kamala Vedanarayanan and Mr. Andhanallur S. Vedanarayanan, Mrs. Alamelu Natarajan and Mr. Lalgudi R. Natarajan.

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# Chapter 1

# Introduction

Louis Pasteur once said, "Messieurs, c'est les microbes qui auront le dernier mot," which translates to "Gentlemen, it is the microbes who will have the last word." We live in a dynamic relationship with microbes, many of which cause disease and many of which share a symbiotic or parasitic relationship with us. How does the immune system distinguish between the various kinds of microbes and how does it eliminate the disease-causing kind? How does the immune system keep up with microbes that are constantly changing and evolving? Answering these questions is what the study of immunology entails. The immune system is comprised of cells that employ several different strategies to keep these foreign agents at bay. The mammalian immune system, in addition, has the unique ability to mount a memory response against specific pathogens, which prevents reinfections and a fact that led to the revolutionary discovery of vaccinations.

## 1.1 How are pathogens recognized by the immune system?

A pathogenic infection can be detected by the innate immune system by pattern recognition receptors (PRRs) that recognize various conserved and unique features of pathogens, which are known as pathogen-associated molecular patterns (PAMPs). These PAMPs are generally essential for the survival of pathogens of which some examples include bacterial flagellin, bacterial cell-wall components lipopolysaccharide (LPS) and peptidoglycan, nucleic acid structures unique to bacteria and viruses like CpG DNA or double and single stranded RNA. One of these PRRs are the Toll-like receptors (TLRs)([41]), which were first identified in Drosophila. TLRs can sense a wide range of pathogenic ligands and are localized either on the cell surface membrane or inside endosomal compartments. When TLRs are activated by their ligands, a signaling cascade is triggered that culminates in the production of inflammatory cytokines or type I interferons ([1]). In addition to the TLRs, there are other innate immune effector molecules that can detect pathogens in the cytoplasm as well. These are the nucleotide binding and oligomerization domain (NOD)-like receptors ([49]) and the retinoid

acid-inducible gene I-like receptors (RLRs)([85]). These receptors are predominantly present in the cytoplasm (or cytosol) for those pathogens that evade the extracellular surveillance system.

Pathogens are not stagnant and unchanging in their morphology and function; they are forever evolving. Therefore, an immune system that detects conserved features of pathogens is not sufficient. The immune system would also have to evolve along with its intruders and hijackers. And this led to the development of, what we call today, the adaptive immune system. Lymphocytes are diverse in their nature and recognize very specific features of pathogens through their receptors. And each lymphocyte differs from the other in its receptor specificity ([21]). This diversity arises during the development and differentiation process of lymphocytes in the bone marrow and in the thymus. These cells are constantly undergoing a process of selection and once the lymphocyte encounters its target antigen, it becomes activated and begins to proliferate.

Lymphocytes are of two kinds: B cells and T cells. B cells differentiate into plasma cells upon activation and produce antibodies that are a secreted form of their specific receptor. T cells upon activation differentiate into different kinds of effector cells. These effector T cells have three main roles killing, activation and regulation. Both B cells and T cells are capable of differentiating into memory cells, which is required for long lasting immunity against a particular pathogen.

## 1.2 Role of antigen presentation in generating an immune response against pathogenic infections

T-cell recognition of peptides relies on the peptide being presented by certain specific membrane glycoproteins known as MHC (Major Histocompatibility Complex) molecules ([7]). The T-cell receptor, therefore, forms a complex with the MHC molecule and its cognate peptide. T cells are comprised of two main classes, one that has the cell surface protein CD8 and one that has the surface protein CD4. These molecules are important for the function of the T-cell and determine what MHC molecule it would bind to and the resulting function. Cytotoxic T cells or T-cells that kill carry CD8 while the T-cells that activate or regulate other cells carry CD4.

CD4 and CD8 T cells bind to distinct MHC molecules. CD4 T cells bind to MHC Class II molecules whilst CD8 T cells bind to MHC Class I molecules. The most important difference between these two kinds of MHC molecules, apart from their structure, is the source of the peptide that they present. MHC Class I peptides are derived from endogenously synthesized proteins and thus MHC I molecules will be able to display peptides of viral origin and the peptide repertoire can indicate a cell undergoing transformation. MHC Class II molecules display peptides that are derived from extracellular sources and end up in intracellular vesicles and thus can be indicative of pathogens living in vesicles or internalized by macrophages or B cells.

MHC Class I binding to CD8 T-cell will result in the killing of the cell that is presenting the peptide since it activates a CD8 specific T-cell response. All nucleated cells in the body present MHC Class I molecules on their cell surface. MHC Class II binding to a CD4 T-cell response triggers the release of inflammatory or suppressor cytokines from the activated CD 4 T-cell which can also activate B-cells to trigger an antibody response. MHC Class II molecules are only presented by professional antigen-presenting cells, which are dendritic cells, macrophages and B-cells.

An additional level of complexity exists in the T-cell recognition process of its cognate antigen. MHC molecules are highly polymorphic, which means that each individual carries different versions of the MHC molecule. Most people are heterozygous and express two types of MHC molecule, which increases the range of foreign peptides that they can present to the immune system. This introduces the dimension of MHC restriction, which means any given T-cell is specific not only for its peptide antigen but also for a particular type of MHC molecule.

There exist different pathways for how these peptides from various sources are generated, processed and presented on MHC molecules. Peptides presented on MHC I molecules are generated endogenously from either new protein synthesis or from degradation of existing proteins by the proteasome ([69]). The proteasome generates the C-terminus of most oligopeptides. These peptides are 4-20 amino acids in length and some of them may require further trimming on the N-terminus by aminopeptidases ([71]). Peptide fragments are then transported into the endoplasmic reticulum (ER) by molecules known as TAP (transporters associated with antigen processing). TAP binds cytosolic peptides and uses ATP to open its pore and translocate the peptide into the ER lumen. TAP is a part of the Peptide Loading Complex (PLC) ([58]; [83]).

The peptides, transported by TAP, are retained in the ER until they are bound by a MHC I molecule. These peptides are further processed in the ER by being trimmed to their optimal size, which is that of 8-10 amino acids in length in order to be able to bind to MHC I molecules. This trimming is performed by a specific aminopeptidase, called ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing), which trims the N-terminus of these peptides. The binding of the peptide to its MHC I molecule is facilitated by the PLC. The PLC consists of many different chaperones and enzymes and increases the efficiency of peptide loading by clustering all the relevant molecules in close proximity. The PLC contains multiple copies of a chaperone protein Tapasin which is bound to a peptide receptive MHC I molecule ([15]). Other chaperones surrounding the MHC I molecule are calreticulin and ERp60. Tapasin binds directly to MHC I and is necessary for optimal peptide loading. Tapasin also binds to ERp60, which then binds to calreticulin. ERp60 is a thioreductase which facilitates the formation of a di-sulfide bridge which connects the walls of the MHC I peptide-binding groove to the base ([11]).

In addition to the proteasome, there are other cytosolic proteases that also contribute to the generation of antigenic peptides. Some of these are the tripeptidylpeptidase II (TPPII) ([83]). Furthermore, internalized MHC I molecules can also bind to antigenic peptides derived from the endosomal pathway  a process that is important for the mechanism of

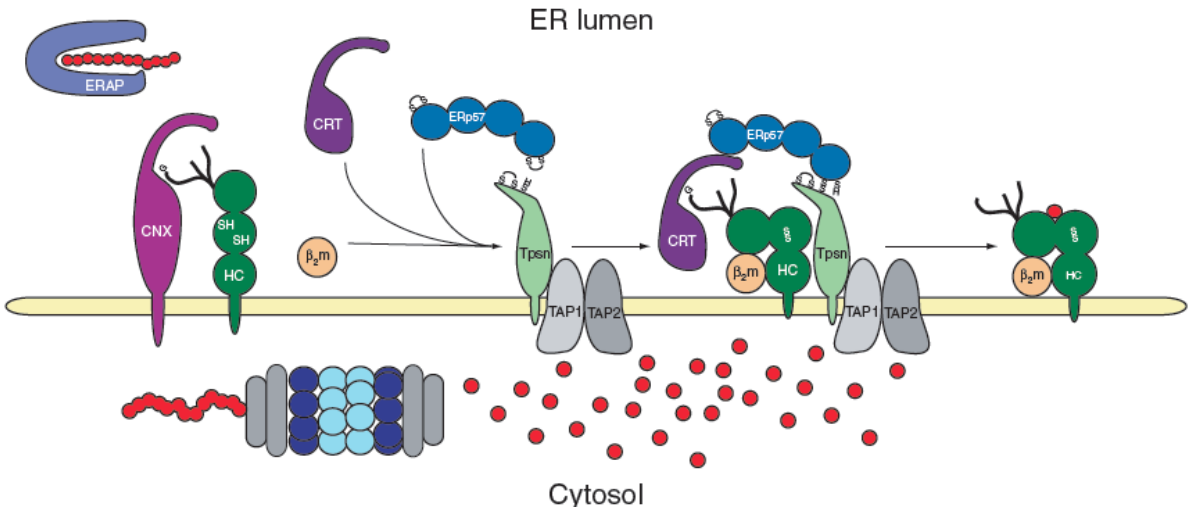


Figure 1.1: **MHC Class I assembly and loading complex (Cresswell et al. 2005)**

cross-presentation. Such peptides are generated by a different set of proteases (cathepsins) present in endosomal compartments ([83]).

## 1.3 How do pathogens evade the immune system?

Virus infections can modulate the signaling pathways involved in pathogen recognition to evade the immune system. Viruses suppress host immune responses like antiviral activity induced by Interferons (IFNs), migration of immune cells induced by cytokines and chemokines, maturation and activation of dendritic cells, and cytokine receptor and co-stimulatory molecule expression. Certain examples are viruses such as HIV, which inhibit TLRs and prevent the activation of NF-kB and thereby suppress TNF-$\alpha$ production and T-cell activation ([84]). Hepatitis C virus (HCV) degrades the TRIF adaptor molecule, downstream of TLR3 to suppress Type I IFN production. Influenza suppresses IRF3/7 activation and thereby inhibits IFN production in plasmacytoid dendritic cells ([84]). Influenza also prevents the detection of its RNA by binding to RIG-I. Finally, vaccinia virus can bind to the TIR domain of TLRs and inhibit TLR signal transduction.

The antigen presentation pathway is also targeted by various pathogens and especially viruses. Viruses employ several strategies to evade the immune system ([56]). Viruses can encode many different inhibitors to various parts of the pathway. These inhibitors ensure that a CD8+ specific T-cell response is not generated against these virally infected cells ([82]). Certain viral proteins are resistant to proteasomal degradation and thereby the immunodominant peptides are not generated. Certain viral molecules prevent peptide translocation into the ER by inhibiting TAP. Other molecules can interfere with the function

of the peptide loading onto MHC Class I molecules by preventing the formation of the tapasin-TAP complex. Herpesviruses encode molecules that reduce the surface expression of certain MHC Class I molecules by retaining them in the ER-cis-Golgi complex (LoPiccolo et al., 2003). Certain viral molecules can induce degradation of MHC Class I by inducing their ubiquitinylation via retrograde translocation and proteasome degradation or targeting them to the lysosome. Some viruses also encode MHC Class I homologs and the reasons for this are still being studied ([14]). This suggests that the immune system must constantly evolve and generate new ways of generating peptides that can be presented to CD8+ T cells.

## 1.4 Sources of peptide for antigen presentation

Peptides that are presented on MHC Class I molecules to CD8+ T cells, are derived mostly from endogenous sources - degradation of endogenously synthesized proteins or newly synthesized proteins. Apart from a couple of viral proteins that are resistant to degradation, all endogenously synthesized proteins contribute to the antigenic peptide repertoire ([69]). The theory in the field is that old proteins towards the end of their life cycle can be degraded and used as peptide precursors ([51]). However, other data indicate that newly synthesized proteins as a source encompass a more thorough representation of all the proteins within the cell and therefore are more likely to be used as peptide ([59]). This would prevent the problem of excluding certain proteins that are compartmentalized, like in the nucleus or other organelles. This also allows early viral proteins to be detected since they would be using host translational machinery. Similarly, misfolded proteins or truncated proteins that would be targeted to degradation would also be able to contribute peptides to the antigen presentation pathway allowing any cellular transformation event to be detected. These misassembled proteins that are degraded are known as defective ribosomal products or DRiPs, which are known to couple protein synthesis to the MHC Class I presentation pathway ([81]). These DRiPs enable rapid T-cell responses to be generated against viral infections. Another source of MHC Class I peptides is through the pioneer round of translation which degrades mRNAs that contain premature termination codons and produce truncated proteins, which are then degraded by the nonsense-mediated decay pathway ([3]). This was discovered by blocking cap-dependent translation, which shut down most of protein synthesis, but did not inhibit the generation of antigenic peptides.

## 1.5 Protein translation: an overview

Previously, we mentioned that many antigenic peptides are generated from new protein synthesis. Here, we review the process of protein translation. The process of mRNA translation in eukaryotes has three phases: initiation, elongation and termination. Each stage is regulated by specific translation factors. The first phase of initiation involves the formation of the 43S ribosomal complex. This step involves the binding of the 40S ribosomal subunit

to the eukaryotic translation initiation factors 1 (eIF1), eIF1A, the eIF3 complex and eIF5. These initiation factors then help load the initiator Met-tRNA (Met-tRNAi) into the P site of the 40S ribosomal subunit, with the help of eIF2-GTP. Subsequently, this complex is loaded onto the 5' end of a capped and polyadenylated mRNA by eIF4F (a multisubunit complex comprising of the cap-binding protein eIF4E, molecular scaffold eIF4G and RNA helicase eIF4A). The polyadenylated 3' end of the mRNA is recognized by a poly(A)-binding protein (PABP) which then binds with eIF4G at the 5' end resulting in a closed loop of the mRNA. An eIF4E kinase (MNK1 or MNK2) then binds eIF4G and phosphorylates eIF4E. This resulting 48S ribosomal complex then begins to scan the mRNA to locate the AUG start codon. After the recognition of the AUG start codon, which is facilitated by eIF3, eIF1 and 1A, the 60S ribosomal subunit joins the 48S complex to form an 80S ribosomal complex. The joining of the 60S subunit also triggers the release of all the initiation factors. This culminates the initiation stage. The elongation phase begins with a charged tRNA being delivered to the A site of 80S complex aided by eEF1A. GTP. A peptide bond is then formed, catalyzed by the ribosome, which is followed by 80S translocation facilitated by eEF2 and transfer of the deacetylated tRNA to the E site. The peptidyl-tRNA is retained in the P site and the A site is open to receiving another charged tRNA. Termination of translation occurs with eukaryotic release factor 1 (eRF1) recognizing a stop codon in the A site. This arrests the 80S complex and releases the polypeptide chain. Subsequently, the ribosome complex is dismantled with the help of eRF3 and other initiation factors and the ribosomal subunits are recycled ([30]).

## 1.6 Signaling pathways that regulate translation initiation

Different growth factors, hormones and mitogens can regulate protein synthesis within the cell by regulating the mRNA translation initiation process. The signal from these growth factors is transduced by the phosphatidylinositol 3-kinase (PI3K) pathway. Multiple players of the PI3K pathway converge on a kinase mTOR (mammalian target of rapamycin). mTOR is a protein that is conserved from yeast to humans and is a major effector of the PI3K pathway. When extracellular signals activate PI3K, it leads to the stimulation of the catalytic activity of Akt, which then causes the phosphorylation of mTOR kinase ([61]). Akt can be negatively regulated by the PTEN phosphatase. Akt controls protein synthesis by inactivating the TSC complex (tuberous sclerosis complex), which negatively regulates the mTOR kinase. Deregulation of the PI3K pathway occurs in many cancers. One of the ways in which Akt regulates translation is by stimulating ribosomal RNA synthesis and ribosomal DNA transcription. This is likely to be mediated by ribosomal proteins S6K1 kinase, which is a target of mTOR. Furthermore, several translation initiation factors are regulated by Akt. All components of the eIF4F complex have been found to be targets of Akt. eIF4E is also regulated by this pathway as are the eIF4E-binding proteins (4E-BPs), which are a

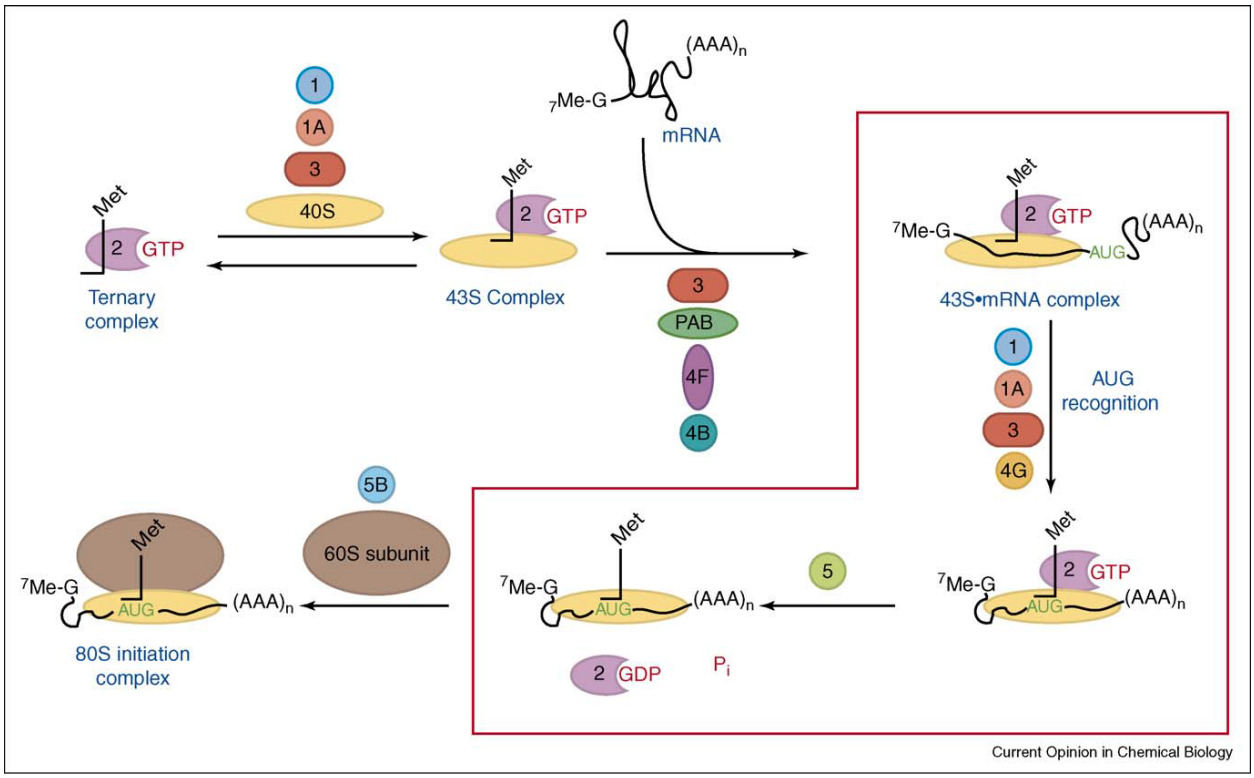


Figure 1.2: **Mechanism of translation initiation.**

family of repressor proteins that negatively regulate eIF4E. ([48]). Moreover, eIF4G1 is also a target of mTOR as rapamycin treatment inhibits its phosphorylation.

## 1.7 Translation initiation regulation

The initiation stage of translation is very highly regulated ([53]). There are many factors regulating codon selection. eIF1 promotes the continued scanning of the 48S ribosome when non-AUG codons occupy the P-site by causing a structural change that stabilizes an open conformation ([30]). eIF1 also prevents initiation at non-AUG codons by preventing the release of Pi from the eIF2-GDP-Pi. These inhibitory actions are neutralized when AUG is present in the P site.

This scanning mechanism predicts that translation mostly initiates at the AUG codon nearest the 5' end of mRNA([37]). However, this is not the only mechanism by which translation initiation events. Since ribosomal scanning is such a dynamic event, there are other phenomenon that may occur like reinitiation and context-dependent leaky scanning that can also give rise to translational products and these can act as regulatory mechanisms too ([38]). Furthermore, ribosomes can also enter at an internal site. A lot of viruses encode internal ribosome entry sites (IRES) to evade cap-dependent translational regulation.

However, there are several cellular IRES that play an important role during conditions of cellular stress, development and diseases like cancer ([27]).

Moreover, studies have shown that different amino-acyl tRNAs are selected uniformly on the ribosome. And all amino-acyl tRNAs have similar intrinsic decoding properties. This suggests that global translational regulation depends on the concentration of active ternary complex to control the rate of protein synthesis ([40]).

## 1.8 Constraints on the scanning mechanism

The 'first AUG rule' is a constraint posed on the scanning mechanism wherein protein translation initiates at the first upstream AUG that it encounters. This is an effective regulatory mechanism whereby the translational product from the upstream-most AUG can act as a agonist or antagonist isoform. Furthermore, splicing may generate an alternative isoform that lacks the upstream-most AUG thereby generating the other isoforms ([38]).

Upstream AUG codons may also act to inhibit or reduce the efficiency of a downstream open-reading frame (ORF). If the ribosome indeed scans the mRNA from the cap onwards, this would mean that the 5' untranslated regions (5' UTR) are also scanned and if there are AUG codons present in the 5' UTR, this can cause short translated peptides and reduce diminish translation downstream. Some upstream AUG codons create small ORFs that are translated and the short peptides may be degraded rapidly. This allows reinitiation at downstream AUG codons. However, the reinitiation events are often inefficient ([38]).

Furthermore, a highly structured 5' UTR sequence can also inhibit translation if it results in the formation of a secondary structure. These structured sequences include GC rich regions, stem-and-loop structures that prevent ribosome binding ([38]).

The scanning mechanism, interestingly, also allows translation initiation to occur at certain non-AUG codons like ACG or CUG. This initiation activity at these codons is generally too weak to inhibit translation at an AUG codon. However, it is not uncommon for translation to initiate upstream at these non-AUG codons in addition to the first AUG start. This is generally thought to occur in mRNAs with highly structured UTRs or leader sequences which would slow down the scanning process allowing time for a mismatched codon (similar to AUG) to pair with the initiator Met-tRNA. A lot of viruses also encode some of their genes to initiate with non-AUG codons for the generation of different protein isoforms.

As a regulatory mechanism, some of these mRNAs are generated to induce inefficient ribosomal scanning, so that the translational products from these mRNAs are low in amount. For example, genes that encode cytokines, growth factors, kinases and transcription factors have to be tightly regulated ([12]). While they need to be constantly generated, there needs to be a shutdown mechanism also (in addition to protein turnover) to limit their synthesis. However, there are other mechanisms that do not support excessive translation.

Context-dependent leaky scanning ([38]) exists as a mechanism to gauge if the AUG start codon lies in an optimal context. A particular consensus sequence immediately upstream and downstream to the AUG codon determines whether it is optimal to initiate translation.

This is knows as the Kozak context. A strong context is defined as GCCRCCaugG where R is a purine. The identity of -3 and +4 positions contributes greatly to the strength of the context. AUGs that lie in a poor context have reduced translation initiation activity where some ribosomes may initiate but others will continue scanning. This can result in the generation of multiple isoforms from the same mRNA. Furthermore, initiation at non-AUG codons also requires excellent context despite which scanning could be leaky to the weak strength of the codon itself. Research shows that this leaky scanning is not just an erroneous occurrence but also a deliberately employed regulatory mechanism ([38]). There are particular N-terminal and C-terminal residues in the eukaryotic initiation factor eIF1A that regulate its binding to the preinitiation complex and this is an important determinant of AUG selection. Certain C-terminal mutants in eIF1A can enhance initiation at UUG codons and decrease leaky scanning at AUG codons. Certain N-terminal mutations in eIF1A increase leaky scanning at AUG codons. ([16])

Reinitiation ([38]), referred to earlier, occurs when there are small open reading frames (ORFs) near the 5' end. This is thought to occur when the 60S subunit detaches upon reaching termination site and the 40S subunit remains bound to the mRNA and continues to scan further along the mRNA and initiate at a downstream AUG codon. For this, the 40S subunit reacquires the initiator Met-tRNA. However, reinitiation can only occur if the upstream ORFs are short, because otherwise initiation factors would dissociate from the ribosome.

All of these different mechanisms of translation initiation can generate antigenic peptides, which can stimulate a CD8+ T-cell response against viruses and tumor cells ([38]). Leaky scanning maybe the source of antigens in cases where the major ORF starts at an AUG codon in a suboptimal context but the antigen peptide is derived from initiation at the downstream and out-of-frame AUG. In one example, a tumor rejection antigen was derived from a translational shift event, which leads to translation from an AUG codon upstream to the in-frame AUG codon. In certain cases, antigenic peptides were derived from an alternative form of mRNA. In other examples, potent tumor rejection antigens were derived from an internal AUG codon in a truncated cDNA. In a ribosomal profiling study ([28]), increased translation initiation activity was found at non-AUG codons. This activity was found to be regulated during times of stress like nutrient deprivation. Since these studies were performed in-vivo in yeast, it confirmed that non-AUG mediated initiation is a regulated event and that can be experimentally detected.

## 1.9 Cryptic translation

Some of the endogenously generated peptides in the MHC Class I pathway can also originate from sources other than translation of the primary open reading frame. These sources were termed cryptic because their origin was unconventional and unknown. These cryptically derived antigenic peptides contribute to the diversity of the peptide repertoire presented on the cell surface making the process of immune surveillance more effective. Cryptically

translated antigenic peptides can arise in many different ways. Some of the sources are alternative reading frames of a gene, intron-exon junctions, and untranslated regions of an mRNA transcript ([69]) or the usage of an alternate initiation codon. There is a study that has curated numerous mRNAs that initiate translation using a non-AUG codon ([74]), some of which have been annotated to use an alternate initiation codon. This study has shown that most mRNAs that use non-AUG start sites are involved in some regulatory function or signaling mechanism and are either kinases, growth factors, DNA/RNA binding proteins or are involved in immune responses or cell proliferation. This study also showed that the codon context surrounding the non-AUG initiation codon is highly conserved and thereby gives rise to an alternative transcript or isoform of the protein.

## 1.10 Characteristics of non-AUG initiated translation

Previous studies by the Shastri laboratory have established that the translation initiation of antigenic peptides from a non-AUG codon can occur in normal cells and is a cell-intrinsic mechanism ([46]). A CUG-initiated peptide, LYL8, derived from the H60 minor histocompatibility gene ([47]) was shown to elicit CD8+ T cell responses and induce self-tolerance in-vivo ([66]). The mechanism of CUG-initiated translation was shown to be distinct from canonical AUG-initiated translation in many ways ([67]). Firstly, the CUG initiation codon was decoded as a leucine and not as the canonical methionine. Ribosomes were found to specifically scan for the CUG initiation codon in a 5' to 3' manner and ribosomal binding to the CUG codon occurred during the initiation phase. More importantly, CUG-initiated translation was resistant to translation inhibitors like bruceantin and edeine that inhibited canonical AUG-initiated translation. Finally, CUG-initiated translation was not inhibited by the phosphorylation of eukaryotic translation factor 2$\alpha$ (eIF2$\alpha$), which leads to the inhibition of canonical translation and global protein synthesis. The phosphorylation of eIF2$\alpha$ occurs under conditions of cellular stress that inhibit host translation. The persistence of CUG-initiated translation under such conditions further suggests that this phenomenon is mechanistically different from canonical translation initiation. EIF2$\alpha$ phosphorylation can be triggered by different stress factors like oxidative stress and ER stress. Previous work by Ow Y. (PhD Thesis. 2008) established that these different stress inducers upregulate CUG-initiated translation while inhibiting AUG-initiated translation.

## 1.11 Mechanism of cryptic translation

Recent studies ([72]) have revealed a novel mechanism for CUG initiated translation. Schwab et al. (2003) alluded to specific ribosomes scanning for the CUG codon versus the AUG codon and that this led to the non-methionine starts. Starck et al. (2012) further reinforced this hypothesis by showing that there was a novel initiator tRNA that was present at CUG start codons and that this initiator tRNA was charged with a Leucine and had the anticodon

CAG. This study also showed the requirement for a particular initiation factor eIF2A for CUG initiation.

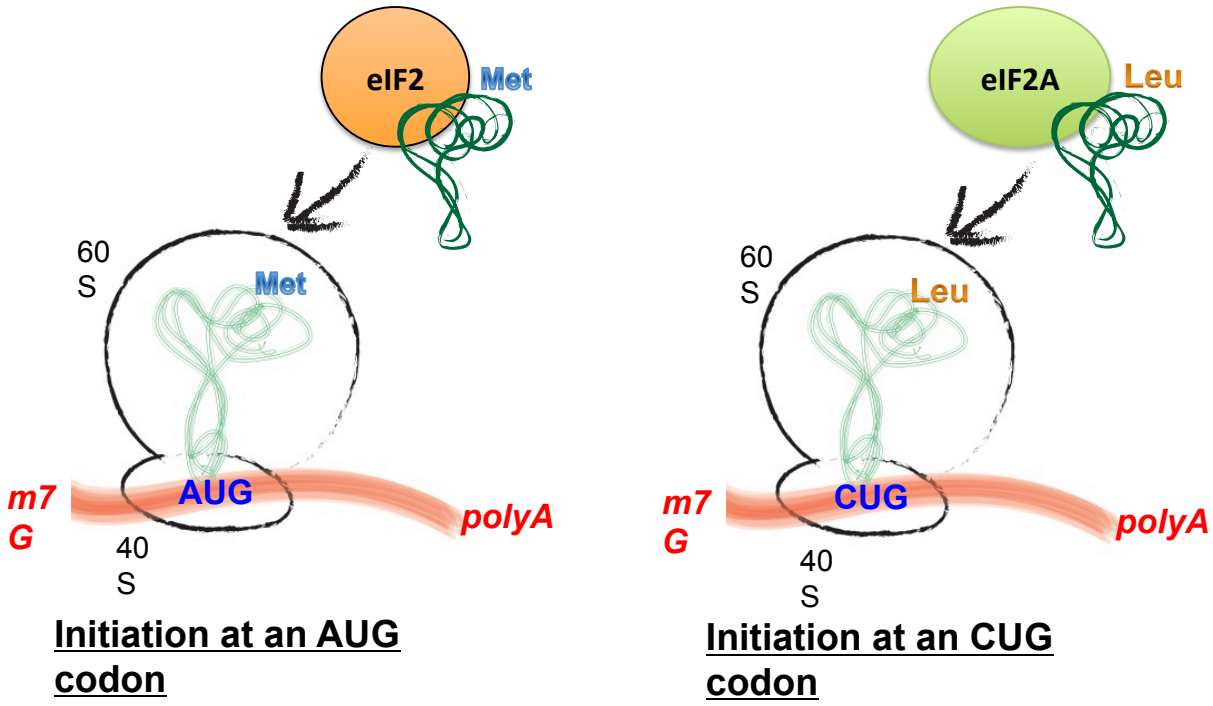


Figure 1.3: **Mechanism of cryptic translation.**

## 1.12 Examples of cryptic translation in immunology

Since viruses pack many genes into their small genomes, many of them are encoded in overlapping reading frames and a variety of unconventional mechanisms are used to translate them. Many viral antigenic epitopes arise from alternative reading frames ([10]), especially in the HIV genome. CD8+ T-cell responses are generated against some of these cryptic epitopes arising from alternative reading frames of HIV-1 genome. Certain cryptic epitopes generated from splice variants of HIV-1 genes were also shown to be targets for T-cell responses. Interestingly, these responses were shown to be required in order to be able to control the disease. Non-controllers did not have T-cell responses to these cryptic epitopes ([20]) arising from antisense transcripts. These T-cell responses generated against the cryptic epitopes are supposed to be essential to generate a wide and robust vaccine response against HIV-1. Moreover, a lot of these cryptic epitopes were shown to evolve during the first year of HIV-1 infection and there is evidence of reversion of the cryptic epitopes to the original consensus sequence, which indicates that these cryptic epitopes might be required for viral fitness ([5]). Thus, targeting cryptic epitopes is important for identifying novel vaccine targets.

Other examples of viral cryptic antigens are that of ones arising from frameshifts introduced into the Influenza nucleoprotein. This was predicted to occur by read-through of the AUG-initiation codon and initiation downstream of the conventional start site ([9]). There is also evidence for a protein arising from an alternative reading frame (ARF) of HCV which leads to oncogenesis progression during HCV infection ([26]). An effective cancer vaccine would seek to target epitopes arising from these ARF proteins. In addition to controlling viral infections and being important in viral vaccine designs, cryptic epitopes were shown to arise from an adenoviral vector used in a gene therapy trial ([42]). In this study, they could not identify the specific source of the cryptic peptide except for that it was from an alternative reading frame and that it was immunogenic during the therapeutic gene transfer. This emphasizes the importance of cryptic epitopes and requires careful analysis of all transgene sequences of viral alternative reading frames.

Furthermore, there are several examples of cryptic peptides arising from endogenous sources also. A CD8+ T-cell epitope arising from VEGF was characterized ([80]) and was shown to arise in a cryptic manner from an alternative initiation codon CUG. The implication of this finding is that the tumor-associated antigens from the vascular endothelial growth factor (VEGF) protein, can be targeted by T-cells and this could be a potential peptide-based anti-VEGF immunotherapy in addition to other anti-VEGF cancer therapies. Furthermore, cryptic epitopes were shown to arise from proteins such as AIM2 ([2]) and NA17-A. T-cell responses to these epitopes were widely detected in melanoma patients, but not in healthy individuals or patients with breast or renal caners. These antigens can be used as model antigens in clinical and preclinical settings for monitoring the progression of the disease. Thus, increasing occurrences of cryptic peptides have been established. However, no mechanism of how these peptides are arising or how their expression can be controlled was established.

## 1.13 Future questions about cryptic translation

Previous studies in the Shastri Laboratory have established that there are specific cellular stresses like oxidative stress, nutrient deprivation and agents that inhibit conventional translation do not affect cryptic CUG initiated translation. However, all of these stresses were induced by chemical agents. The question remains if there are any physiological stimuli that can enhance or differentially regulate cryptic translation in comparison with canonical translation.

The purpose of this study is to investigate if antigenic peptides that are translated in a cryptic manner can be differentially presented to CD8+ T cells under conditions when conventional translation is inhibited. This continued presentation of cryptically translated peptides would be indicative that the cell is under stress. This is particularly important for viral infections where viruses can inhibit host translation and or inhibit antigen presentation to T-cells. The increased repertoire of cryptically translated peptides on the cell surface could be indicative that the cell is under the stress of a viral infection and would act to

alert the immune system. For this purpose, instead of using cryptic peptides arising from viral genomes, we are using a model that utilizes a self-model of peptide presentation. The assumption is that these cryptic peptide-specific T-cells would not be negatively selected against during T-cell development. If inflammatory stimuli can increase cryptic peptide presentation, then this would be a novel way to alert the immune system of an infection, which attempts to evade immune-surveillance.

In this thesis ,we address how inflammatory stimuli can regulate cryptic peptide presentation. (By cryptic peptide presentation, we mean presentation of cryptically translated peptides). In chapter 2, we address whether the Toll-like receptor (TLR) signaling pathway can regulate cryptic peptide presentation. In chapter 3, we determine how pathogen infection affects cryptic peptide presentation. In chapter 4, we establish that cytokines can also enhance cryptic peptide presentation and that the effect of pathogens is an indirect one. In chapter 5, we attempt to define the mechanism by which enhanced cryptic peptide presentation occurs. In chapter 6, we describe an alternate way of studying cryptic translation of antigenic peptides. In chapter 7, we propose further studies that can be undertaken to learn more about cryptic translation of antigenic peptides, in-vivo. Finally in chapter 8, we describe the techniques that were used to perform all the experiments in this study.

# Chapter 2

# Toll-Like Receptor (TLR) Ligands Can Enhance Presentation Of Cryptically Translated Antigenic Peptides

**Summary**

Cryptic translation is known to be a distinct mechanism from conventional translation. It has been established that different kinds of cellular stresses, which inhibit conventional translation, do not inhibit cryptic translation. However, the question that remains is, whether pathogen infection can affect cryptic translation. Different pathogens affect translation differentially. Certain viruses are known to hijack the translation machinery in order to preferentially produce their own proteins ([8]) and prevent anti-viral genes from being generated. Some viruses tend to inhibit the host translational machinery ([77]). In order to assess how virus infection affects cryptic translation, pathogen associated molecular patterns (PAMPs) were used to stimulate cells that can respond to pathogen infection. For this purpose, macrophages and dendritic cells were chosen since they are the first line of defense against infection([86]). These PAMPs activate particular receptors localized on the cell surface or in endosomes and they are known as Toll-like receptors (TLRs)([41]). Activation of these TLRs would trigger the same signaling pathways that would be triggered during a pathogen infection. Our hypothesis is that presentation of peptides that arise from cryptic translation would not be inhibited by any stimulus that diminishes presentation of peptides generated from conventional translation.

## 2.1 Toll-like receptor ligands can enhance cryptic peptide presentation in an in-vitro model system

### Pam3CSK4 and CpG can enhance cryptic peptide presentation in immortalized macrophages

Preliminary studies involving vaccinia virus were performed in immortalized macrophages (unpublished data). Vaccinia virus was selected because it is known to inhibit host translation ([65]).This study showed that the presentation of the cryptic CUG-initiated antigenic peptide was unaffected by vaccinia virus infection whilst the presentation of the canonical AUG-initiated peptide was severely inhibited. Vaccinia virus is detected by Toll-like receptor 2 (TLR2) on macrophages ([87]). This led to the question of whether innate immune signaling pathways can be used to regulate cryptic non-AUG initiated translation.

An in-vitro model system was used to study the effect of TLR signaling on cryptic translation of antigenic peptides. This model system involves the use of cDNA constructs encoding the same peptide with a conventional initiation mechanism through the AUG codon and with a cryptic initiation mechanism through the CUG codon. The peptide LTFNYRNL (abbreviated as LYL8 from here on) is the naturally processed product of the H60 minor histocompatability locus ([47]). This peptide binds to the $K^b$ MHC class I molecule. The first N terminal amino acid of this peptide can be replaced with a methionine, to render the MYL8 peptide, without compromising its ability to bind to $K^b$. The cDNA constructs encoding these peptides will be described as [ATG]-YL8 and [CTG]-YL8, giving rise to MYL8 and LYL8 respectively.

Immortalized macrophages of the C57BL/6 (B6) background were obtained from the laboratories of Dr. Greg Barton and Dr. Russell Vance (UC Berkeley). mRNA generated from the [ATG]-YL8, [CTG]-YL8 and [CCC]-YL8 cDNA constructs were transfected into these macrophages which are of the $H2^B$ haplotype. mRNA was used in order to evade any transcriptional or post-transcriptional regulation. The transfection was performed for a duration of 4 hours, after which the cells were stimulated with different TLR ligands for a duration of 4-6 hours. The amount of translated peptide was measured using a T-cell hybridoma assay in which the amount of peptide was quantitated by a colorimetric assay. The T-cell hybridomas are immortalized T cells, specific for a particular peptide-MHC Class I complex. They also contain an inducible lacZ reporter downstream of the nuclear factor of activated T cells (NFAT) enhancer element of the IL-2 gene ([64]). Therefore, upon T-cell receptor (TCR) stimulation (which leads to T-cell activation and production of IL-2), lacZ would be induced. Addition of a substrate CPRG (chlorophenol red-$\beta$-D-galactopyranoside) leads to its cleavage by the $\beta$-galactosidase protein to render a colored product that can be quantified by a colorimetric assay. The T-cell hybridoma, BCZ103, is specific for the LYL8 peptide (translated product of [CUG]-YL8) bound to the $K^b$ MHC Class I molecule. However, this hybridoma can also detect the MYL8 peptide (translated product of [AUG]-YL8) at a lower sensitivity ([46]). When Pam3CSK4, a bacterial lipopeptide and a TLR2 ligand, was

added to macrophages transfected with [CUG]-YL8, which is representative of a cryptically translated peptide, the BCZ103 response to [CUG]-YL8 was enhanced in comparison to the untreated condition (Figure 2.1). [AUG]-YL8 is representative of a conventionally generated peptide. When the mRNA of this construct was transfected into immortalized macrophages and the cells were treated with Pam3CSK4, there was no difference in the BCZ103 response to the MYL8 peptide between the untreated and Pam3CSK4 treated conditions. [CCC]-YL8 was used as a negative control in each of these experiments since [CCC] does not elicit much translation initiation activity. Therefore, no BCZ103 response was detected to the the peptide product of [CCC]-YL8.

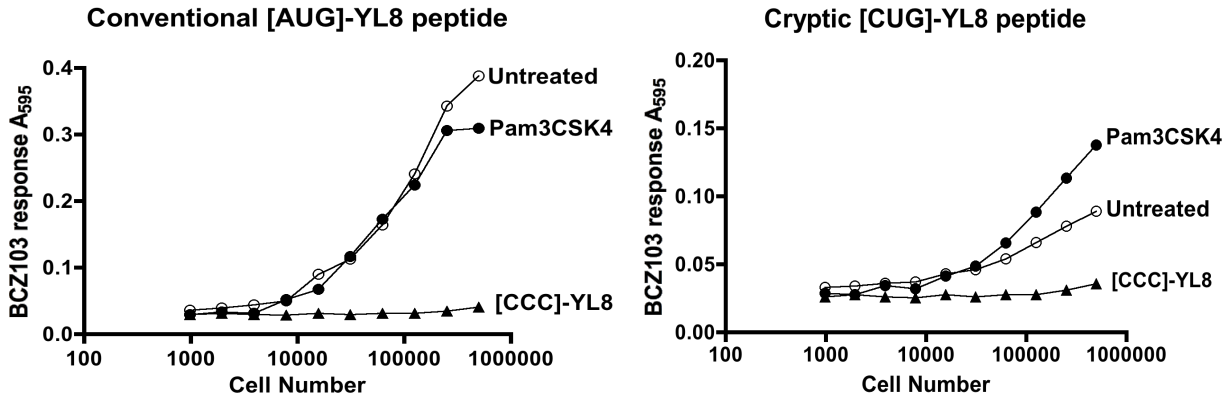


Figure 2.1: **Pam3CSK4 enhances the T-cell response to the cryptic [CUG]-YL8 peptide.** mRNA encoding [AUG]-YL8, [CUG]-YL8 or [CCC]-YL8 constructs were transfected into C57BL/6 (B6) immortalized macrophages for a duration of 4 hours. The macrophages were then stimulated with Pam3CSK4 for duration of 4-6 hours. The macrophages were then harvested and cultured with the BCZ103 hybridoma. Peptide presentation is represented as a function of the $\beta$-galactosidase activity from the activated T-cell hybridoma, which is measured through a colorimetric assay and recorded at an absorbance of 595nm. (Data is representative of three independent experiments).

In addition to Pam3CSK4, another TLR ligand CpG (unmethylated CpG, which is a TLR9 ligand) was used in a similar experimental setup as described above. When macrophages were transfected with [CUG]-YL8, an increased BCZ103 response to the LYL8 peptide was observed in the presence of CpG, compared to the untreated condition. The BCZ103 response to MYL8 in cells transfected with [AUG]-YL8 and treated with CpG remained similar to that of the untreated condition.(Figure 2.2)

Each experiment measures the T-cell response as a function of the lacZ activity, measured as absorbance at a wavelength of 595nm. Therefore, every experiment would have varying levels of lacZ activity depending on the sensitivity of the T-cell hybridoma in that experiment. In order to statistically quantify the effects from multiple experiments, it is not appropriate to group multiple experiments and average the T-cell response, because of the increased

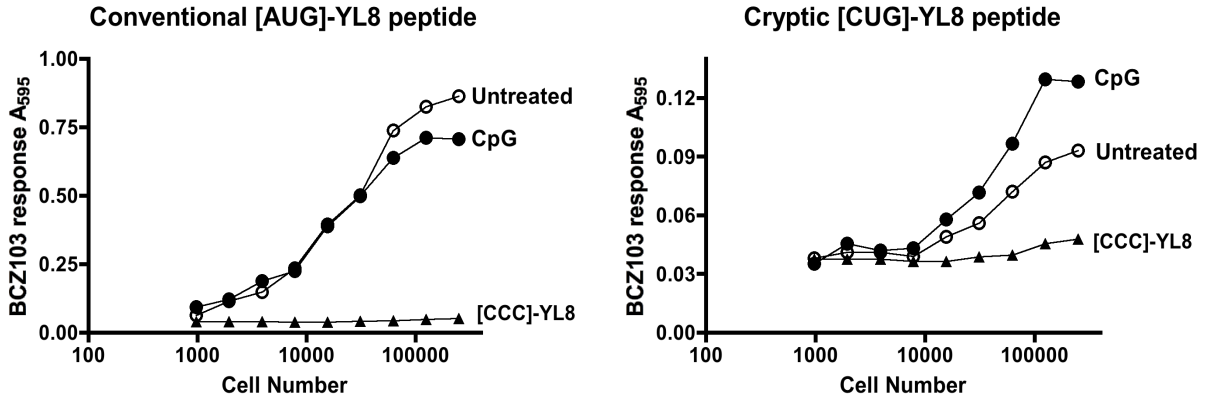


Figure 2.2: **CpG enhances T-cell response to the cryptic [CUG]-YL8 peptide.**: mRNA encoding [AUG]-YL8, [CUG]-YL8 or [CCC]-YL8 constructs were transfected into C57BL/6 (B6) immortalized macrophages for a duration of 4 hours. The macrophages were then stimulated with CpG for duration of 4-6 hours. The macrophages were then harvested and cultured with the BCZ103 hybridoma and peptide presentation was measured. (Data is representative of three independent experiments).

variability in responses. Therefore, a way, in which multiple experiments were combined for statistical purposes was, to obtain the area under the curve for each of the different conditions in an experiment. The value obtained for the TLR ligand treated condition was represented as a percentage of the untreated condition, with the untreated condition being 100%. Thus, values for each condition, across multiple experiments were averaged and represented as a bar graph shown in Figure 2.3.

For cells transfected with [CUG]-YL8, the samples that were treated with Pam3CSK4 and CpG had a greater value for area under the curve, since the T-cell response curves were enhanced. However, for the cells transfected with [AUG]-YL8, there was minimal difference in the values for area under the curve, between the untreated and TLR-ligand treated conditions. This showed, that in these in-vitro mRNA transfection experiments, the T-cell response to the cryptically translated peptide was differentially enhanced compared to the T-cell response to the conventionally translated peptide.

Interestingly, when two other TLR ligands, LPS (lipopolysaccharide, a TLR4 ligand) and PolyI:C (dsRNA and a TLR3 ligand), were used in these in-vitro experiments, similar effects as reported above were not observed. An increased T-cell response to the LYL8 peptide in the presence of LPS was not observed (Figure 2.4). The tested concentration of LPS for most of the experiments was 1ug/mL. This may have been too high a concentration, therefore, lower concentrations of LPS were tested. When lower concentrations of LPS were used, there seemed to be slight increases in the T-cell responses to the LYL8 peptide (Figure 2.5), but not as pronounced as what was seen with Pam3CSK4 or CpG. The reason for this is unknown since it was expected that LPS would be able to enhance cryptic peptide

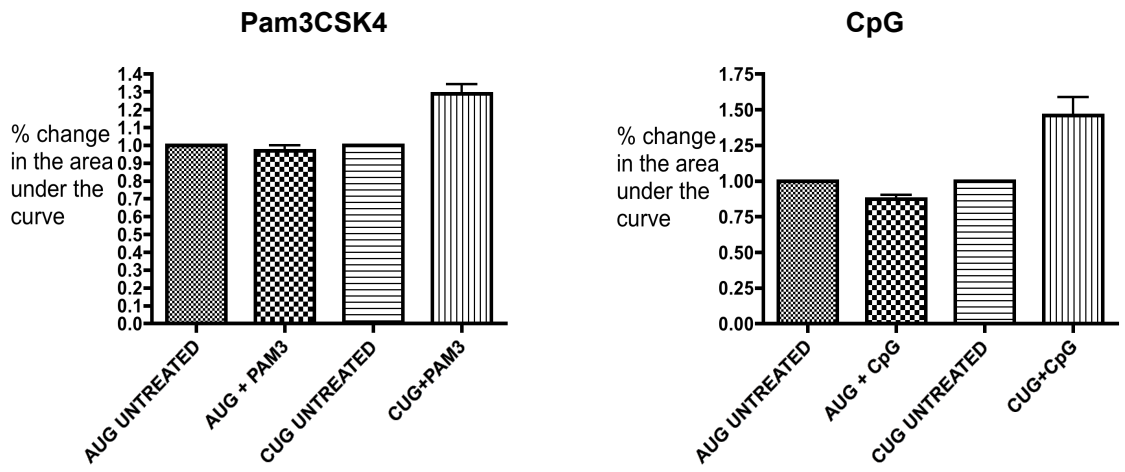


Figure 2.3: **Pam3CSK4 and CpG enhance cryptic peptide specific T-cell responses more than conventional peptide specific T-cell responses.**: The area under the curve was calculated for every T-cell response curve in each experiment. These values were represented as a percentage of the untreated condition and averaged across 11 independent experiments for Pam3CSK4 and 8 independent experiments for CpG.

presentation. In this particular experiment, we also observed slight increases in the T-cell responses to the MYL8 peptide. This enhancement of the T-cell response to the MYL8 peptide was not reported before, for any of the other TLR ligands.

With respect to PolyI:C, it was expected that this TLR3 ligand would activate PKR (Protein Kinase R) ([19]), which would then trigger apoptosis of the cell, since dsRNA is representative of a viral ligand. This would mean that presentation of the conventionally translated peptide would decrease with PolyI:C stimulation. But given that cryptic translation is resistant to many such stimuli, it would be resistant to the effects of PolyI:C. The T-cell response to the MYL8 peptide was inhibited in the presence of PolyI:C (Figure 2.6), however the T-cell response to the LYL8 peptide was unchanged in the presence of PolyI:C. Therefore, in this in-vitro system PolyI:C does not act to enhance the presentation of the cryptically translated peptide. However, given the unique properties of cryptic translation it is evidently resistant to the inhibitory effects of PolyI:C.

## Increased presentation of the cryptically translated peptide is observed by quantifying the amount of peptides through HPLC analysis.

In addition to T-cell presentation assays, an alternate method to assess peptide amounts is to transfect the cDNA constructs into cells, extract the peptides from the cells and fractionate them via reverse-phase high performance liquid chromatography (RP-HPLC). In this experi-

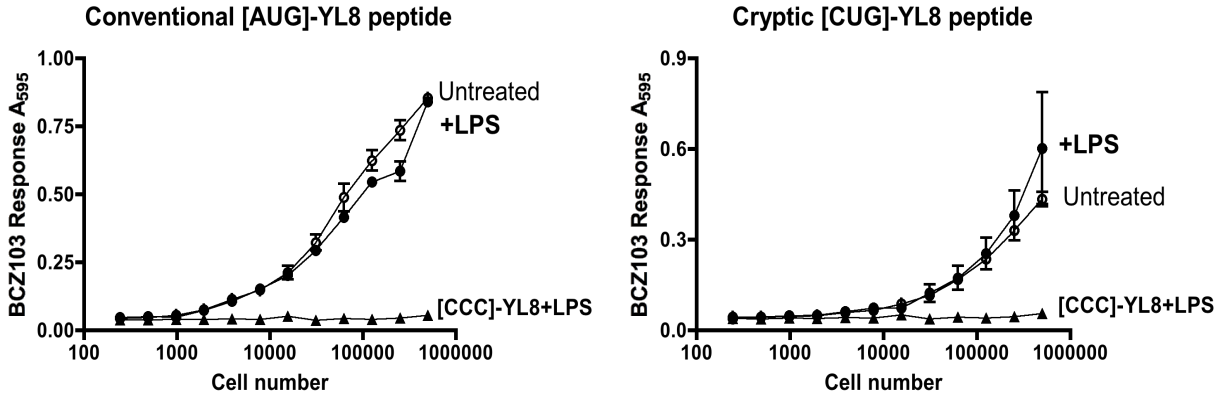


Figure 2.4: **LPS does not enhance cryptic peptide presentation in the in-vitro model system.**: mRNA encoding [AUG]-YL8, [CUG]-YL8 or [CCC]-YL8 constructs were transfected into C57BL/6 (B6) immortalized macrophages for a duration of 4 hours. The macrophages were then stimulated with LPS for duration of 4-6 hours. The macrophages were then harvested and cultured with the BCZ103 hybridoma and peptide presentation was measured. (Data is representative of three independent experiments).

ment, we used the cDNA constructs encoding [CTG]-YL8 and [ATG]-YL8. These constructs were transfected into Cos7 cells, which are African green monkey kidney fibroblast-like cell line and are suitable for transfection. After 24-48 hours post transfection, the peptides were extracted using 10% acetic acid and then filtered through a 10kDa filter to exclude all the large molecules above 10kDa. This filtered extract is then injected into an RP-HPLC column. The HPLC column is a C18 hydrophobic column, where the more hydrophobic peptides will get retained for a longer duration in the column due to hydrophobic attraction forces. The LYL8 peptide, owing to the leucine being more hydrophobic than the methionine, fractionates later than the MYL8 peptide.

When [CTG]-YL8 cDNA is transfected into Cos7 cells, there are two forms of the peptide that arise from it. The [CUG] codon gets decoded as a methionine residue due to wobble base pairing, which gives rise to the MYL8 peptide. In addition, the [CUG] codon also gets cryptically decoded into a leucine, which gives rise to the LYL8 peptide. This can be seen when the peptides are fractionated by RP-HPLC; the more hydrophobic LYL8 elutes later between fractions 35-38, while the MYL8 isoform elutes earlier between fractions 25-30. The more interesting aspect is the relative amounts of the MYL8 and LYL8 peptides that arise from the [CUG]-YL8 gene. Most of it is cryptically decoded as LYL8 as observed by the high peak of LYL8 at fraction 35-38 whilst the MYL8 peak is fairly small in comparison. The [AUG]-YL8 gene gives rise just to one peptide product, which is the MYL8 peptide.

Cos7 cells do not express TLRs endogenously, therefore TLR2 was transfected along with the $K^b$ MHC Class I molecule and the cDNA constructs for the antigenic peptide. Upon Pam3CSK4 treatment of the [CTG]-YL8 transfected cells, there was an increase in

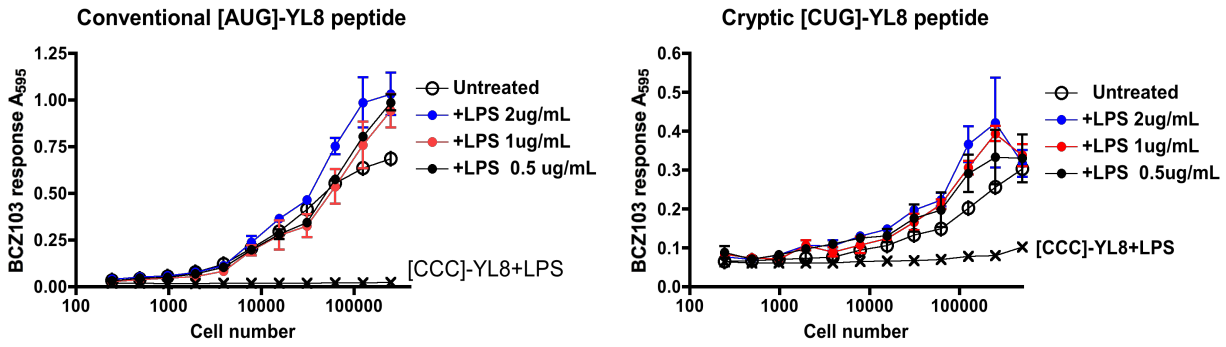


Figure 2.5: **Varying the doses of LPS slightly enhances cryptic peptide presentation.**: mRNA encoding [AUG]-YL8, [CUG]-YL8 or [CCC]-YL8 constructs were transfected into C57BL/6 (B6) immortalized macrophages for a duration of 4 hours. The macrophages were then stimulated with LPS (at either 1ug/mL 1X, 0.5ug/mL 0.5X or 2ug/mL 2X) for duration of 4-6 hours. The macrophages were then harvested and cultured with the BCZ103 hybridoma and peptide presentation was measured.

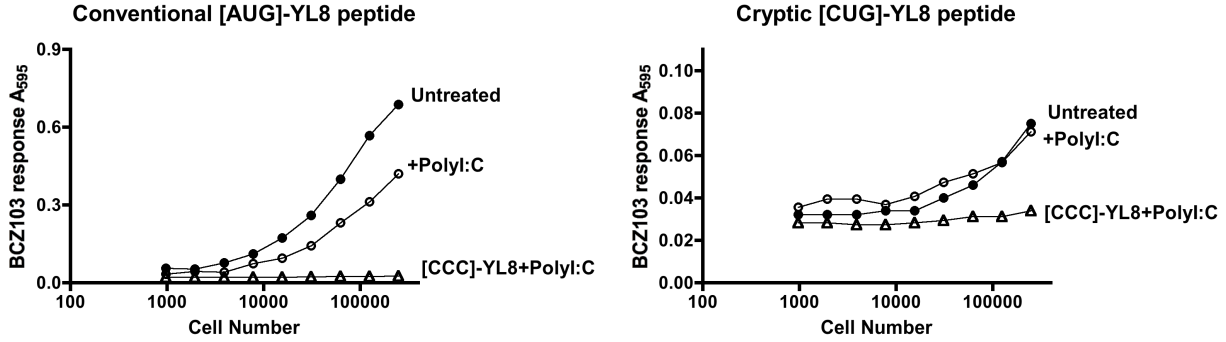


Figure 2.6: **PolyI:C does not inhibit [CUG]-YL8 specific T-cell responses**: mRNA encoding [AUG]-YL8, [CUG]-YL8 or [CCC]-YL8 constructs were transfected into C57BL/6 (B6) immortalized macrophages for a duration of 4 hours. The macrophages were then stimulated with PolyI:C for duration of 4-6 hours. The macrophages were then harvested and cultured with the BCZ103 hybridoma and peptide presentation was measured. (Data is representative of two independent experiments).

the LYL8 peak whilst the MYL8 peak remained unchanged in the same sample. Therefore, this acted as an internal control within the experiment. When Pam3CSK4 was added to the [ATG]-YL8 transfected cells, the MYL8 peptide amount remained unchanged (Figure 2.7). This confirms the result observed in the mRNA transfection experiments, that Pam3CSK4 enhances the amount of cryptic peptide presented to CD8+T cells.

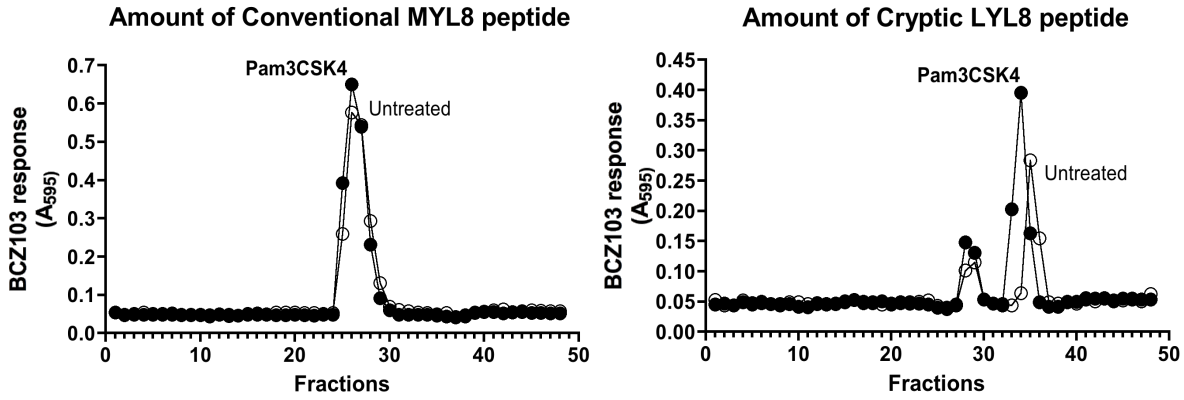


Figure 2.7: **Analysis of peptide amounts by extraction of peptides and fractionation by RP-HPLC.**: cDNA constructs encoding [ATG]-YL8 or [CTG]-YL8, along with the $K^b$ MHC Class I molecule and TLR2 were transfected into Cos7 cells. These cells were then treated with Pam3CSK4. Peptides were extracted by acid and fractionated by RP-HPLC. K89 antigen presenting cells and BCZ103 hybridoma cells were added to the fractionated peptide samples to measure the amount of peptide through the T-cell assay.

## GFP expression from a CUG initiation codon was not enhanced upon Pam3CSK4 stimulation

All of the experiments described above are assessing peptide antigens that were translated in a cryptic manner, but not of cryptically translated proteins. In order to assess the effect of TLR ligands on cryptically translated proteins, mRNA constructs encoding [AUG]-GFP, [CUG]-GFP or [CCC]-GFP were used, wherein GFP is the reporter protein being assessed. These constructs were transfected into immortalized macrophages. GFP expression was analyzed by flow cytometry (Figure 2.8). The mean fluorescent intensity of the cells transfected with [AUG]-GFP was almost 30 times greater than that of the cells transfected with [CUG]-GFP. However, Pam3CSK4 treatment was not able to enhance the amount of GFP initiated with a CUG. Levels of AUG initiated GFP were also unchanged upon Pam3CSK4 treatment. This suggested that the TLR ligands were more effective in enhancing antigenic peptide amounts than overall translation. However, the reason for not seeing changes in the GFP levels could also be because the rate at which it is degraded or generated may not be as responsive to TLR ligands. Therefore, this assay may not be sensitive enough to detect those changes.

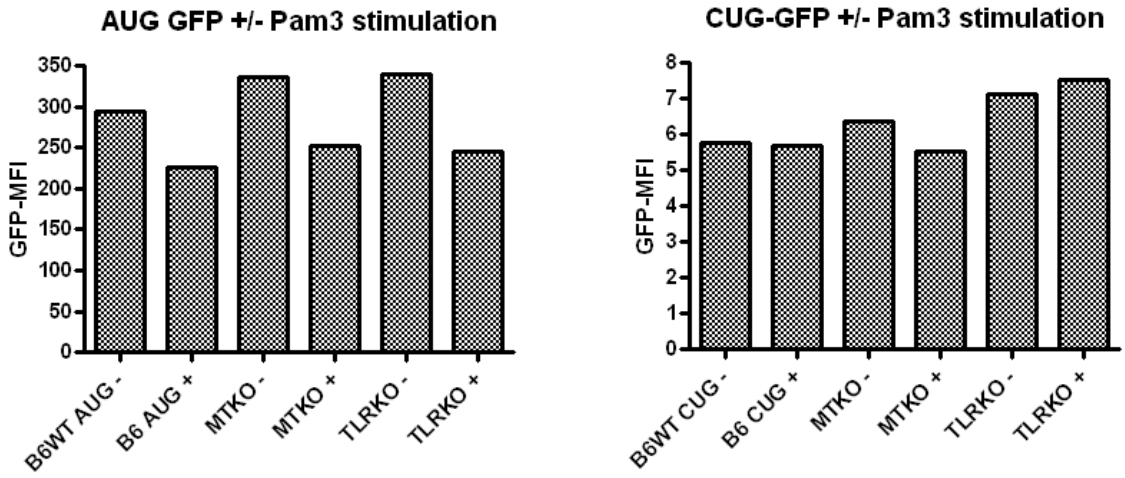


Figure 2.8: **Pam3CSK4 does not enhance the amounts of [AUG]-GFP or [CUG]-GFP**: Immortalized macrophages were transfected with either [AUG]-GFP or [CUG]-GFP mRNA for a duration of 4 hours and then stimulated with Pam3CSK4 ligand for 3 hours. Cells were than harvested and analyzed by flow cytometry. The mean fluorescence intensity of GFP expression is plotted as bar graph.

## 2.2 Toll-like receptor ligands can enhance cryptic peptide presentation in an ex-vivo model system in primary bone-marrow derived macrophages and splenocytes.

### Toll-like receptor ligands can enhance the presentation of cryptically translated peptides in primary bone-marrow derived macrophages.

TLR ligands could enhance the T-cell responses to the cryptic LYL8 peptide in immortalized macrophages through the use of an in-vitro model system. We transitioned to an alternate model system which would not rely on the transfection efficiencies of the mRNA constructs encoding the conventionally and cryptically translated peptides. This model system is more physiologically relevant since it involves the use of a mouse model that expresses a transgene endogenously (described in Schwab et al. 2003, [66]). This transgene encodes a biscistronic transcript, whereby there is the [AUG]-initiated WMHHNMDLI (abbreviated as WI9) antigenic peptide arising from a conventional reading frame followed by a stop codon. Downstream of this stop codon, encoded in a cryptic reading frame is the [CUG]-initiated LYL8 peptide. WI9 is a peptide derived from the Uty gene on the Y chromosome and is

presented on the $D^b$ MHC Class I molecule, whilst LYL8 is presented on $K^b$. Both these peptides are recognized by two different T-cell hybridomas, 11p9Z recognizes the WI9-$D^b$ complex and BCZ103 recognizes the LYL8-$K^b$ complex. Therefore, this one transcript (in a single mRNA) provides a way to assess both conventional and cryptic translation of antigenic peptides. This transgenic mouse model will, henceforth, be referred to as the WI9.LYL8 transgenic system.

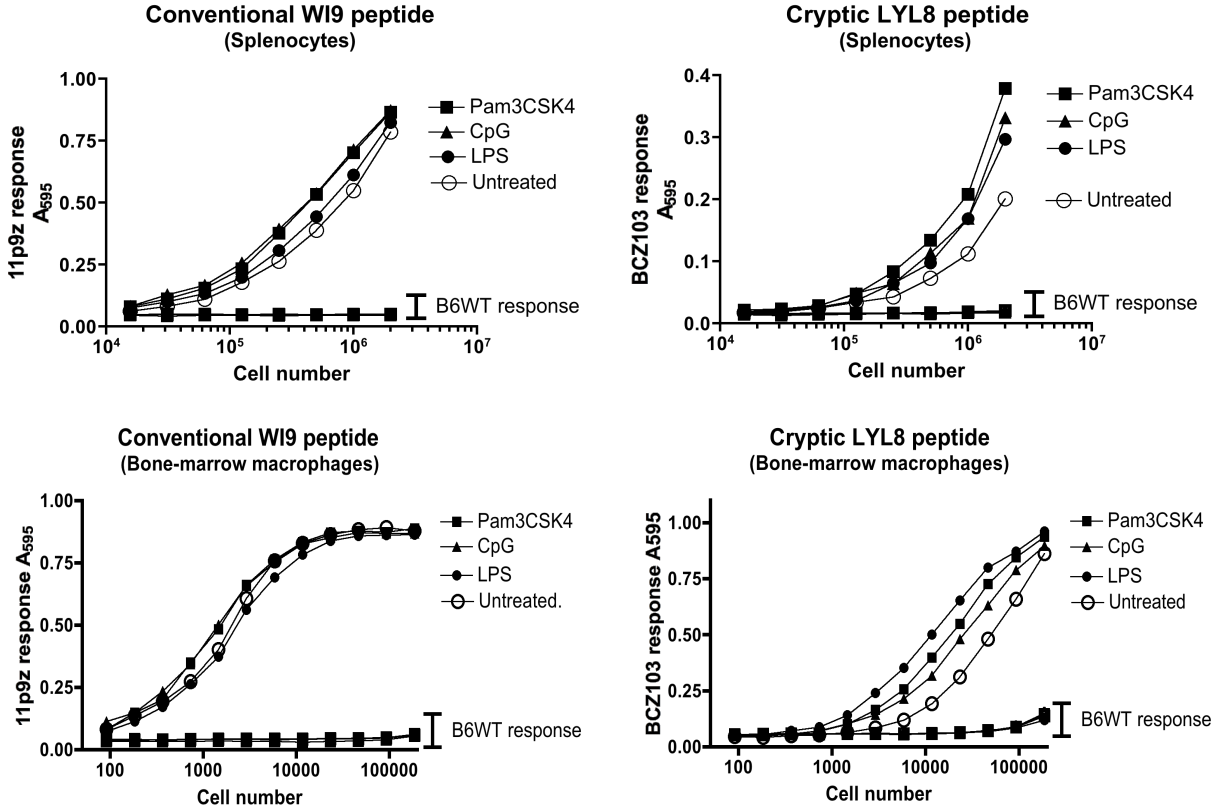


Figure 2.9: **Toll-like receptor ligands enhance cryptic peptide presentation in splenocytes and primary macrophages**: Splenocytes (top panel) and bone-marrow derived primary macrophages (bottom panel) were generated from the WI9.LYL8 transgenic mice and from C57BL/6 mice (as a negative control). Different toll-like receptor ligands were added to these cells for a duration of 6 hours after which the cells were harvested for a T-cell assay with either the LYL8 specific BCZ103 hybridoma or the WI9 specific 11p9z hybridoma. (Data is representative of three independent experiments).

Splenocytes were obtained from the WI9.LYL8 mice. These cells were washed with a citric acid buffer. Acid-washing removes all the cell surface MHC Class I molecules. Any antigen presentation response can now be analyzed from a clean slate. TLR ligands were than added to the supernatant and 6 hours later, the cells were harvested and a T-

cell assay was performed. The transgenic WI9.LYL8 splenocytes displayed a very robust enhancement of the T-cell response to the cryptically translated LYL8 peptide, upon TLR ligand treatment using ligands such as Pam3CSK4, CpG and LPS (Figure 2.9). In contrast to the mRNA transfection experiments, LPS robustly enhanced the cryptic peptide specific T-cell responses and was more potent than ligands such as Pam3CSK4 or CpG. The reason for more robust responses could be because every primary cell, from the WI9.LYL8 transgenic mice, expresses a cryptic and a conventional peptide whereas in the immortalized macrophage system, the mRNA transfection efficiency of the constructs would have been only 30-40%. The conventional peptide specific T-cell responses were only subtly enhanced, in the presence of TLR ligands, or not at all. Wildtype splenocytes from C57BL/6 (B6) mice, lacking the WI9.LYL8 transgene, were used as a negative control in the experiments as they would not elicit any T-cell hybridoma response. In this way, we could be certain that the enhanced T-cell responses were specific to the peptide and were in response to the TLR ligand treatment.

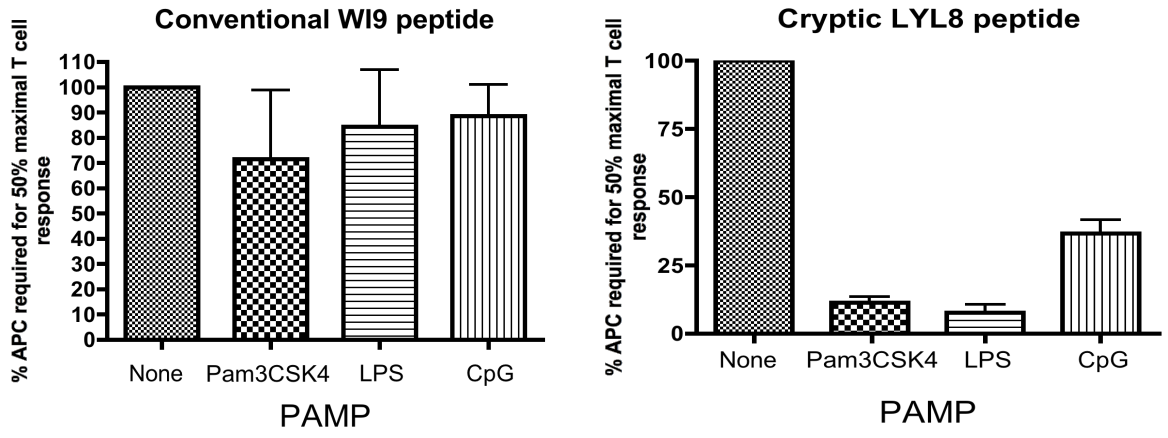


Figure 2.10: **Cryptic peptide presentation is enhanced more than conventional peptide presentation, as determined by a statistical test.**: Sigmoidal curves were fit for all the T-cell assay experiments. Based on the sigmoidal curve, number of APC required for half maximal T-cell response was determined by obtaining the EC50 value. This was obtained and averaged out across multiple experiments. TLR treated conditions were normalized to untreated conditions. The response of the untreated condition was represented as 100, and the TLR treated conditions were represented as a % of that. The lower the % of APC required for half maximal T-cell response, the higher the T-cell response.

An alternate way in which multiple experiments were combined for statistical purposes was to obtain the cell number needed to attain the half maximal T-cell response in each experiment. This cell number was normalized as a percentage of the cell number obtained for the untreated condition. Percentages obtained from each condition were then averaged across multiple experiments and plotted as a bar graph. Transgenic cells treated with ligands required fewer cells to attain the half maximal T-cell response to the cryptic peptide, compared to the untreated condition. This indicated that the T-cell responses to the LYL8

peptide, in the presence of the TLR ligands, was enhanced compared to the untreated condition. For the conventionally translated WI9 peptide, however, there seemed to be no difference in the number of cells required to achieve half maximal T-cell response in the untreated and TLR-treated conditions (Figure 2.10).

In addition to splenocytes, bone-marrow derived macrophages were generated from the transgenic WI9.LYL8 mice. The transgenic macrophages, like the transgenic splenocytes, also displayed a robust enhancement of T-cell responses to the cryptically translated LYL8 peptide upon treatment with TLR ligands, with LPS causing the largest enhancement. (Figure 2.9)

Furthermore, peptides were extracted from splenocytes that were treated with either LPS or Pam3CSK4 and fractionated via RP-HPLC. Upon detection by a T-cell assay, LYL8 and WI9 peptides from the TLR treated sample fractionated at the same position as those from the untreated sample. This showed that the peptides were not modified in any way upon TLR ligand treatment. However, we were unable to detect any enhancements of the LYL8 peptide upon TLR ligand treatment and fractionation by RP-HPLC. This could have been because three spleens were required for each condition and these might have been too many cells to extract peptide from. Using fewer than three spleens did not give rise to a detectable amount of LYL8. (Figure 2.11)

## 2.3 Discussion

Cryptic translation is known to be a distinct mechanism from conventional translation because of its resistance to different inhibitors that cause various cellular stresses like ER stress and oxidative stress and translation inhibition. The mechanism by which translation initiation occurs at CUG codons is also very distinct, through the usage of a Leu-initiator-tRNA as opposed to the conventional Met-initiator-tRNA. However, it remained to be seen how cryptic translation and more specifically how antigenic peptides that were generated in a cryptic manner, were regulated under the stress of a pathogenic infection. Pathogen infection can be simulated through the usage of pathogen associated molecular patterns (PAMPs), which trigger various receptors like TLRs, which are situated on the cell surface membrane or within endosomal membranes. Using in-vitro mRNA transfection assays and an ex-vivo transgenic model, we have established that induction of the Toll-like receptor signaling pathway through the use of various ligands can enhance cryptic peptide presentation. This was detected through a T-cell assay whereby a T-cell hybridoma specific for the cryptic peptide was used. RP-HPLC analysis also showed that peptide amounts may be increased in the presence of TLR ligand treatments. With respect to the robustness of the T-cell responses, usage of primary cells that endogenously expressed the bicistronic transgene yielded more consistent and robust T-cell responses than using mRNA constructs encoding the LYL8 and MYL8 peptides and transfecting them into immortalized macrophages.

In this study, in the in-vitro model system, Pam3CSK4 and CpG induced an enhanced T-cell response to the LYL8 peptide whilst LPS was not able to potently enhance the T-

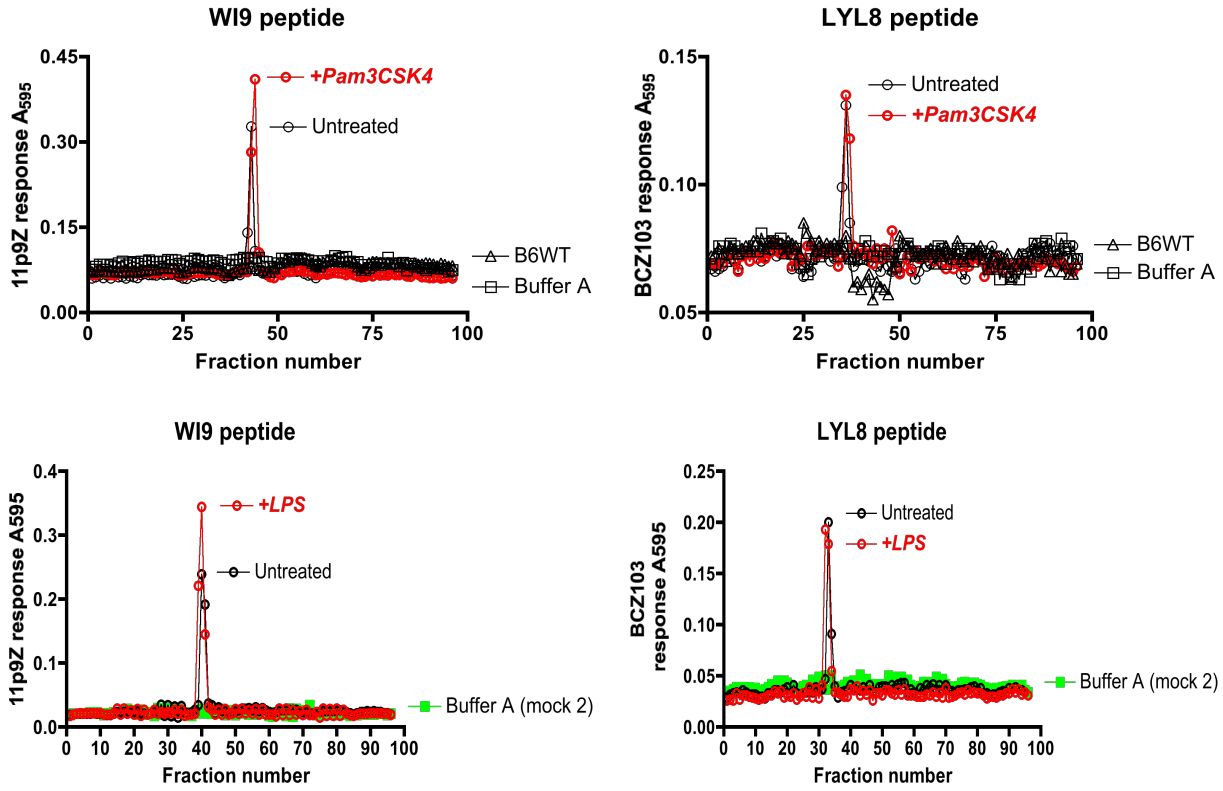


Figure 2.11: **Peptides, upon fractionation by RP-HPLC, are not modified upon TLR ligand treatment.**: Whole spleens from WI9.LYL8 mice were isolated, were treated with RBC lysis buffer and cultured in complete RPMI media. Spleens were treated with either Pam3CSK4 or LPS for a duration of 6 hours. Splenocytes were then harvested and acid extracted with 10% acetic acid and fractionated by RP-HPLC. Peptide amounts were analyzed by a T-cell assay (3 spleens were used for each condition.)

cell response to the LYL8 peptide. However, in the ex-vivo transgenic mouse model system using primary macrophages, LPS and PolyI:C induced the largest enhancements in T-cell responses to the LYL8 peptide. Though the reason for this not known, it can be presumed that the LPS-TLR4 signaling pathway was more effective in activating both the Myd88 dependent and the Myd88 independent pathways downstream of TLR4 ([44]). This would have led to the induction of both Type I Interferons and pro-inflammatory cytokines such as TNF-$\alpha$ resulting in an enhanced T-cell response against the cryptically translated peptide. The TLRs have also been implicated in regulating adaptive immune responses through the activation of T-cells ([29]). TLRs are known to upregulate co-stimulatory responses in order to induce T-cell activation. Therefore, it is very fascinating that TLR ligands can enhance the presentation of cryptically translated peptides on MHC Class I molecules to activate CD8+T cells. This valuable finding can be used to further establish and understand the link

between TLRs and antigen presentation on MHC Class I molecules.

# Chapter 3

# Pathogen infection enhances presentation of cryptically translated peptides

**Summary**

Previously, we established that Toll-like receptor signaling can enhance presentation of cryptically translated peptides. This effect was observed in-vitro through mRNA transfection assays and also ex-vivo in primary splenocytes and macrophages. In order to examine the effect of pathogens on cryptic translation of antigenic peptides, live virus and bacterial infections were performed. The pathogens chosen for infection were Murine cytomegalovirus (MCMV), Influenza, and the bacterium Legionella Pneumophila. Influenza and Legionella are known to inhibit translation ([70]), ([18]), ([4]), therefore, the aim of these experiments was to determine how cryptic peptide translation is affected by these different pathogens. Our hypothesis was that if viruses inhibit conventional translation, cryptic translation would be resistant to those effects.

## 3.1 Virus infection enhances presentation of cryptically translated peptides.

### Murine cytomegalovirus (MCMV) infection enhances cryptic peptide presentation

MCMV is a $\gamma$-herpesvirus that is used to model CMV infection and immune responses. Cytokines like TNF-$\alpha$ and Type I IFN ([25]) are secreted in response to MCMV infection. With respect to translation, herpesviruses are known to induce host translation by activating mTORC1, 4EBP1 phosphorylation and eIF4F assembly. This is required for the translation of viral protein synthesis. Human cytomegalovirus, in particular, is known to stimulate

cap-dependent translation and increases initiation factor concentration ([78]). MCMV also encodes MHC Class I immune evasion genes ([14]). Therefore, this virus seemed like an interesting candidate to assess the effect of pathogen infection on cryptic translation and peptide presentation.

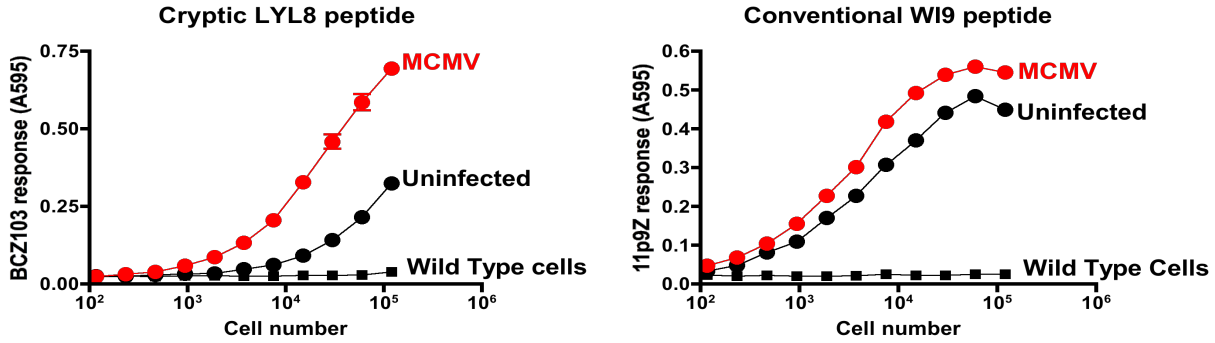


Figure 3.1: **MCMV infection can enhance cryptic peptide presentation.** Primary bone-marrow derived macrophages from WI9.LYL8 transgenic mice and B6 mice were obtained and infected with MCMV at an MOI of 0.5 or 1.0 for a duration of 6 hours. The cells were than harvested for a T-cell assay with either the BCZ103 or 11p9Z hybridoma. (Data is representative of more than three independent experiments).

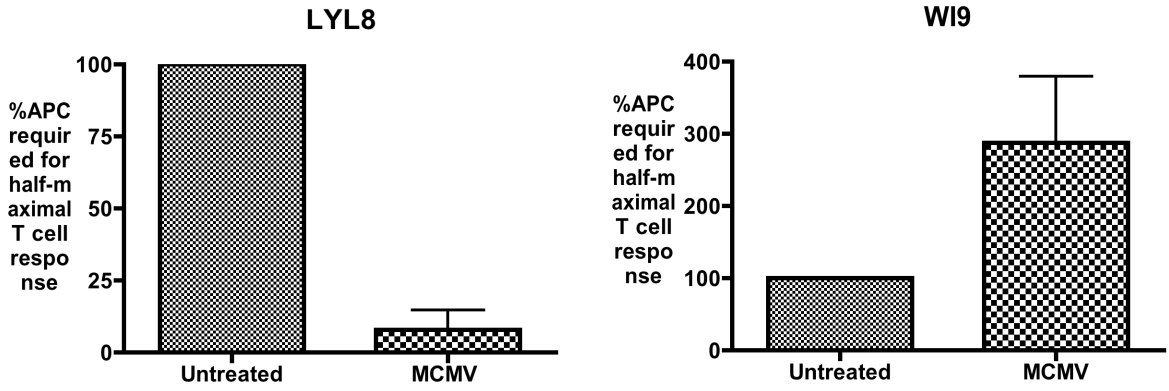


Figure 3.2: **MCMV infection enhances cryptic peptide presentation more than conventional peptide presentation.** % APC required for half maximal T-cell response was determined for 4 independent experiments and averaged. The fewer the % of cells required for half maximal T-cell response, the higher the T-cell response.

Primary bone-marrow derived macrophages were obtained from the WI9.LYL8 transgenic mice and also from C57BL/6 (B6) mice. MCMV supernatant was generated from NIH3T3 cells that were infected with MCMV. This viral supernatant was filtered through

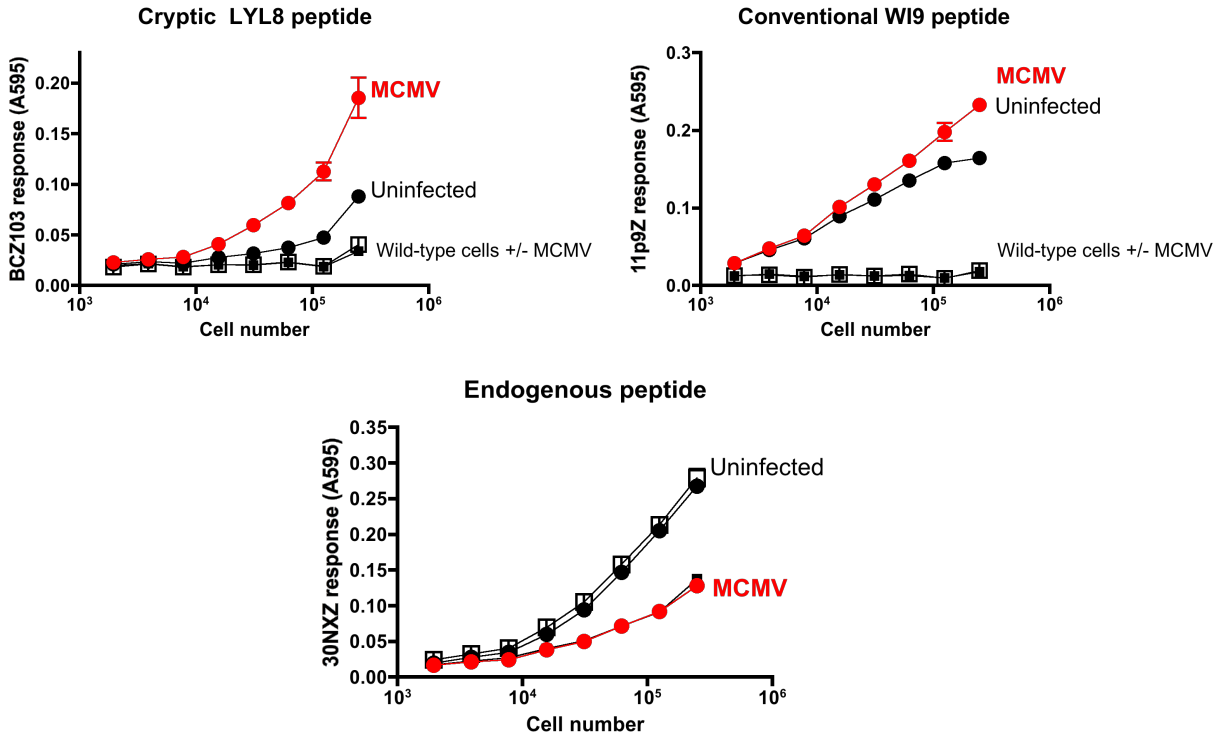


Figure 3.3: **MCMV infection enhances T-cell responses to the cryptic peptide more than the conventional peptide or an endogenous peptide.** Primary WI9.LYL8 and B6 macrophages were infected with MCMV at an MOI of 1.0 for a duration of 6 hours. The cells were harvested and cultured with either the BCZ103, 11p9Z or the 30NXZ T-cell hybridoma. (Data is representative of more than three independent experiments).

a 0.45um filter, to exclude any floating cells in the supernatant, before use. The WI9.LYL8 macrophages were infected with this MCMV supernatant at an MOI (multiplicity of infection) of 1.0, which would mean that 1,000,000 cells would receive virus at a concentration 1,000,000 plaque forming units (pfu)/mL or an MOI of 0.5. After an infection of 6 hours, macrophages were harvested and cultured with either the BCZ103 or the 11p9Z hybridoma. We observed that B6 macrophages did not elicit any T-cell response with or without the MCMV supernatant. The transgenic macrophages, infected with MCMV, elicited an increased T-cell response to the LYL8 peptide compared to the uninfected samples. The transgenic macrophages infected with MCMV did not, however, exhibit increased conventional WI9 peptide specific T-cell response (Figure 3.1). This effect was consistent over several experimental repeats (Figure 3.2). [The statistical analysis performed in this figure is similar to the one that was done in Chapter 2 by measuring the number of cells required to attain half-maximal T-cell response.] We would have expected the conventional peptide specific T-cell response to be inhibited in the presence of MCMV. Instead, the conventional

T-cell response was unchanged in the presence of this stimulus and in certain instances slightly enhanced. This was intriguing and this could have been due to the saturated nature of the 11p9Z response or that presentation of the conventionally translated peptide was indeed unchanged. In addition to these two hybridomas that were specific for peptides that arose from a transgene, we tested how MCMV affects the T-cell response of a hybridoma that recognizes an endogenous peptide arising from the H13 gene and which is presented on the $D^b$ molecule. This hybridoma was named as 30NXZ. When cells infected with MCMV were harvested, they were divided equally into three plates; one set was cultured with BCZ103, one set was cultured with 11p9Z and the other with 30NXZ (Figure 3.3). This T-cell response was detected in both B6 and transgenic cells, since an endogenous peptide was being recognized. In the presence of MCMV, the 30NXZ response decreased in both B6 and transgenic cells. This showed that the enhancement observed for the BCZ103 response was specific to the LYL8 peptide in response to MCMV infection.

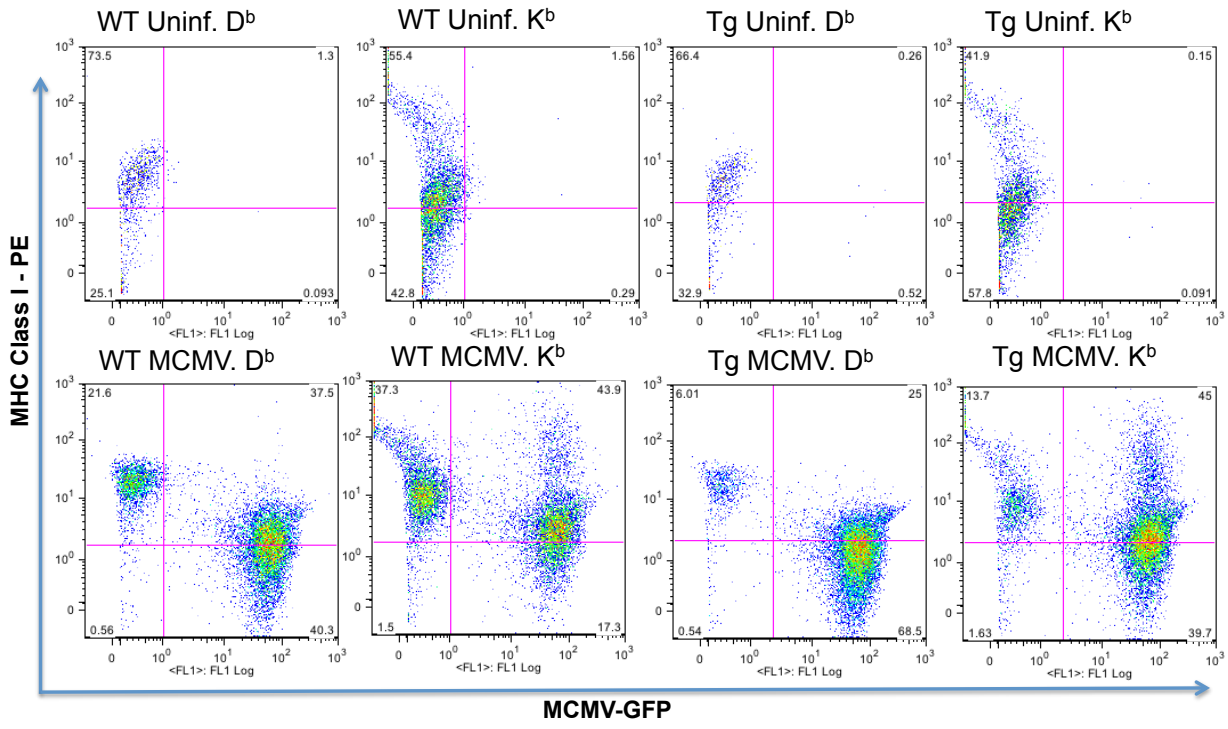


Figure 3.4: **MHC Class I levels are not inhibited, upon MCMV infection.** Primary WI9.LYL8 macrophages were infected with MCMVGFP at an MOI of 1.0 for a duration of 6 hours. Cells were then harvested and stained with surface MHC Class I antibodies ($K^b$-PE, Clone - AF6-88.5 , $D^b$-PE, Clone KH95)

MCMV encodes for multiple MHC Class I inhibitors and is known to inhibit Class I expression. Therefore, we tested the expression of MHC Class I on the cell surface, in the presence and absence of MCMV infection to determine if there was any inhibition. For this

experiment, macrophages were stained with $K^b$ and $D^b$ antibodies and analyzed by flow cytometry. Interestingly, MHC Class I expression was unchanged in the presence of MCMV infection (Figure 3.4). Furthermore, when the cells were examined under the microscope to gauge their morphology, MCMV infected cells were activated and looked healthier than uninfected cells. This could be because a lot of signaling pathways are activated in macrophages by MCMV and this could have led to an increase in transcription and translation overall. Moreover, the inhibition of MHC Class I expression by MCMV was caused by a very high MOI of MCMV ([43]). To ensure that MCMV infection had occurred, macrophages were stained intracellularly for TNF-$\alpha$ production by using anti-TNF-$\alpha$ antibodies and analyzed by flow cytometry. Additionally, MCMV-GFP (MCMV with a GFP coding sequence) was also used for infection of macrophages. This MCMV-GFP allowed infected cells to be visualized by flow cytometry.

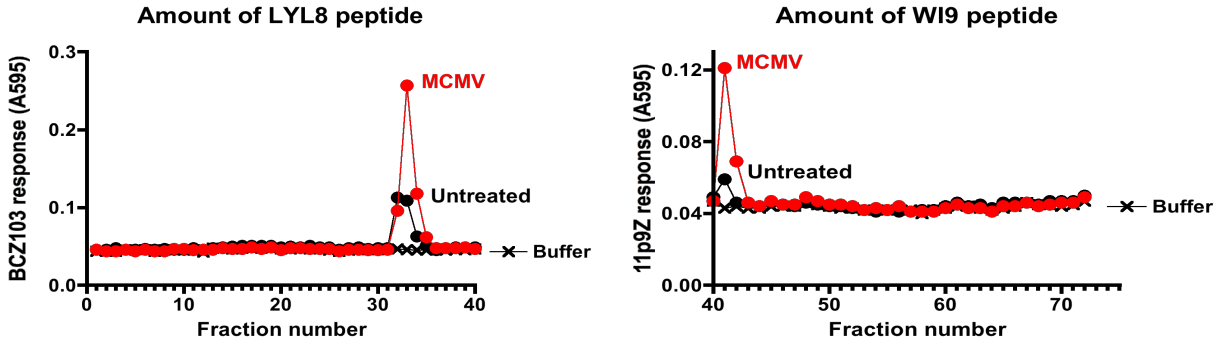


Figure 3.5: **Peptide amounts are increased upon MCMV infection.** WI9.LYL8 Bone-marrow macrophages were infected with MCMV. The peptides were then extracted using acid and fractionated via RP-HPLC. Peptide amounts were determined by addition of antigen presenting cells and T-cell hybridomas, through a T-cell assay. (Data is representative of two independent experiments).

Apart from T-cell presentation assays, the peptide amounts in the presence of MCMV infection were also determined by extracting peptides from uninfected and infected macrophages and fractionating them via RP-HPLC. Since WI9 and LYL8 fractionate at different times, we could test for both WI9 and LYL8 from the same injected sample. This was done by adding BCZ103 and the K89 antigen-presenting cell (which expresses the $K^b$ molecule) to the fractions in which LYL8 would be found and adding 11p9Z and the $D^b$L antigen-presenting cell (which expresses the $D^b$ molecule) to the rest of the fractions. This experiment showed that both LYL8 and WI9 peptide amounts were greatly increased in the presence of MCMV compared to the uninfected samples ((Figure 3.5)). This meant that in the T-cell presentation assay, the 11p9Z response was saturated in the presence of WI9 because there was an overwhelming amount of WI9 peptide to begin with. Therefore, the differences were not getting detected in that particular assay. This RP-HPLC experiment was insightful in show-

ing that MCMV was able to increase T-cell responses to both the conventionally translated and cryptically translated peptide, in a duration of 6 hours. This finding was also consistent with an RT-PCR analysis that was performed to determine the level of WI9.LYL8 transcript levels in the presence of MCMV infection. We found WI9.LYL8 transcript levels to be greatly enhanced in the presence of MCMV compared to the uninfected levels. The level of transcripts in the infected cells were 5 times greater than those in uninfected cells (Figure 3.6). Given that both the controls L32 and GAPDH also were slightly increased, the level of WI9.LYL8 transcript was normalized to the expression of L32 and GAPDH. After the normalization, WI9.LYL8 transcript levels were still significantly enhanced in the MCMV infected samples.

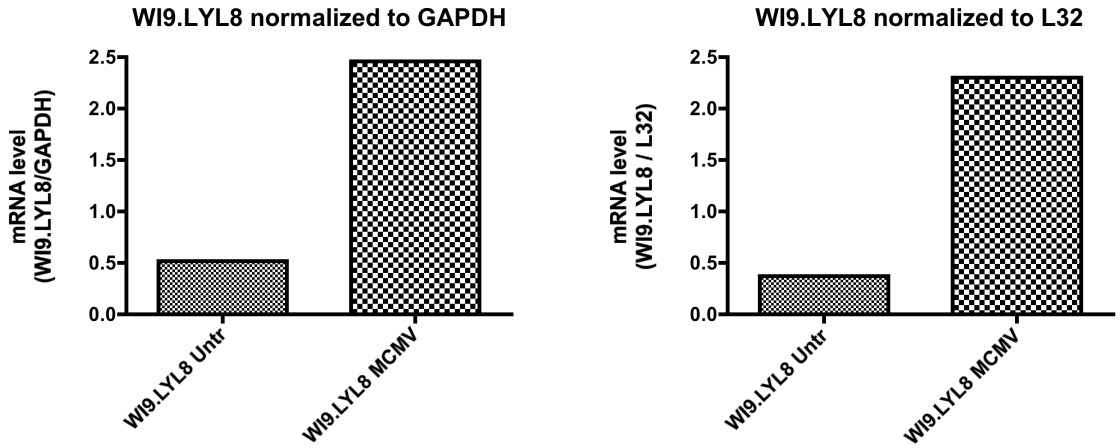


Figure 3.6: **WI9.LYL8 mRNA transcript levels are enhanced upon MCMV infection.** RNA was isolated from macrophages that had been infected with MCMV. cDNA was generated from this RNA and a quantitative RT-PCR was performed using GAPDH and L32 as controls. The CT values of the WI9.LYL8 samples were normalized to that of GAPDH and L32.

## Brefeldin A inhibits the enhancement of cryptic peptide presentation

In order to determine if the presentation pathway is important for this phenomenon of enhanced T-cell responses to cryptically translated peptides, we used Brefeldin A, which is used as the Golgi-plug and prevents molecules in the ER from reaching the cell surface. Macrophages were acid-washed to remove all the cell surface molecules and then infected with MCMV. The cells were cultured with Brefeldin A at different time points; right after acid-wash before infection, at the same time of infection, 1 hour, 2 hours and 4 hours after infection for a total duration of 6 hours. The cells were then harvested and a T-cell assay was performed. When Brefeldin A was added right after the acid-wash, the presentation levels of the peptides, both conventional and cryptic, were the lowest. The presentation level

correlated with the time after which Brefeldin A was added to the cells. The earlier the Brefeldin A was added, the more the molecules were retained in the Golgi and the lower the cell surface presentation. A similar phenomenon was observed for conventional peptide presentation as well. With no inhibition of presentation, the enhancement of cryptic peptide presentation after MCMV infection was greater than with Brefeldin A. This showed that efficient transport of MHC Class I molecules from the ER to the cell surface is required for enhanced cryptic antigen presentation to occur, in response to MCMV infection (Figure 3.7). Also, this result shows that cryptically translated peptides are transported in exactly the same way as conventionally translated peptides, from the ER to the cell surface.

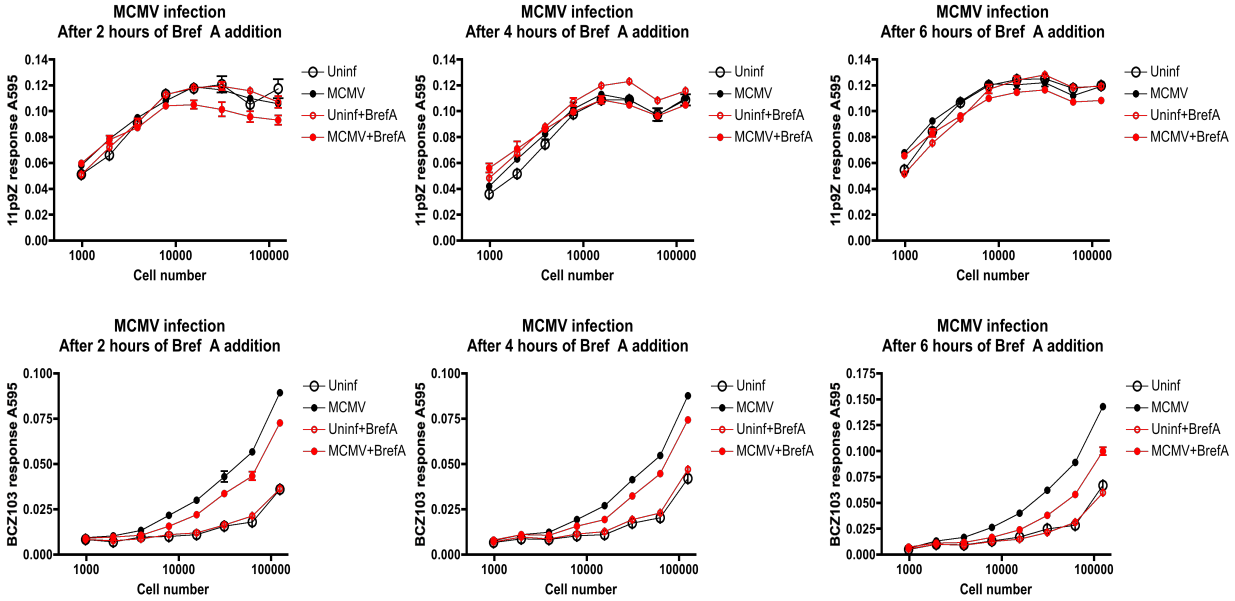


Figure 3.7: **Brefeldin A inhibits enhanced cryptic peptide presentation upon MCMV infection.** Primary WI9.LYL8 macrophages were infected with MCMV and incubated with Brefeldin A at different time points (2 hours, 4 hours and 6 hours post infection). Cells were then harvested and a T-cell assay was performed

## Influenza enhances presentation of cryptically translated peptides.

In addition to MCMV, we also tested the effect of the Influenza virus on peptide presentation. Influenza encodes some translation inhibitors ([63]) and can inhibit conventional host translation ([33]). Bone-marrow derived macrophages (WI9.LYL8 and C57BL/6) were infected with Influenza at an MOI of 0.5 for a duration of 6 hours and 24 hours. The cells were then harvested and cultured with the BCZ103, 11p9Z or the 30NXZ hybridoma. In the MCMV infected condition in the transgenic cells, the BCZ103 response was enhanced compared to the uninfected samples, the 11p9Z response was slightly enhanced and the

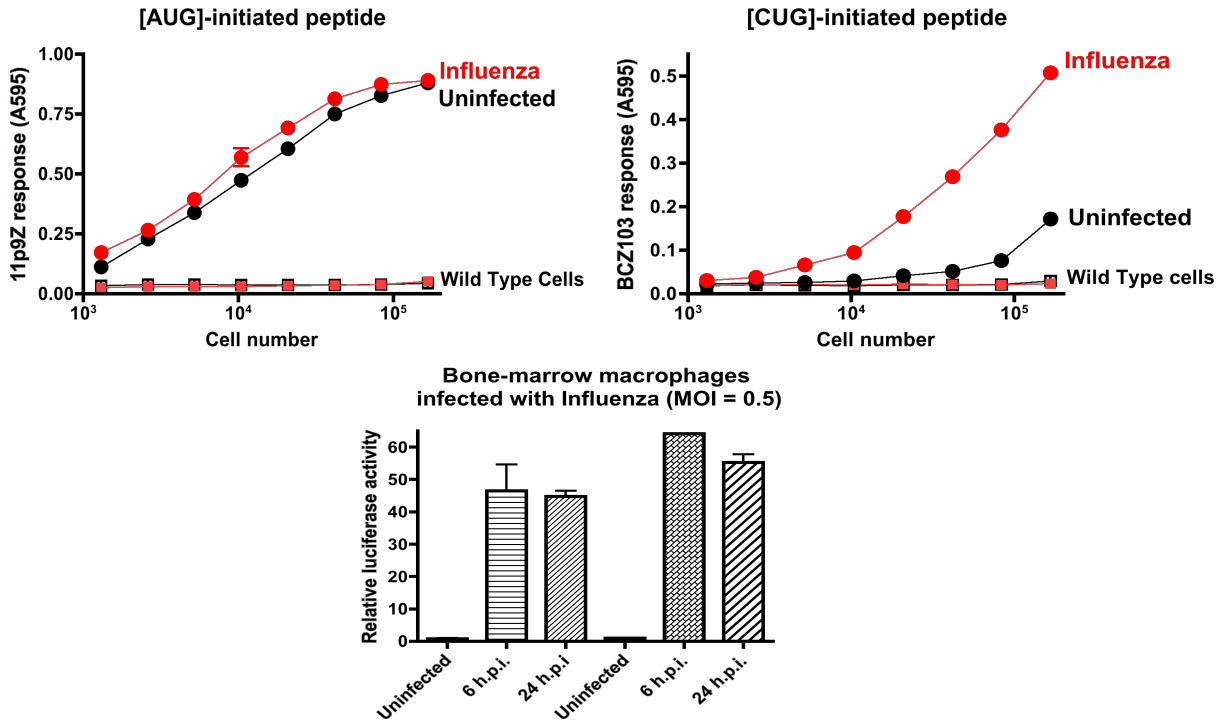


Figure 3.8: **Influenza infection enhances cryptic peptide presentation.** WI9.LYL8 macrophages were infected Influenza at an MOI of 1.0 for a duration of 6 hours. The cells were then harvested for a T-cell assay. The supernatants from the infected cells were added to L929-ISRE cells that contained an Interferon-$\beta$ reporter. The presence of luciferase indicated that the cells had been infected with Influenza. (Data is representative of three independent experiments).

30NXZ response remained unchanged. In B6 cells, only a 30NXZ response was detected and that remained unchanged between infected and uninfected samples. To test for Influenza infection, an IFN-$\beta$ dependent luciferase assay was performed. Influenza is said to induce IFN-$\beta$ production in macrophages ([36]). There is a reporter cell line (L929) that contains a luciferase reporter that is downstream of an Interferon-stimulated-response-element (ISRE). The supernatant from the infected cells was added to these L929 cells. The L929 cells were then lysed and incubated with the luciferin substrate to test for luciferase activity. Supernatant from the Influenza infected cells induced a significant amount of luciferase whereas cells that were uninfected did not exhibit any luciferase activity. (Figure 3.8) This confirmed that the enhanced cryptic peptide presentation was an effect of Influenza infection of the macrophages. This experiment also suggested that this was not an effect that was specific to MCMV, but an effect that was caused by live virus infection that activated macrophages and induced them to produce inflammatory cytokines.

## MCMV deletion mutants can enhance presentation of cryptically translated peptides.

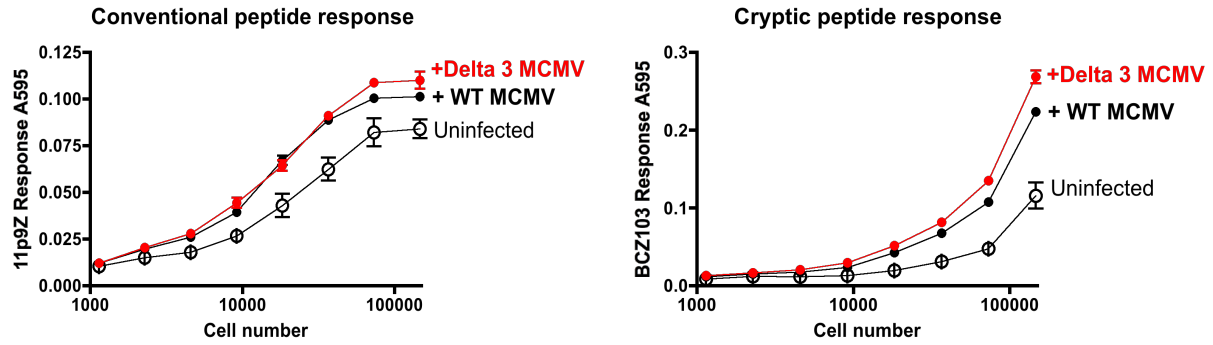


Figure 3.9: **Infection with MCMV deletion mutant Delta 3 enhances cryptic peptide presentation.** Primary WI9.LYL8 macrophages were infected with wild-type MCMV and the Delta3 mutant MCMV for a period of 6 hours. Cells were then harvested and a T-cell assay was performed. (Data is representative of two independent experiments).

Previously, we established that live virus infection can induce enhanced cryptic peptide presentation. The next question to be addressed was if the virus had to be of a wild-type genotype in order to be able to cause this phenomenon. Another question to be addressed was if there was a specific kind of viral gene that was responsible for this effect of enhanced cryptic peptide presentation. To address these questions, we obtained several MCMV deletion mutants that were lacking in multiple open-reading frames of the MCMV genome (obtained from the laboratory of Dr. Laurent Coscoy, UC Berkeley). The mutants that were used were Delta 3, Delta 8, Delta 19, Delta 20 and Delta 152. Apart from Delta 152 all of the other mutants lacked large segments of the MCMV genome. Delta 152 lacked the m152 gene that encoded for the MHC Class I inhibitor. We hypothesized that this specific mutant Delta 152 would enhance the T-cell responses for both the cryptically and conventionally translated peptides since there would be no Class I inhibition. After the cells had been infected with these various mutants, and T-cell assays were performed, we found that cryptic peptide presentation was enhanced in the presence of these different mutants, irrespective of what MCMV gene they lacked. T-cell responses to the conventional peptide remained unchanged between infected and uninfected samples (Figure 3.9). However, for the delta 152 mutant, the T-cell response to the WI9 peptide was also enhanced, much more than what was seen with just MCMV infection. This experiment showed that no particular viral gene was required for the enhancement of cryptic peptide presentation and that live or mutant viruses were able to stimulate a signaling pathway that led to increased cryptic peptide presentation. (Figure 3.10). One aspect that was not examined in this experiment was the $K^b$ and $D^b$ expression in the presence of these various mutants and whether there was increased $K^b$ and $D^b$ levels in the presence of the delta 152 virus.

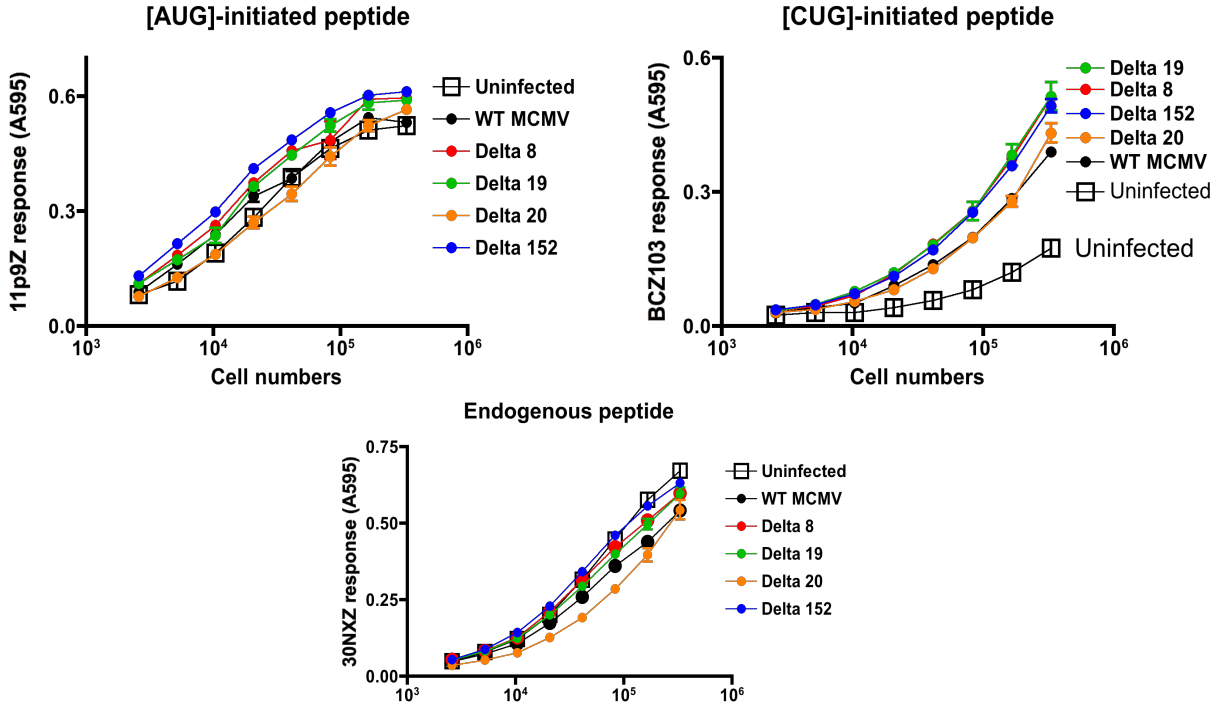


Figure 3.10: **Infection with various MCMV deletion mutants also enhances cryptic peptide presentation.** WI9.LYL8 macrophages were infected wild-type MCMV and MCMV mutants lacking multiple open-reading frames of the MCMV genome. The mutant Delta 152 indicates that it lacks the m152 gene that encodes for MHC Class I inhibitors. The cells were harvested and incubated with either the 11p9Z, BCZ103 or the 30NXZ hybridoma that is specific for an endogenous peptide. (Data is representative of two independent experiments).

## UV-inactivated MCMV does not enhance presentation of cryptically translated peptides.

Previously, we showed that infection of mutant viruses was able to enhance cryptic peptide presentation. Therefore, we wanted to determine whether the virus had to be live for this phenomenon to occur. Since we previously showed that Toll-like receptor ligands could also enhance cryptic peptide presentation, our hypothesis was that inactivated virus would also be able to enhance cryptic peptide presentation. In order to determine this, MCMV viral supernatant was exposed to Ultra-violet (UV) light, which could cross-link the DNA, for a period of 30 minutes. Transgenic macrophages were then infected with either WT MCMV supernatant or the UV-inactivated MCMV supernatant for the same duration as the previous experiments. Cells were then harvested and cultured with T-cell hybridomas BCZ103 and 11p9Z. The inactivated status of the virus was determined by its inability to replicate from cells, which can be ascertained using a plaque assay. Neat supernatant added to cells caused

the lysis and death of the cells, however in the diluted samples, there were no plaques observed showing that the virus was replication deficient caused by the cross-linking of its DNA. In comparison, the WT supernatant produced plaques in all of the dilutions showing that it was replication competent and was able to efficiently lyse the cells it infected. Furthermore, MCMV-GFP was used to infect the cells. Inactivation of MCMV-GFP does not produce any GFP in the virally infected cells and this was determined by flow cytometry analysis of the cells (Figure 3.11).

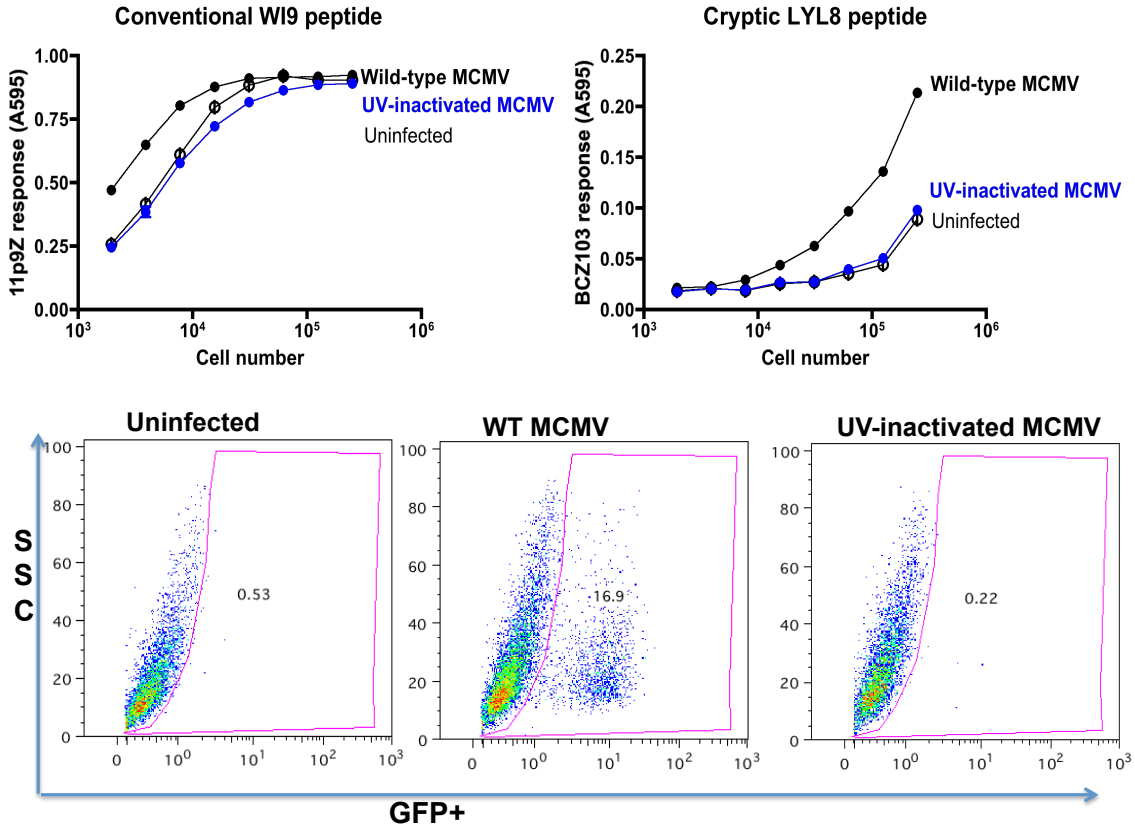


Figure 3.11: **Infection with UV-inactivated MCMV does not enhance cryptic peptide presentation.** MCMV-GFP supernatant was exposed to UV for a period of 30 minutes. This supernatant was used to infect WI9.LYL8 macrophages along with wild-type MCMV-GFP supernatant. Cells were harvested after 6 hours for a T-cell assay. UV-inactivated MCMV does not produce any GFP compared to wild-type MCMV as shown by flow cytometry. (Data is representative of three independent experiments).

In the T-cell presentation assay, the BCZ103 response to the cells infected with WT MCMV was significantly enhanced like all the previous experiments. However, the BCZ103 response to the cells infected with UV-inactivated virus was similar to that of the uninfected samples and not enhanced at all. The 11p9Z response was slightly enhanced in the cells

infected with WT MCMV, but the 11p9Z response to the cells infected with UV-inactivated virus was same as that of the uninfected cells. This showed that something about the UV-inactivated virus was not able to enhance the T-cell response to the cryptic and conventional peptides, in contrast to the Toll-like receptor ligands or the live-virus. Since virus infection was determined by the production of TNF-$\alpha$, we tested for TNF-$\alpha$ production in the cells infected with UV-inactivated virus. Interestingly, cells infected with UV-inactivated virus did not produce any TNF-$\alpha$, as determined by flow cytometry (Figure 3.12). UV-inactivation seems to abolish the ability of MCMV infected cells to produce TNF-$\alpha$ and therefore the enhanced cryptic peptide presentation phenomenon does not occur when cells are infected with UV inactivation. This was a very important observation and leads to the hypothesis that the production of inflammatory cytokines by these pathogenic stimuli or PAMPs could be critical in generating enhanced presentation of cryptic peptides.

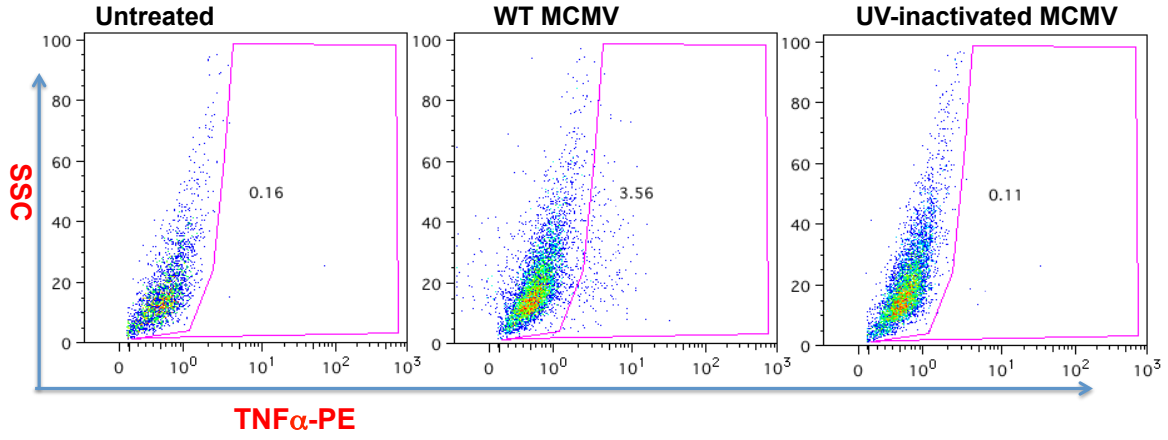


Figure 3.12: **Infection with UV-inactivated MCMV does not induce TNF-$\alpha$ production in primary macrophages.** TNF-$\alpha$ production by macrophages infected with MCMV/UV-inactivated MCMV was measured by intracellular cytokine staining for TNF-$\alpha$ and analysis by flow cytometry. (Data is representative of two independent experiments).

## 3.2 Bacterial infection of macrophages also enhances presentation of cryptically translated peptides

In addition to viruses, we wanted to determine if other pathogens can also lead to this increased presentation of cryptically translated peptides. We selected Legionella pneumophila, a bacterium that infects macrophages. Legionella also encodes for a translation elongation/initiation inhibitor ([18]) and therefore we wanted to assess how this would impact cryptic translation of antigenic peptides. Wild-type Legionella causes the lysis of macrophages through the activation of the inflammasome ([6]), therefore transgenic WI9.LYL8 macrophages

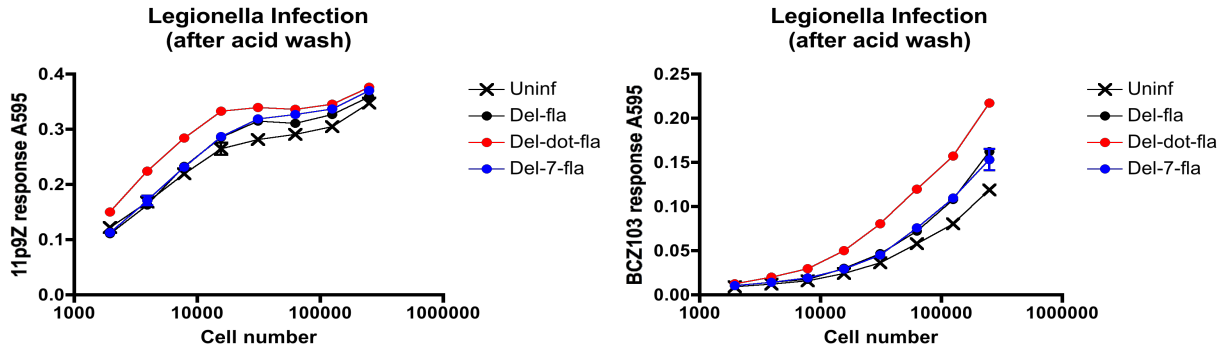


Figure 3.13: **Infection with Legionella pneumophila enhances cryptic peptide presentation** WI9.LYL8 macrophages were washed with acid to remove cell surface MHC Class I molecules and then infected with Legionella mutants lacking flagellin. The other mutants were made in this delta-fla background. Delta-dot-fla lacked the Type IV secretion system and Delta-7-fla lacked some of the host translation inhibitors encoded by Legionella. After 6 hours, the cells were harvested for a T-cell assay. (Data is representative of two independent experiments).

were infected with a mutant of Legionella that lacks the flagellin and this prevents activation of the inflammasome since the flagellin cannot be sensed. All of the other mutants were generated in this Delta-fla background. Delta-dot-delta-fla lacks the Type IV secretion system as well along with lacking the flagellin and the Delta-7-delta-fla mutant lacks some, but not all, of the host translational inhibitors encoded by Legionella. All of these mutants were obtained from the Vance Lab and the infection of macrophages was also performed in the Vance Lab. All of the macrophages were acid washed before infection. This would mean that all of the existing cell surface MHC Class I molecules were removed. This experiment showed that all of the Legionella mutants were able to enhance cryptic peptide presentation compared to the uninfected cells.(Figure 3.13) The Delta-dot-delta-fla mutant was able to significantly enhance both cryptic and conventional peptide presentation. The mutant that was deficient in TNF-$\alpha$ production was not able to strongly enhance the cryptic peptide presentation. This was consistent with our hypothesis that cytokine production is critical for the enhancement of cryptic peptide presentation. Interestingly, we expected the Delta-7-delta-fla mutant to be the strongest enhancer of cryptic peptide presentation since it lacks the host translational inhibitor. However, that was not the case. The reasons as to why this could have happened are not clear, since we did not test for how global translation was affected in the presence of these Legionella mutants. To be able to do this, labeling proteins with radioactive methionine (S35) during mRNA translation would identify if there was any host translational inhibition caused by Legionella.

## 3.3 Discussion

Pathogen infection can have a variety of effects on cellular functions ranging from evading immune responses, establishing latency to hijacking cellular machinery for the survival and propagation of the pathogen itself. To establish a physiological importance for the generation and existence of cryptic peptides, it is important to determine the effect that pathogen infection can have on the amount of cryptic peptides. It is also important to determine if these self-cryptic peptides can be useful in eliciting a host immune response in the event that the pathogen evades the immune system. This study shows that pathogen infection can enhance presentation of peptides generated in a cryptic manner, more than conventional peptide presentation. When primary macrophages were infected with either MCMV, Influenza or Legionella, cryptic peptide presentation was enhanced more than conventional peptide presentation. Interestingly, when UV-inactivated MCMV was used to infect primary macrophages, enhancement of cryptic peptide presentation was not observed and this could have been because UV-inactivated MCMV was unable to induce any TNF-$\alpha$. This indicated that the effect of pathogen infection induces a host immune response or triggers a signaling pathway, which is sufficient to cause the enhancement of cryptic peptide presentation. Furthermore, our studies showed that all of the MCMV deletion mutants were able to enhance the presentation of cryptically translated peptides. From this, we concluded that the enhancement was not mediated by any particular viral gene. But this could mean that all of those mutants were able to effectively trigger the signaling pathway or host immune response that was causing the enhancement of cryptic peptide presentation. We have yet to test the effect of an MCMV mutant, which is unable to trigger the host immune response, on cryptic peptide presentation.

The importance of cryptic antigens in the context of viral infections is gradually increasing. More groups, in recent studies, have reported CD8+T-cell responses to epitopes arising from alternative reading frames in the simian-immunodeficiency virus (SIV) infection of macaques and HIV infection of humans. ([76]) This study reported a high level of CTL (cytotoxic T lymphocyte) responses against these cryptic epitopes. Given the high magnitude of responses against these epitopes, mutations found in these epitopes correlated with viral escape. This shows the important of developing T-cell responses to these cryptic epitopes, since some of them may be conserved and may be required for viral fitness ([5]) and thereby will revert to the original sequence after transmission. This emphasizes the importance of studying T-cell responses to cryptically translated peptides. Our study can aid other groups in generating more effective vaccines against chronic viral infections, whereby TLR ligands or cytokines can be used in an analogous manner to adjuvants, to enhance presentation of cryptically translated peptides. This also provides a way for the cell to alert the immune system of infection, when conventional translation is being inhibited by the pathogen. For this purpose, we have used a self-peptide model whereby the cryptically translated peptides arise from endogenous genes. The assumption is that T-cells specific for the self-cryptic peptides would not get negatively selected in the thymus. Therefore, when the presentation of these self-cryptic peptides is increased, the T-cells would be able to mount an immune

response against them.

# Chapter 4

# Inflammatory stimuli enhance presentation of cryptically translated peptides

**Summary**

Previously, we established that pathogen infection could cause a robust enhancement in the presentation of cryptically translated peptides. These pathogens could be viruses, bacteria and even mutant viruses. Interestingly, however, UV-inactivated virus was unable to enhance cryptic peptide presentation. This could have been due to the inability of the UV-inactivated virus to produce TNF-$\alpha$. In this chapter, we investigate this indirect effect of pathogen infection on cryptic peptide presentation. The aim is to determine if indeed, secreted factors from infected cells is sufficient to enhance cryptic peptide presentation. Here, we show that enhanced cryptic peptide presentation is indeed an indirect effect of pathogen infection and relies on the ability of the pathogen to induce cytokine production. Moreover, stimulating macrophages with different inflammatory cytokines enhanced presentation of cryptically translated peptide antigens. Blocking the effect of these inflammatory cytokines diminished the enhancement of cryptic antigen presentation.

## 4.1 Enhanced cryptic peptide presentation is caused by host immune responses and signaling pathways induced by virus infection

In order to reaffirm that the enhanced cryptic peptide presentation was occurring because of secreted factors from infected cells, a co-culture assay was designed and set up. Primary macrophages of a different MHC haplotype - $H2^D$ - were used for MCMV infection. These cells were infected with either WT MCMV-GFP or UV-inactivated MCMV-GFP. Af-

ter 6 hours of infection, these infected cells were co-cultured with WI9.LYL8 macrophages. Additionally, the supernatant from the infected $H2^D$ macrophages were filtered with a 0.2$\mu$m filter (to remove any cells and most large virions) and were added to the WI9.LYL8 macrophages. After a co-culture of 3-6 hours, T-cells were added to the mixture of $H2^D$ and WI9.LYL8 macrophages. We found that WT-MCMV-infected $H2^D$ macrophages co-cultured with WI9.LYL8 macrophages induced a potent BCZ103 and 11p9Z response. (Figure 4.1) The BCZ103 response in the WT-MCMV infected samples were enhanced compared to the uninfected samples. However, there was minimal difference in the 11p9Z response between infected and uninfected samples. UV-inactivated-MCMV-infected $H2^D$ macrophages co-cultured with WI9.LYL8 macrophages induced a much-diminished BCZ103 and 11p9Z response similar to that of the uninfected samples.

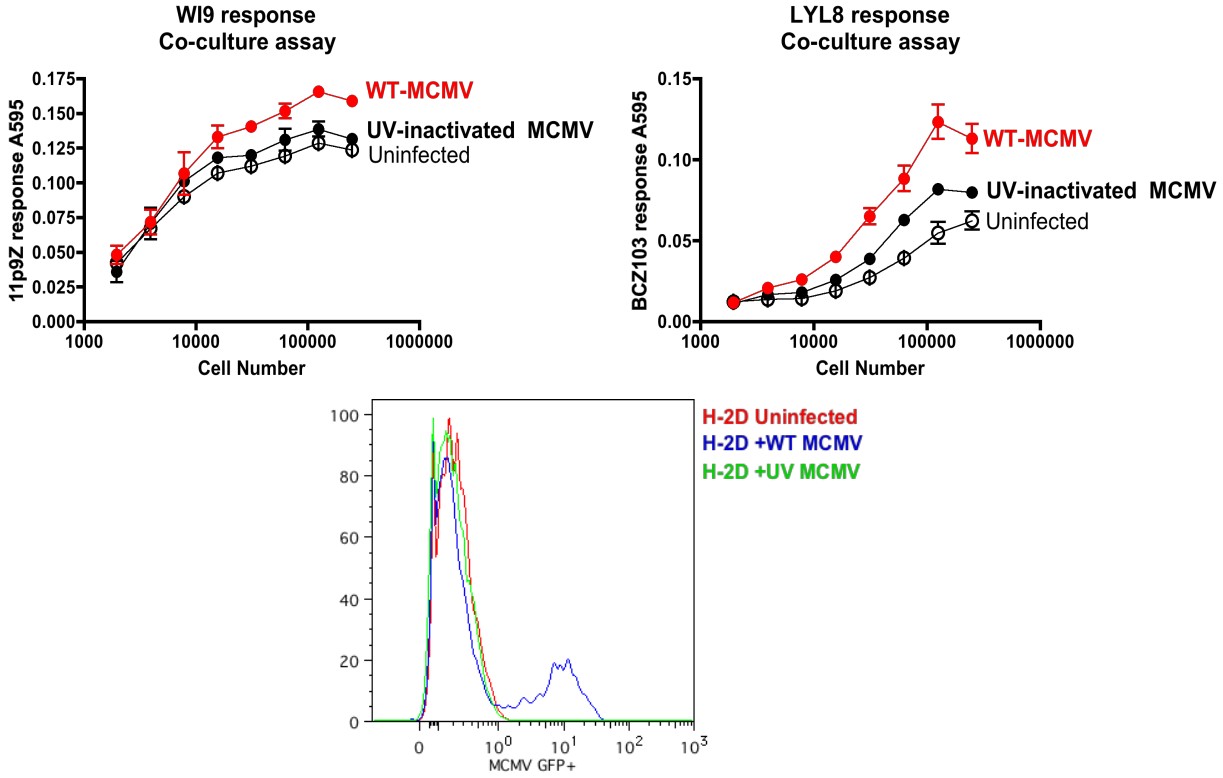


Figure 4.1: **Enhancement of cryptic peptide presentation occurs through host immune signaling pathways induced by virus infection.** $H2^D$ macrophage were infected with wild-type or UV inactivated MCMV for 6 hours and then co-cultured with WI9.LYL8 macrophages. After 4-6 hours, T-cell hybridomas were added to the co-culture. The top panel indicates T-cell response to the co-cultured cells. The bottom panel indicates MCMV infection of the $H2^D$ macrophages. (Data is representative of three independent experiments).

When the filtered supernatant from the infected cells were added to the WI9.LYL8 macrophages, increased cryptic peptide presentation was observed compared to the super-

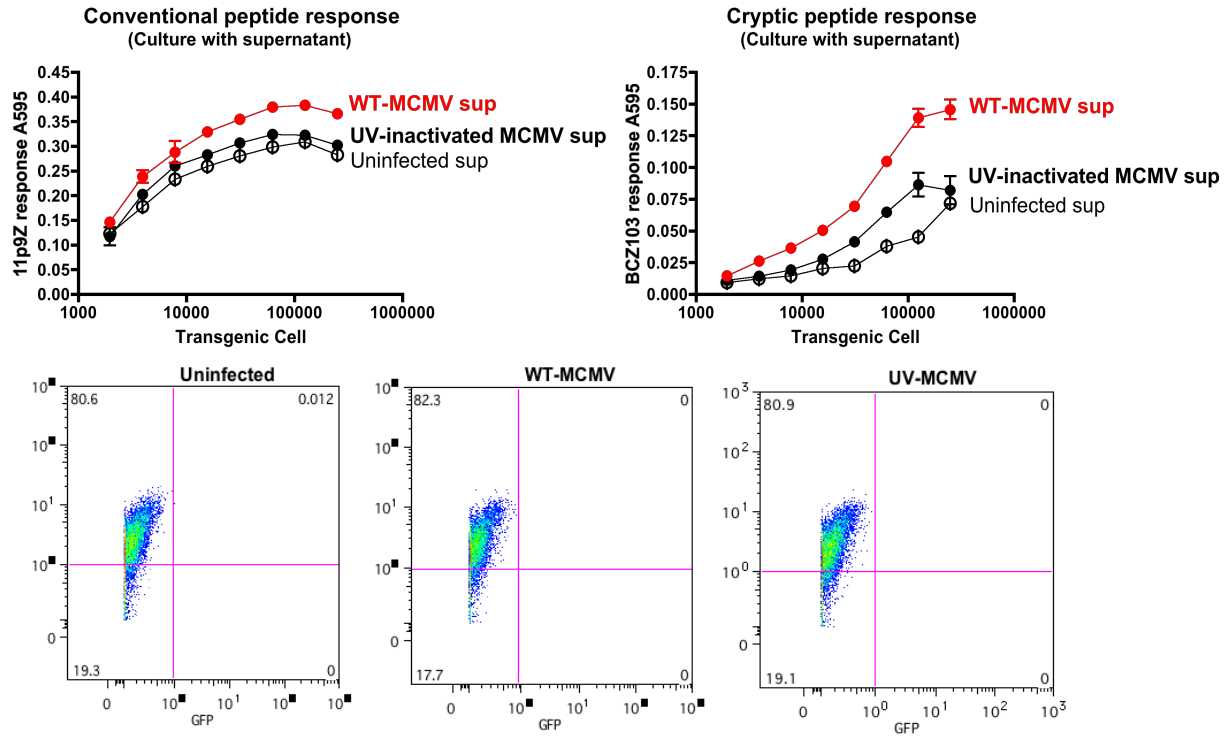


Figure 4.2: **Supernatant from infected $H2^D$ macrophages is sufficient to induce enhanced cryptic peptide presentation in WI9.LYL8 macrophages.** The supernatant from $H2^D$ macrophages, either infected or uninfected, were filtered through a 0.2um filter and added to the WI9.LYL8 macrophages. The supernatant was removed and T-cells were than added to these cells. The bottom panel indicates the WI9.LYL8 macrophages stained with a $D^b$-APC antibody and assessed for any GFP expression. (Data is representative of three independent experiments).

natant from uninfected cells, as was seen by the increased BCZ103 response (Figure 4.2). The 11p9Z response of the samples with infected supernatant was comparable to that of the samples with uninfected supernatant. UV-inactivated viral supernatant caused some enhancement of the BCZ103 response but much less potent than that induced by the wild-type viral supernatant. Finally, as a negative control, uninfected and infected $H2^D$ macrophages cultured with T-cell hybridomas did not stimulate any T-cell response, showing that this is a peptide-specific response and not random stimulation of the hybridoma (Figure 4.3).

In order to determine if there was any reinfection of the WI9.LYL8 macrophages during the co-culture and the overnight incubation with T-cells, the co-cultured cells were stained with various different surface MHC Class I antibodies and the co-expression of these Class I molecules with GFP (which is indicative of the infected cells) was determined. All of the $D^d$ positive cells were expected to be GFP positive, as the $H2^D$ macrophages were the ones that were infected with the virus. However, $D^b$ positive cells that are GFP positive would indicate that there was some reinfection of the WI9.LYL8 macrophages. This is

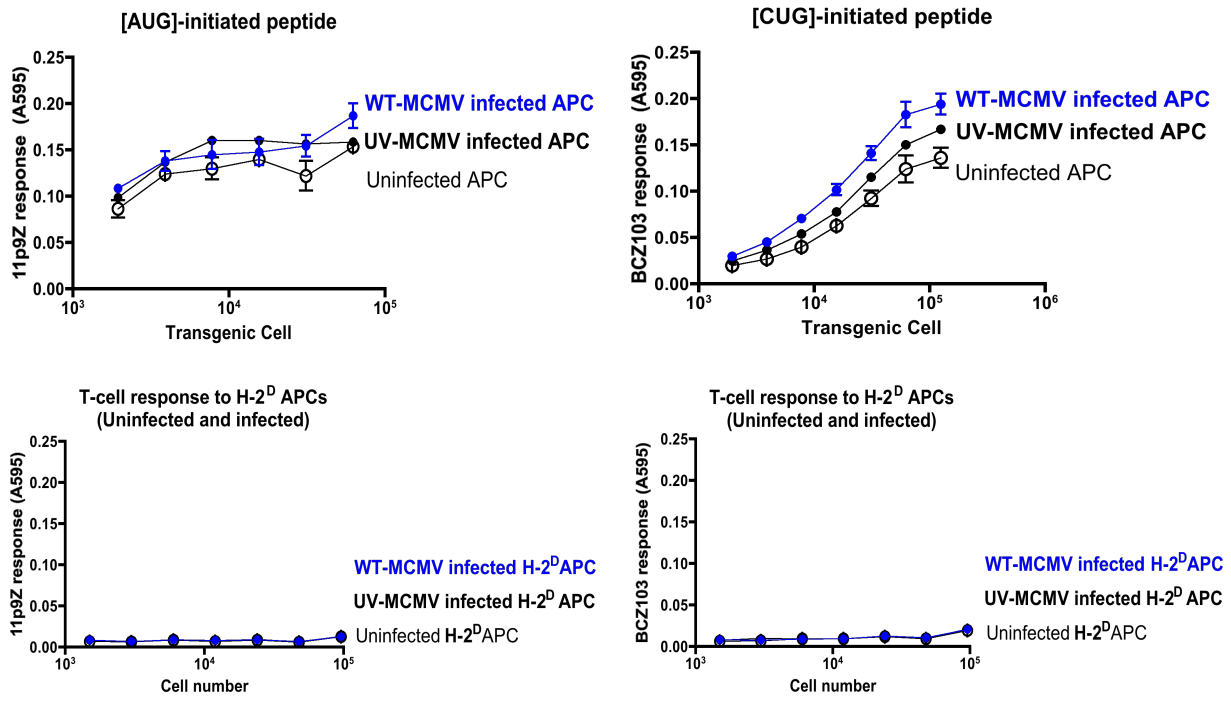


Figure 4.3: **Infected $H2^D$ macrophages do not induce any T-cell hybridoma response.** $H2^D$ macrophage were infected with wild-type or UV inactivated MCMV for 6 hours and then co-cultured with WI9.LYL8 macrophages. After 4-6 hours, T-cell hybridomas were added to the co-culture. The top panel indicates T-cell response to the co-cultured cells. The bottom panel indicates T-cell responses to the $H2^D$ macrophages alone.

exactly what we observed that the all of the GFP positive cells were $D^d$ positive, however there was only 1% of GFP positive cells that were also $D^b$ positive (Figure 4.4). There was mostly a distinct population of GFP positive cells and $D^b$ positive cells and very little overlap between those two populations. Furthermore, WI9.LYL8 cells that were cultured with the filtered supernatant from infected and uninfected cells did not express any GFP, even in the samples that were cultured with supernatant from WT-MCMV GFP (Figure 4.2). This suggested, that there were minimal virion particles in the supernatant and they were completely removed by filtration with 0.2$\mu$m filter. Furthermore, the enhancement observed in the T-cell assay where WI9.LYL8 macrophages were cultured with the supernatant was only due to secreted factors from infected cells. This co-culture assay does show that a majority of the enhancement of cryptic peptide presentation does occur by secreted factors from the infected cells. An alternate way to perform this experiment would be to perform this assay using trans-wells, which might prevent the passage of certain large particles like virions.

In summary, this experiment showed us two things firstly, cells do not have to be directly

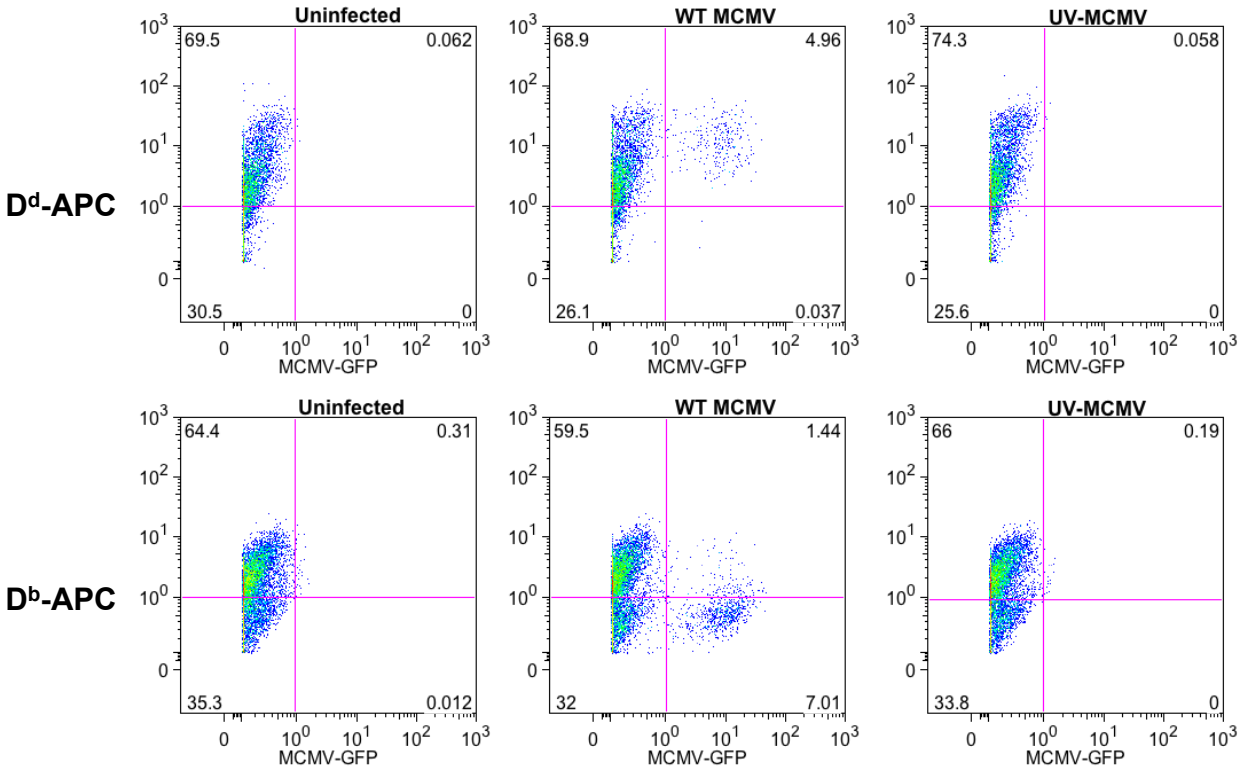


Figure 4.4: **Co-cultured cells, stained with surface MHC Class I antibodies, show minimal re-infection of WI9.LYL8 macrophages.** Co-cultured WI9.LYL8 macrophages and $H2^D$ macrophages were stained with either a $D^d$ antibody or a $D^b$ antibody and analyzed by flow cytometry to determine if there was any reinfection of WI9.LYL8 macrophages with MCMV-GFP. (Data is representative of two independent experiments).

infected with the virus to induce enhanced cryptic peptide presentation. It also showed that secreted factors from the infected cells are necessary and sufficient to induce enhanced cryptic peptide presentation. This experiment also helped identify one of the factors that are required for enhancing cryptic peptide presentation, which is TNF-$\alpha$ .

## 4.2 Inflammatory cytokines are able to enhance cryptic peptide presentation

Our next aim was to confirm that TNF-$\alpha$ does indeed enhance cryptic peptide presentation. For this purpose, we obtained WI9.LYL8 macrophages and stimulated them with TNF-$\alpha$ (0.1ug/mL) for a duration of 6 hours. As a negative control, we also stimulated the cells with IL-10 (0.1 ug/mL), which is not an inflammatory cytokine. The results showed that TNF-$\alpha$ was able to successfully enhance cryptic peptide presentation. (Figure 4.5)

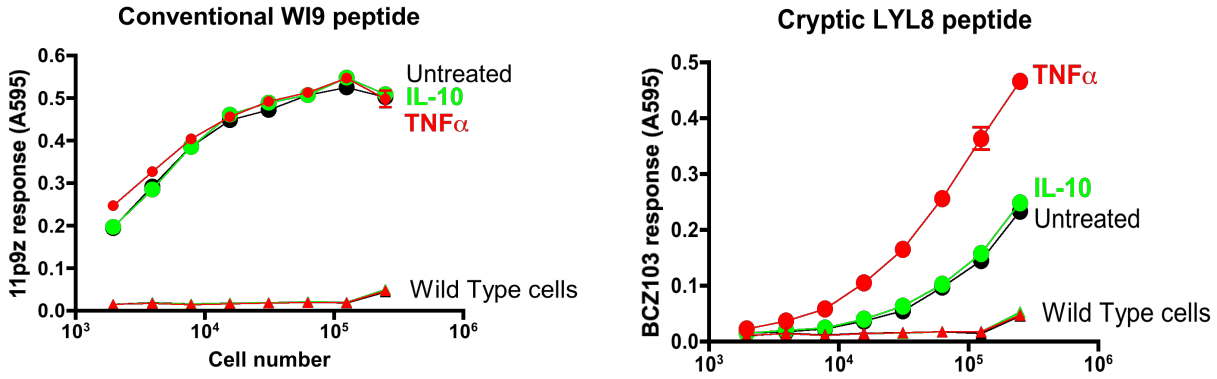


Figure 4.5: **TNF-$\alpha$ can enhance cryptic peptide presentation.** WI9.LYL8 or C57BL/6 primary macrophages were cultured with either TNF-$\alpha$ or IL-10 at a concentration of 0.1ug/mL for a duration of 6 hours. These cells were than harvested and cultured with T-cell hybridomas. (Data is representative of two independent experiments).

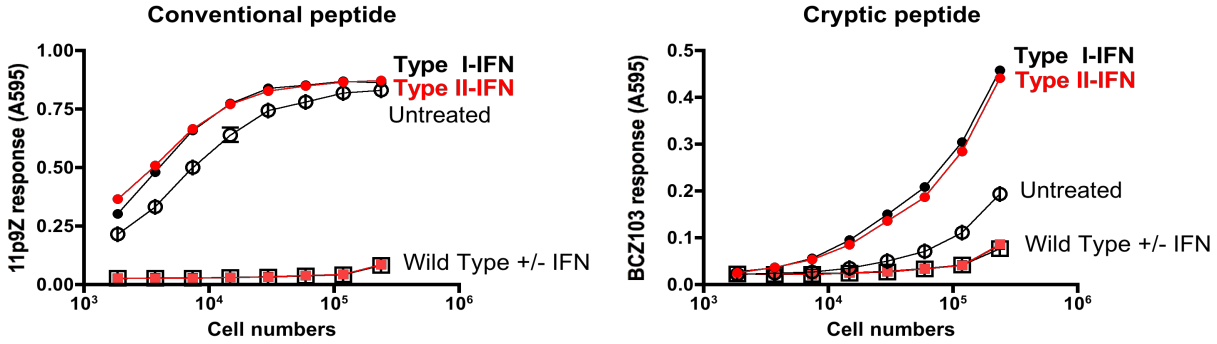


Figure 4.6: **Type I and Type II Interferons can also enhance cryptic peptide presentation.** WI9.LYL8 primary macrophages were stimulated with either Type I IFN (IFN$\beta$) or Type II IFN (IFN$\gamma$)for a duration of 6 hours. Cells were harvested and then cultured with T-cell hybridomas.

However, IL-10 was not able to enhance cryptic or conventional peptide presentation. As an additional control, we incubated the T-cell hybridomas with each of the cytokines to ensure that the hybridoma response was peptide specific. Neither TNF-$\alpha$ nor IL-10 stimulated the hybridomas non-specifically. Conventional peptide response in response to TNF-$\alpha$ also remained unchanged.

In addition to TNF-$\alpha$, there are other inflammatory cytokines which can activate macrophages and which can also be secreted by macrophages also. So, we tested the effect of Type I (Interferon-$\beta$) and Type II Interferon (Interferon-$\gamma$). Primary WI9.LYL8 macrophages were stimulated with IFN-$\beta$ and IFN-$\gamma$ for a duration of 6 hours. Cells were than harvested and

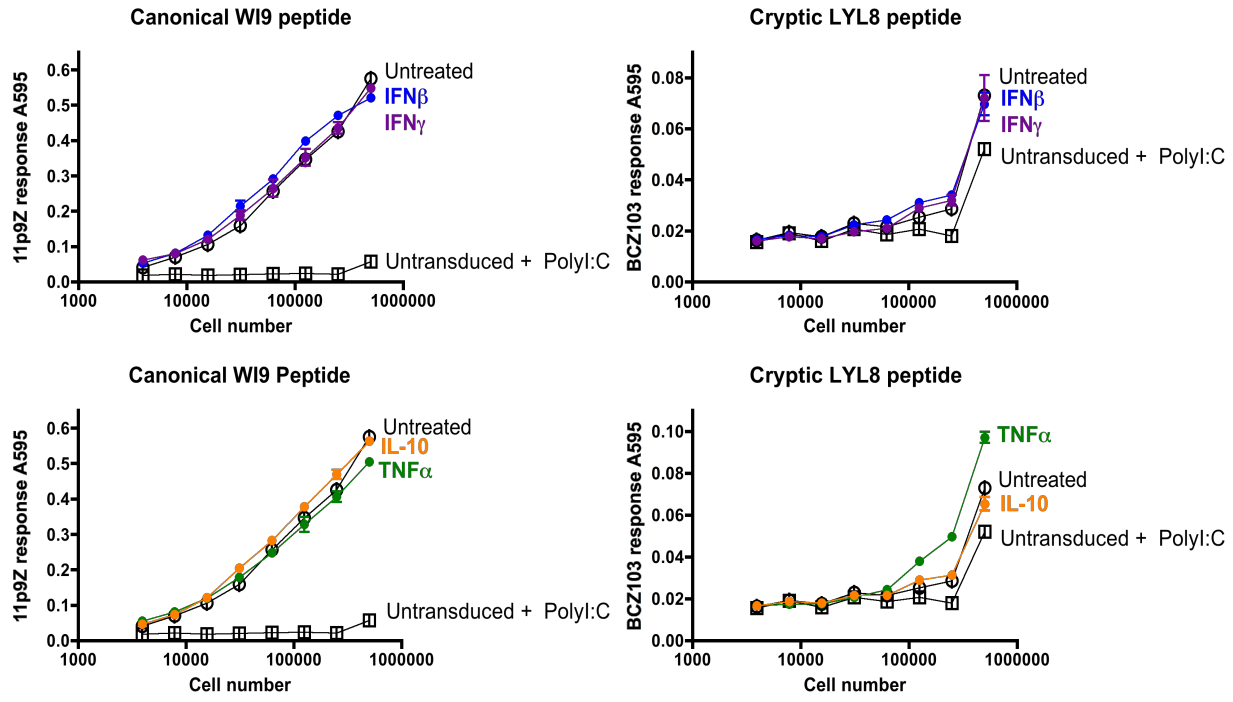


Figure 4.7: **TNF-$\alpha$ enhances cryptic peptide presentation in an alternative model system.** Primary C57BL/6 macrophages were transduced with a retroviral vector encoding the WI9.LYL8 transgene. The transduced macrophages were then stimulated with either Type I IFN, Type II IFN, IL-10 or TNF-$\alpha$, for a duration of 6 hours. Cells were harvested and then cultured with T-cell hybridomas.

incubated with T-cell hybridomas. Expectedly, IFN-$\beta$ and IFN-$\gamma$ were both able to enhance cryptic peptide presentation but they also enhanced conventional peptide presentation as is common with IFN stimulation, which can affect protein translation in a cell. (Figure 4.6). Therefore, this showed that inflammatory cytokines are able to induce enhanced cryptic peptide presentation. Some of them like Interferons are able to enhance conventional peptide presentation as well. Furthermore, this finding was tested in an alternate model system, whereby primary C57BL/6 macrophages were transduced with a retroviral vector (MSCV) expressing WI9.LYL8. After successful transduction, which was determined by the GFP expression in the macrophages from the MSCV vector, these cells were treated with different cytokines, Type I and Type II IFN, TNF-$\alpha$ and IL-10. In this model, it seemed that TNF-$\alpha$ was able to enhance cryptic peptide presentation. However, conventional peptide presentation was not enhanced by any of the cytokines, possibly due to the lack of the MHC Class I promoter in this model system (Figure 4.7). So, this model system confirmed that TNF-$\alpha$ was able to enhance presentation of cryptically translated antigenic peptides.

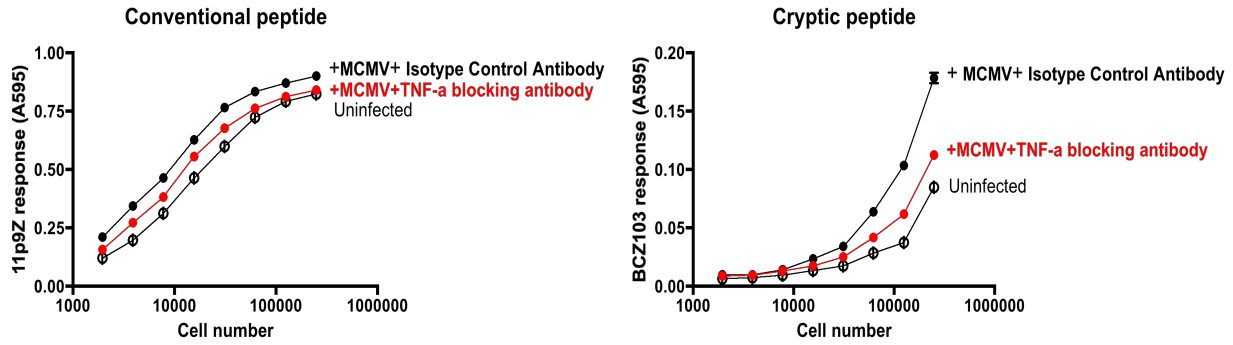


Figure 4.8: **Blocking TNF-$\alpha$ signaling inhibits the enhancement of cryptic peptide presentation upon MCMV infection.** WI9.LYL8 primary macrophages were infected with MCMV and cultured with either the TNF-$\alpha$ blocking antibody (MP6XT22) or the Isotype control antibody. This experiment performed for a duration of 6 hours. Macrophages were harvested and incubated with T-cell hybridomas along with either the TNF-$\alpha$ blocking antibody or the Isotype antibody. (Data is representative of two independent experiments).

## 4.3 Blocking the effect of cytokines with neutralizing antibody inhibits the enhanced presentation of cryptic peptides.

The next question that these results raised was, if the effect of these cytokines were blocked, does that abolish the phenomenon of enhanced cryptic peptide presentation? To answer this question, we used TNF-blocking antibodies to assess if this could reverse the enhancement of cryptic peptide presentation. Initially, we used a TNF-blocking antibody along with IgG1 Isotype control antibody. Primary WI9.LYL8 macrophages were infected with MCMV and concurrently cultured with the blocking antibody or the Isotype control antibody for a period of 6 hours. Cells were than harvested and a T-cell assay was performed. This particular experiment showed no effect of the blocking antibody on cryptic peptide presentation or conventional peptide presentation. We then switched to using an alternate clono-type of the TNF-$\alpha$ blocking antibody (MP6XT22) at a concentration of 1.0ug/mL and 2.0 ug/mL. Again primary WI9.LY8 macrophages were infected with MCMV and cultured with increasing doses of the new TNF-$\alpha$ blocking antibody or the isotype control antibody. (Figure 4.8) This experiment showed that with increasing concentration of the blocking antibody there was decreased enhancement of cryptic peptide presentation. Any increases in conventional peptide presentation were also blocked through the use of TNF-$\alpha$ blocking antibody. However, even at a high concentration of TNF-$\alpha$ blocking antibody like 2 ug/mL, there was still some enhancement of cryptic peptide presentation. This could be due to two possible reasons (which are not mutually exclusive). When the harvested macrophages are incubated with T-cells overnight along with blocking antibody, the effect of the antibodies might not have

lasted for 15 hours and the cells might have overcome the effect of the blocking antibodies. Another reason is that there might be other factors or cytokines in addition to TNF-$\alpha$ that could be causing the increased presentation of cryptically translated peptides.

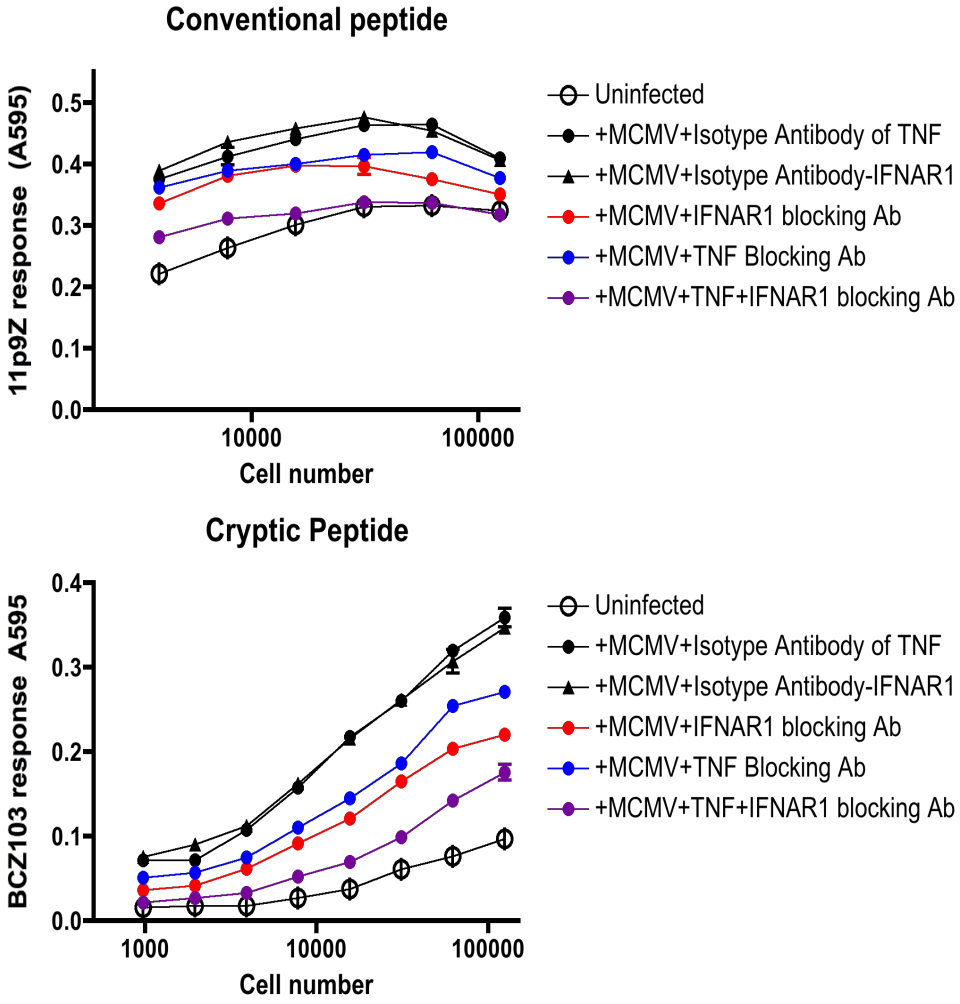


Figure 4.9: **Blocking TNF-$\alpha$ and Type I Interferon signaling further inhibits the enhancement of cryptic peptide presentation upon MCMV infection** WI9.LYL8 macrophages were infected with MCMV and then cultured with either TNF-$\alpha$ blocking antibody, IFNAR 1 blocking antibody or Isotype control antibodies or both TNF-$\alpha$ and IFNAR 1 blocking antibodies for a period of 6 hours. The cells were then harvested and cultured with T-cell hybridomas along with the respective antibodies. (Data is representative of two independent experiments).

So, we went on to test if IFN stimulated responses could also be blocked. IFNAR1 blocking antibody was obtained along with the Isotype antibody. Similar to the experiment with

the TNF blocking antibody, primary macrophages were infected with MCMV and simultaneously cultured with IFNAR1 blocking antibody at a concentration of 2ug/mL and Isotype antibody. As an additional control, cells were cultured with both TNF blocking antibody and IFNAR1 blocking antibody. This experiment clearly showed that blocking IFNAR1 alone diminished the enhancement of cryptic peptide presentation. Moreover, blocking TNF and IFNAR1 was able to further diminish the enhancement of cryptic peptide presentation close to the level of T-cell response towards uninfected cells. The remnant enhancement of cryptic peptide presented could be attributed towards incomplete blocking by the antibodies or cells overcoming the blocking in the overnight incubation or other inflammatory cytokines such as IL-6 (which were not tested for) (Figure 4.9).

In addition, WI9.LYL8 macrophages infected with MCMV were also treated with a compound known as Epigallocatechin-3-gallate (EGCG). This compound was shown to inhibit Myd88 and TRIF dependent signaling downstream of the Toll-like receptors ([73]). Therefore, we decided to test the effect of EGCG on the MCMV-induced enhancement of cryptic peptide presentation. (Figure 4.10). Cells treated with MCMV alone were able to elicit enhanced BCZ103 responses compared to the untreated cells. Cells treated with MCMV along with EGCG, showed mild reductions in the enhancement of cryptic peptide presentation. However, this was not a significant reduction in cryptic peptide presentation. This suggested that EGCG was not potent enough to completely shut down the signaling pathway that induced enhanced cryptic peptide presentation. However, EGCG probably acted to diminish some cytokine production downstream of Myd88 signaling and this led to the diminished cryptic peptide presentation that was observed in this experiment. The luciferase assay also shows lower levels of IFN-$\beta$ produced in samples treated with EGCG. This further confirms the findings observed above that cytokines regulate cryptic peptide presentation.

## 4.4 Discussion

In this study, we observed that inflammatory cytokines were able to enhance presentation of cryptically translated antigenic peptides. TNF-$\alpha$ and Type I and Type II Interferon were able to enhance the T-cell response to the LYL8 peptide. However, interestingly, IL-10 was not able to do so. It is known that IL-10 is a non-inflammatory cytokine. Studies have also shown that IL-10 is able to inhibit LPS induced TNF-$\alpha$ production in macrophages ([17]). There is also an interplay between IFN-$\gamma$ signaling which induces inflammatory cytokine production in macrophages but at the same time suppresses IL-10 production. It would have been interesting to test the effect of IL-10 on MCMV induced enhancement of cryptic peptide presentation along with the effect of both Type I and Type II Interferon.

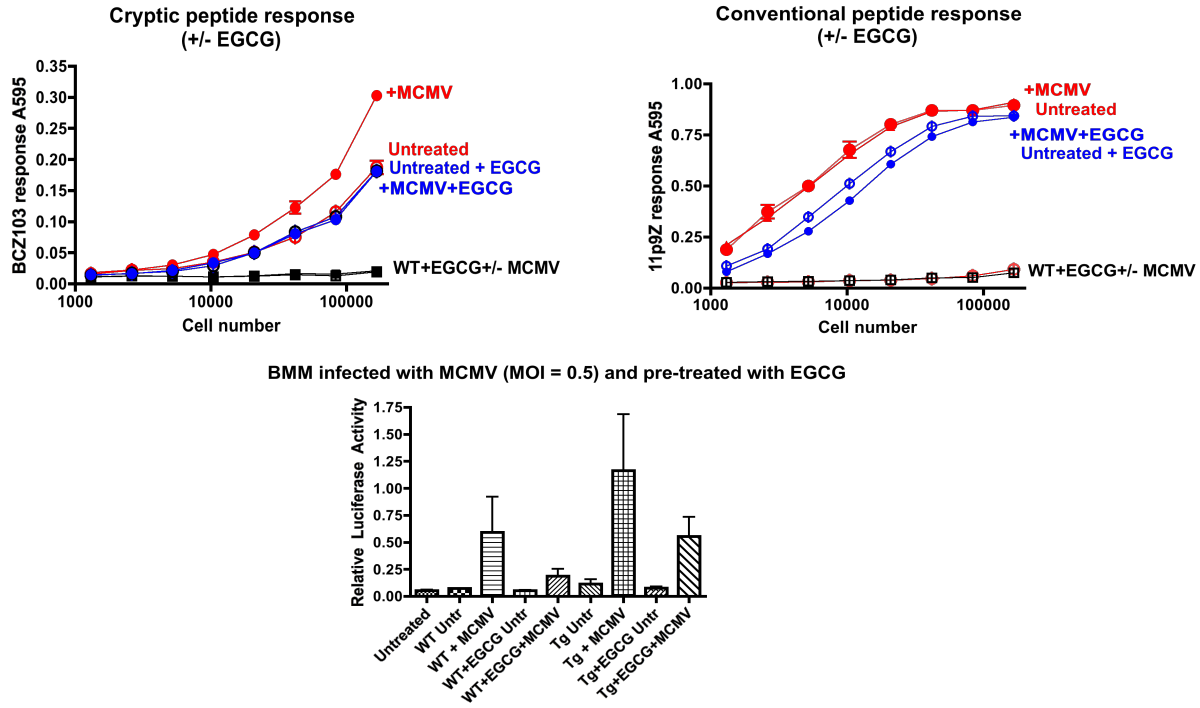


Figure 4.10: **Inhibitor of Myd88 inhibits enhancement of cryptic peptide presentation upon MCMV infection.** Top panel: Primary WI9.LYL8 macrophages were infected with MCMV at an MOI of 0.5 with or without the inhibitor EGCG (Epigallocatechin-3-gallate) for a duration of 6 hours and were then harvested for a T-cell assay Bottom panel: Supernatant from the infected and uninfected cells were cultured with the L929 cells that contain an ISRE-Luciferase reporter. These cells were harvested and lysed for a luciferase assay.

If a cell is increasing the presentation of cryptically translated antigenic peptides, in response to virus infection and inflammatory cytokines, what physiological advantage can that render? It could be a part of an antiviral response being generated against the virus, or it could be a by-product of the viruses controlling the cellular translation machinery in order to translate viral gene products. A virus infection or cytokine stimulation of a cell activates many different signaling pathways within the cell, some of which enhance translation. Previous studies have only looked at the effects of virus infection on conventional translation; however, this is the first study that establishes that cryptic peptides are also significantly increased during such events. Moreover, when the effect of cytokines is blocked by using different cytokine blocking antibodies, such as anti- TNF-$\alpha$ or IFNAR1 antibodies, cryptic peptide presentation is diminished. This shows that cryptic peptide presentation is directly linked to the activation status of the cell.

A study ([57]) showed that MCMV infection in macrophages acts to inhibit TNF-$\alpha$ signaling in an attempt to diminish the antiviral immune responses. Our findings, interestingly, show that MCMV induces macrophages to produce TNF-$\alpha$, as an antiviral response and

thereby increases cryptic peptide presentation as well. The MCMV infection in the referred study was performed at an MOI of 10 for a duration of 18 hours. It would be of interest to test if higher MOIs of MCMV lead to diminished TNF-$\alpha$ production and if that leads to decreased cryptic peptide presentation. It would also be interesting to test the effect of MCMV and TNF-$\alpha$ on cryptic peptide presentation.

If TNF-$\alpha$ and Interferons are able to enhance presentation of cryptically translated peptides, there must be a downstream factor that can transduce the signal and affect the translation pathway. A factor that is common to both the IFN and TNF signaling pathway is the p38 MAP kinase. The p38 MAP kinase pathway can be induced by different kinds of cellular stresses, osmotic shock, heat, and proinflammatory cytokines. ([34]) Some of the targets of p38 MAP kinase that are of interest to our study are MNK1 (MAP kinase interacting kinase) and RSK-B (ribosomal protein kinase B). MNK1 regulates cap-dependent translation by phosphorylation eIF4E. RSK-B is a nuclear protein that is associated with the regulation of glycogen metabolism. Interestingly, RSK-B was one of the genes that gets inhibited by EGCG (epigallocatechin-3-gallate), a chemotherapeutic agent derived from green tea, in a study that was used to assess the cell growth inhibition properties of EGCG by looking at the gene expression profiles in prostrate cancer cells ([79]). [Interestingly EGCG also played a role in diminishing the enhancement of cryptic peptide presentation in our study]. Therefore, this p38 MAP kinase pathway could have played a role in enhancing cryptic peptide presentation in response to TNF and IFN signaling.

Our study suggests that this increased phenomenon of cryptic peptide presentation can be exploited for vaccines and therapy against viruses, especially those that are latent. The immune system may not be tolerant towards many self-cryptic peptides and therefore the T-cells specific for these cryptic peptides may not have been negatively selected. Therefore, it is useful to identify alternative reading frames or initiation codons that could give rise to peptide products and develop T-cell responses against these peptides. In terms of developing more effective vaccines against latent viruses, it has been shown that despite robust acute CTL responses against cryptic epitopes arising from viral genomes, the memory response to these cryptic epitopes are impaired. The memory CTLs have reduced cytolytic capabilities and cytokine production ([62]) . Therapeutic strategies are currently being designed to improve the memory T-cell response. If cryptic peptides can be enhanced by inflammatory stimuli like cytokines, this could be a way to prime CD8+T cells and enhance T-cell responses.

# Chapter 5

# Investigating the mechanism of enhanced presentation of cryptically translated antigens

**Summary**

CUG-initiated translation is known to be a distinct mechanism that is resistant to a variety of cellular stresses and is also known to use a distinct initiator tRNA to begin translation at the CUG codon. This study has shown that virus infection and inflammatory cytokines are able to enhance presentation of cryptically translated peptide antigens to T-cells, significantly more than conventionally translated peptide antigens. However, the mechanism behind how this happens is still unclear. Do cryptic peptides get delivered to the antigen presentation pathway in a distinct manner? Are all cryptic peptides generated in the same location as conventional peptides? Are the same signaling pathways used to induce cryptic translation of mRNAs or are there distinct factors involved. This chapter attempts to address some of these questions. Here, we show that both Met and Leu initiator tRNAs are enhanced upon MCMV infection. Moreover, eIF2A protein levels remain unchanged upon MCMV infection. However, the PI3 kinase pathway seems to be required for both cryptic and conventional peptide presentation.

## 5.1 eIF2A levels are unchanged in macrophages upon stimulation with TLR ligands

To delineate the mechanism of this enhanced presentation of cryptically translated peptides, we determined what eukaryotic initiation factors may be involved in this process. We decided to use this approach since it was established by Starck et al. 2012, that eIF2A was required for cryptic initiation to take place. So our hypothesis was that eIF2A may be enhanced under inflammatory conditions explaining the enhanced cryptic peptide presentation. Primary

WI9.LYL8 macrophages were infected with MCMV or treated with LPS or PolyI:C, for a duration of 6 hours. Cells were then harvested and re-suspended in lysis buffer for analysis by western blotting. After running the samples on a gel and transferring to a nitrocellulose blot, the samples were stained with an anti-eIF2A primary monoclonal antibody. The samples were then stained with an appropriate secondary polyclonal antibody and visualized by enhanced chemiluminescence (ECL). Upon repeated attempts of this experiment, we concluded that there was no significant difference in eIF2A protein expression upon MCMV infection or TLR ligand treatment. (Figure 5.1).This did not rule out the fact that there could be differential activity of the eIF2A protein upon MCMV infection or TLR ligand treatment. This means that there could be differences in phosphorylation, for example, that could change the enzymatic activity of this protein and how it regulates translation initiation. The only way to evaluate that would be to further characterize eIF2A and the nature of its activity, since the protein was discovered very recently ([88]). In addition to eIF2A, we tested the expression of some other initiation factors like eIF2-$\alpha$ as a control since we wanted to establish that there are differences only in eIF2A levels and not in other initiation factors as well. There was no visible change observed in the levels of eIF2-$\alpha$ either upon MCMV infection or TLR ligand treatment.

In addition, eIF2A was knocked-down in primary macrophages, by using the INTERFERin transfection reagent to introduce the siRNA against eIF2A into cells. The expression of eIF2A was determined by western blots, to ensure that eIF2A was indeed knocked-down. This was followed by a T-cell assay to determine the expression of WI9 and LYL8 in the presence of the siRNA against eIF2A. No changes were observed in the T-cell responses to WI9 or LYL8 in the presence of the eIF2A siRNA. Interestingly, the LYL8 specific T-cell response seemed to be enhanced in the presence of the eIF2A siRNA. Therefore, this further confirmed that in primary macrophages, eIF2A was not involved in regulating cryptic peptide presentation. (Figure 5.2)

## 5.2 Methionine and Leucine tRNA levels are enhanced upon MCMV infection.

In humans, tissue-specific differences in tRNA expression are observed ([13]) and these differences are as high as ten-fold between different tissue types. In the same study, a correlation between relative tRNA abundance and codon usage was observed in the regulation of tissue-specific genes. Hence, this suggested that tRNA levels could play a unique role in regulating cellular development and differentiation. Therefore, we decided to test the amount of tRNAs in macrophages with and without MCMV infection to determine if Met and Leu tRNAs are differentially expressed.

RNA was extracted from cells that were infected with MCMV or cells that were uninfected. This RNA was used in a Northern Blot analysis, where the RNA was run out on a gel, transferred onto a membrane. The membrane was then blotted with tRNA probes

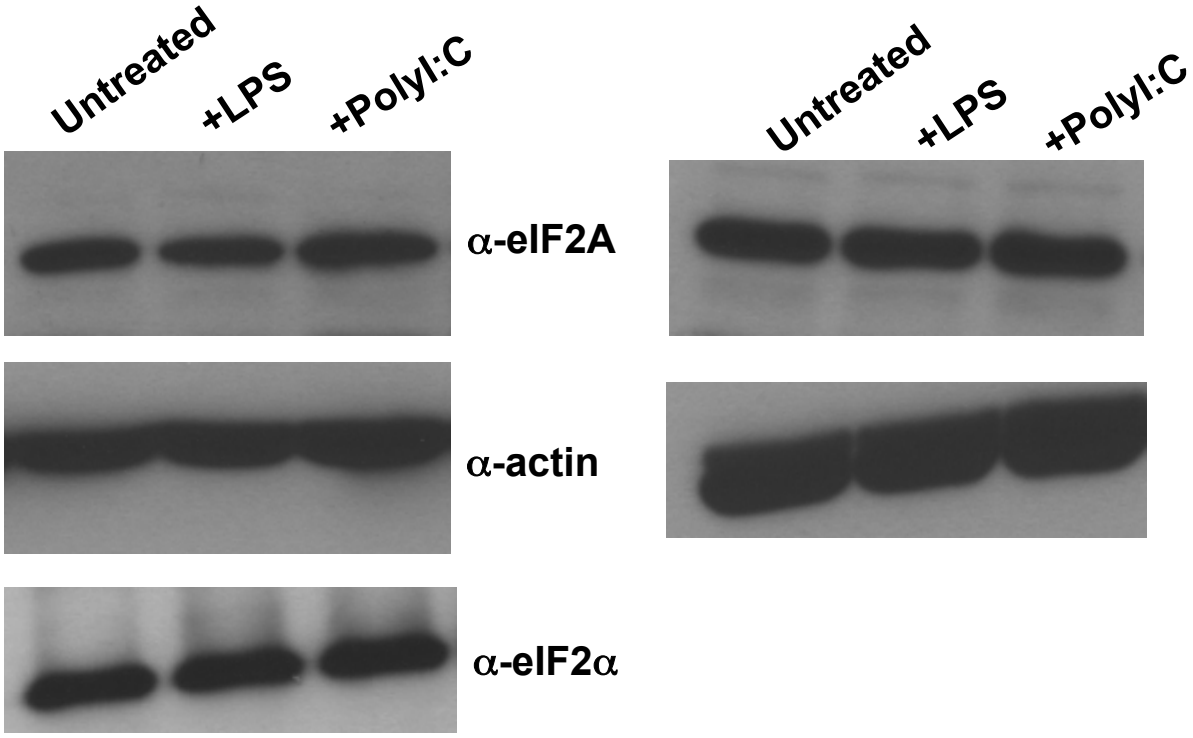


Figure 5.1: **eIF2A levels are unchanged upon TLR ligand stimulation** WI9.LYL8 macrophages were stimulated with LPS and PolyI:C for duration of 6 hours. The cells were then lysed and run on an SDS-PAGE gel, transferred onto a nitrocellulose membrane and then blotted with different antibodies (which are indicated above) and detected by chemiluminescence. The panel on the right is one trial and the panel on the left is a second trial. (These data are representative of multiple trials).

that were specific for Met-tRNA, Leu-tRNA. Upon visualization of the membrane, we observed that both Met-tRNA and Leu-tRNA were enhanced upon MCMV infection. There was no differential enhancement of the Leu-tRNA that could explain the enhancement of cryptic peptide presentation. However, we did lack a loading control to ensure that the same amount of RNA was loaded for each sample (Figure 5.3).

## 5.3 PI3-kinase pathway is required for cryptic peptide presentation.

The PI3-kinase pathway is important in regulating cell proliferation and survival upon any growth factor stimulation or immune signaling pathway activation ([61]). We decided to investigate if this pathway plays a role in regulating cryptic peptide presentation in a sim-

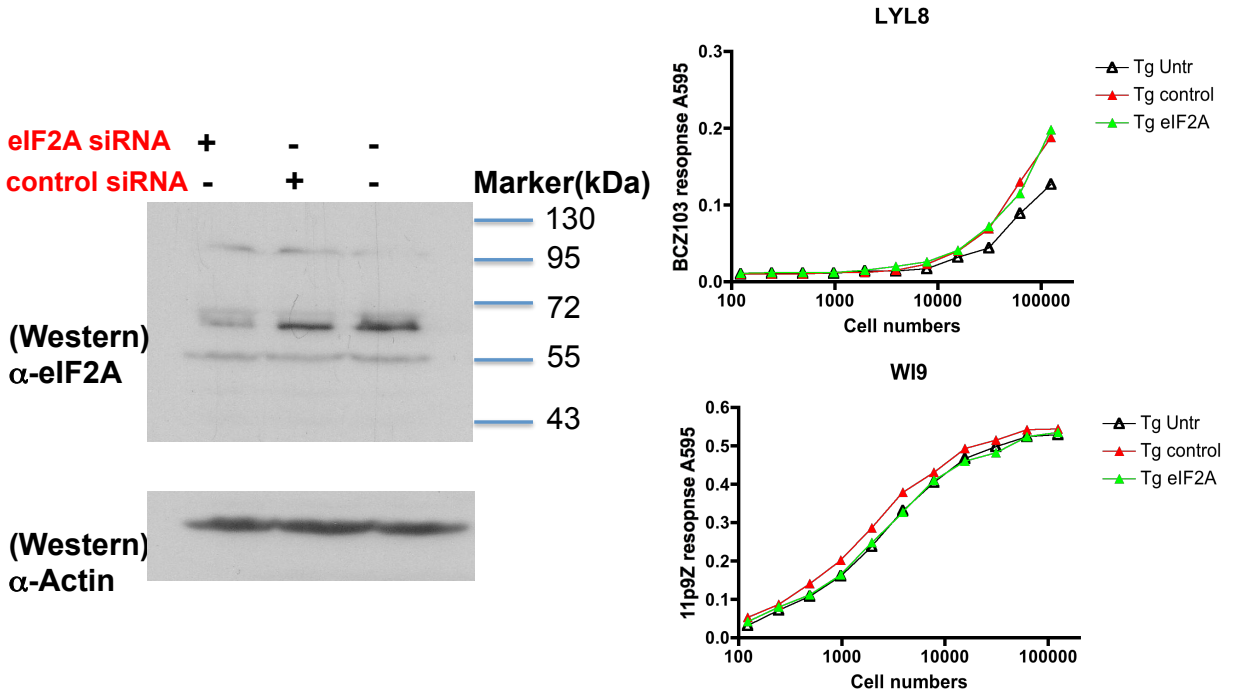


Figure 5.2: **eIF2A knock-down in primary macrophages does not affect cryptic peptide presentation** eIF2A was knocked down using siRNA eIF2A, which was transfected into primary macrophages using the INTERFERin reagent. Cells were than harvested and used for a T-cell assay or for western blotting to determine the level of eIF2A protein within the cell.

ilar way to conventional peptide presentation. WI9.LYL8 macrophages were infected with MCMV and at the same time treated with 2 different PI3-kinase/Akt inhibitors Ly294002 ([22]) and Wortmannin for a duration of 6 hours. The cells were then cultured with T-cell hybridomas. We observed that MCMV infection enhanced both cryptic and conventional peptide presentation. Upon Ly294002 treatment, conventional peptide presentation was diminished compared to the presentation levels of the uninfected cells. Cryptic peptide presentation was also diminished upon Ly294002 treatment, however, the level of cryptic peptide presentation was still significantly more than that of the uninfected cells. Similarly, Wortmannin also diminished conventional peptide presentation to that of uninfected cells. (And this was after a 6 hour period of inhibition and 16 hour period of recovery when the macrophages were cultured with T-cells. The inhibitors were excluded during the T-cell assay, lest they act on the T-cell hybridomas). Cryptic peptide presentation again was inhibited upon Wortmannin treatment, but this inhibition was not as severe as compared to the Ly294002 treatment and the cryptic peptide specific T-cell response was significantly enhanced compared to that of uninfected cells. This showed that cryptic peptide presentation was more resistant to these inhibitors than was conventional peptide presentation. However,

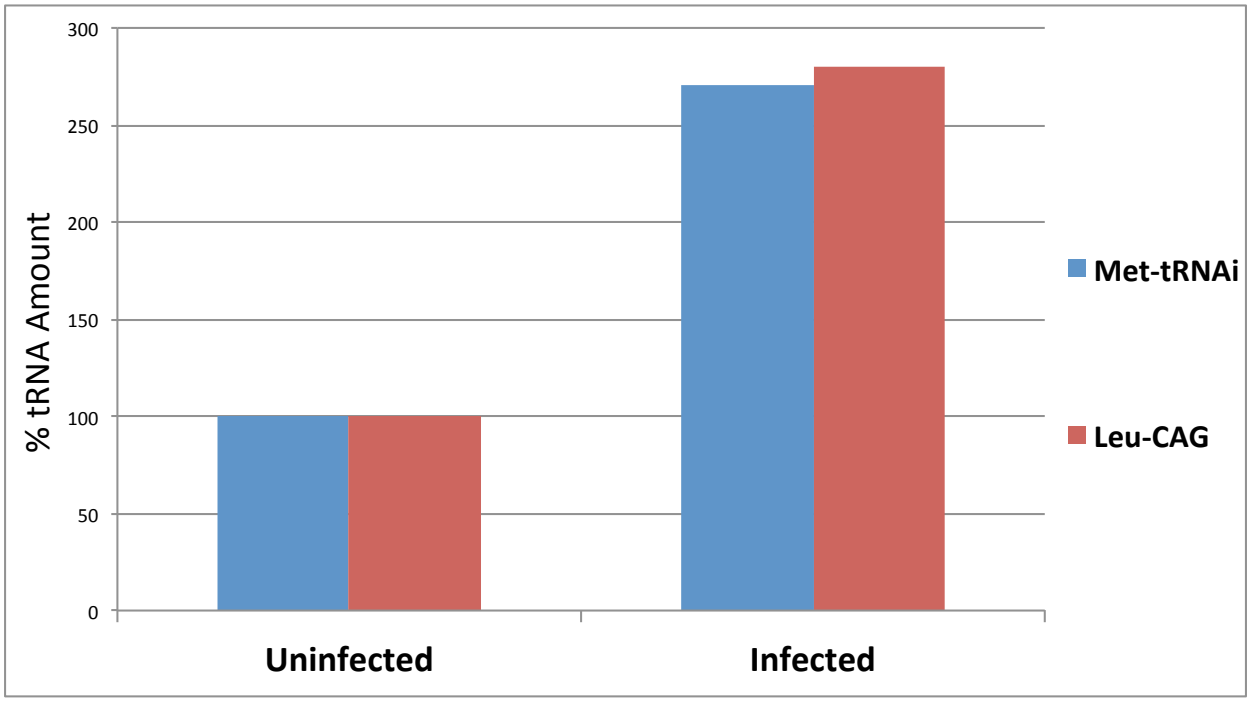


Figure 5.3: **Both Methionine and Leucine tRNA amounts are enhanced upon MCMV infection** RNA was isolated from macrophages that were infected with MCMV. This RNA was run on a polyacrylamide gel, transferred onto a membrane and blotted with different anti-Met and anti-Leu tRNA probes.

the PI3-kinase pathway is required for enhancing cryptic peptide presentation to the same level as that with MCMV (Figure 5.4).

## 5.4 Discussion

We determined in the previous chapter that cytokines were required for the enhancement of cryptic peptide presentation. Studies have shown that the PI3-kinase pathway is important for the generation of cytokines in antigen-presenting cells ([23]). The PI3-kinase pathway has also been shown to be important in regulating NKG2D ligands, especially upon MCMV infection ([75]). Moreover, the PI3-kinase pathway has been implicated in contributing to the generation of cytosolic bodies known as DALIS (Dendritic cell Aggresome like Induced Structures). DALIS is a structure that arises during an adaptive stress response when there is a large increase in protein synthesis and a massive build-up of DRiPs ([54]). DALIS formation is shown to be resistant to PI3-kinase inhibitors ([55]). All of these findings led us to believe that the PI3-kinase pathway might be important in regulating cytokine production in macrophages. Inhibition of the pathway led to lesser production of cytokines and thereby a lower cryptic peptide presentation response.

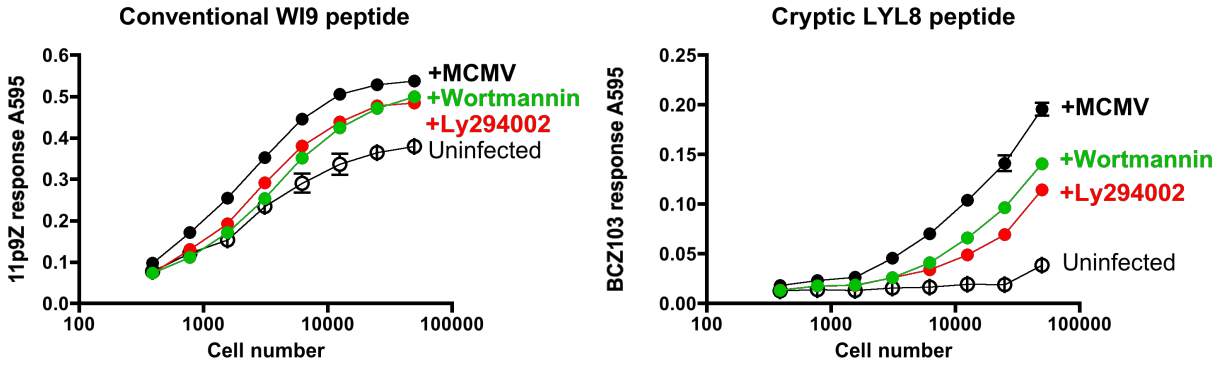


Figure 5.4: **PI3-kinase pathway inhibits both cryptic and conventional peptide presentation**WI9.LYL8 macrophages were infected with MCMV and treated with either Ly294002 or Wortmannin for a duration of 6 hours and then a T-cell assay was set up. (Data is representative of three independent experiments).

In order to rationalize this phenomenon of enhanced cryptic peptide presentation in terms of cellular mechanism, the ways in which antigens are generated would have to be assessed. The antigen generation pathway begins with protein translation. Here, we have established a difference between cryptic and conventional translation by showing that there is a novel tRNA initiating at CUG codons. However, both Leu tRNAs and Met tRNAs are enhanced upon MCMV infection and there was no differential enhancement of Leu tRNAs. Therefore, enhanced cryptic peptide presentation is not regulated at the tRNA level. However, there are studies of proteins that can regulate the efficiency with which Met-initiator tRNA is bound to the eIF2 complex and thereby control the efficiency of translation initiation. One such protein is the AIMP3/p18 ([31]). This protein is anchored to the methionyl tRNA synthetase complex. AIMP3 knock down resulted in reduced delivery of Met-initiator tRNA to eIF2 and reduced protein synthesis. AIMP3 is found in a complex with AIMP1 and AIMP2 proteins ([52]). AIMP2 can act as a critical mediator of TNF signaling and induce apoptosis by downregulating TRAF2 (TNF receptor associated factor 2). AIMP1 can be released from the multi-tRNA synthetase complex upon cellular stress and activate macrophages by the p38 MAPK pathway and induce the production of TNF-$\alpha$. Given the localization of these AIMP proteins within the tRNA synthetase complex and their diverse functionality, it would be of interest to test the expression of these AIMP proteins in the context of MCMV infection to determine if they can regulate cryptic peptide presentation. Are AIMP proteins required for cryptic translation to take place? Will an overexpression of AIMP3/p18 cause decreased initiation at CUG initiation codons? Upon cytokine stimulation, does the functionality of these AIMP proteins change? These would be worthwhile questions that can be addressed, to figure out the mechanism by which cryptic peptide presentation occurs.

Translation initiation is regulated by numerous eukaryotic initiation factors, which can control the efficacy of translation initiation. Starck et al. (2012) showed that the unique

factor eIF2A was required for initiation at CUG codons. eIF2A was shown to be required for the translation of an HCV mRNA ([35]). Translation of HCV mRNA was shown to be resistant to the effects of eIF2-$\alpha$ phosphorylation. Under such stressful conditions, eIF2A was shown to interact with an HCV IRES element and enabled the loading of Met-tRNA to the P site on the 40S ribosomal subunit. Since CUG initiated translation was also shown to be resistant to eIF2-$\alpha$ phosphorylation, we had decided to test the eIF2A levels in macrophages with and without TLR ligand stimulation. We had expected to see an increase in the eIF2A protein levels upon LPS and PolyI:C stimulation, which cause increased cryptic peptide presentation. However, there were no differences in the total eIF2A protein level suggesting that this was also not the mechanism by which cryptic peptide presentation was regulated.

In addition to the mechanism of translation, the location of protein translation could also be an important determinant in figuring out how cryptic peptide presentation is enhanced. Are these cryptic ribosomes situated in the cytoplasm, in the nucleus or in specialized compartments such as the DALIS? Do they get degraded at the same rate as conventional proteins or are there special chaperones that protect them? The proteolytic pathway has been shown to play an important role in the generation of proteins. The proteolytic pathway targets a host of cytosolic and nuclear proteins and these proteins can include abnormal, short-lived and long-lived proteins. There is a special 20S proteasome that is interferon inducible. Components of this immunoproteasome such as LMP2 and LMP7 are encoded in the MHC locus ([60]). We have assumed that all of these factors contribute equally to the generation of cryptic peptides. But these have not been tested. Moreover, the proteasome has been known to selectively destroy certain antigenic epitopes more than others. This could have also led to the increased representation of cryptically translated peptides.

The next question that arises is whether these cryptically translated peptides get translocated into the ER at the same rate as conventional peptides? These aspects can be easily tested with the use of the WI9.LYL8 model system and more light can be shed on the mechanism by which enhanced cryptic peptide presentation occurs.

# Chapter 6

# Alternate ways to study cryptic translation of antigenic peptides

**Summary**

The previous studies focused primarily on using two model systems to study cryptic translation. One model was the in-vitro [AUG]-YL8/[CUG]-YL8/[CCC]-YL8 mini-gene constructs which could be transfected into cells and the other model was using the WI9.LYL8 transgenic mouse model. We, briefly, also examined the usage [AUG]-GFP/[CUG]-GFP/[CCC]-GFP. However, an alternate model system to study cryptic translation would be to combine these peptide antigen and generic GFP protein-based models. This would enable us to study a peptide antigen and a generic protein arising from the same initiation codon. In this chapter, we describe the development of a fusion DNA construct that combines the [X]-YL8 antigen (X representing an initiation codon) and GFP. These constructs were transfected into different cell types and the expression of the peptide antigens and GFP was examined by a T-cell assay and flow cytometry respectively.

## 6.1 Generation of Peptide-GFP-fusion constructs

In a system devised by Schwab et al. (2004), each initiation codon has repeats of a particular codon upstream and out of frame with it, which can either enhance or inhibit translation initiation at the downstream start site. So as the ribosome scans for the start, it can halt at these upstream codons depending on the strength of the initiation codon. For example, if CUG is the initiation codon, having AUGs upstream of it will enhance cryptic initiation at the CUG, since the ribosomes would tend to halt at the upstream AUGs and this would facilitate the binding of the cryptic ribosome to the CUG codon. However, having AUGs upstream of an AUG start site will inhibit translation initiation at the AUG start site for the same reason; ribosomal scanning. We used these constructs, which contained the (AUG)-YL8 or (CUG)-YL8 gene downstream of the scanning codons and decided to fuse this to a

GFP reporter. In this way, this construct would encode an antigenic peptide and a generic protein, both initiated by the same initiation codon (Figure 6.1).

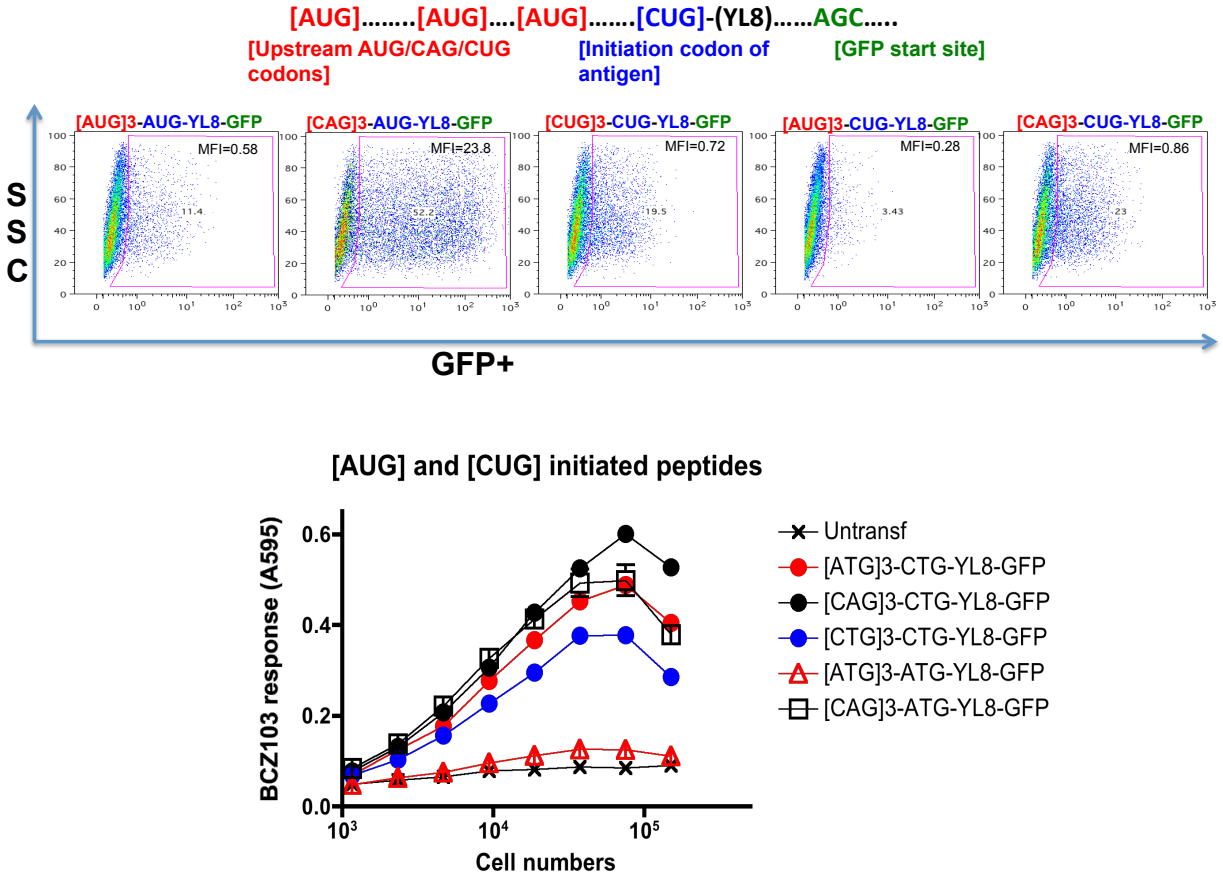


Figure 6.1: **GFP was fused to the (X)-YL8 peptide constructs to generate a fusion construct.** The schematic on top shows the kind of constructs that were made. Upstream codons will strengthen or weaken the translation initiation at the initiation codon of the antigenic peptide. The initiation codon is either ATG or CTG and downstream of that is the GFP sequence which lacks the ATG initiation codon and therefore relies on the initiation codon of the antigenic peptide. The FACS plots show the GFP expression from each of the different initiation codon and its corresponding upstream codons. The cDNA of these different constructs were transfected into K89 cells and harvested for a T-cell assay with the BCZ103 hybridoma.

For this purpose, five different constructs were made with GFP downstream of the (X)-YL8 antigenic peptide. The initiation codon of GFP was removed so as to couple the translation initiation of GFP to the initiation codon of the antigenic peptide. The 5 different constructs that were used are [ATG]3-CTG-YL8-GFP (1492), [CAG]3-CTG-YL8-GFP (1493), [CTG]3- CTG-YL8-GFP (1495), [ATG]3-ATG-YL8-GFP (1549) and [CAG]3-ATG-YL8-GFP (1554). The codon in parentheses is the upstream codon for ribosomal scanning.

The purpose of having different scanning codons is to determine the effect of the scanning codon on the strength of translation initiation. CAG is a non-specific codon that will not have any effect on translation initiation. AUG is the most widely used initiation codon and CUG is the next best initiation codon as characterized by Malarkannan et al. (1999)

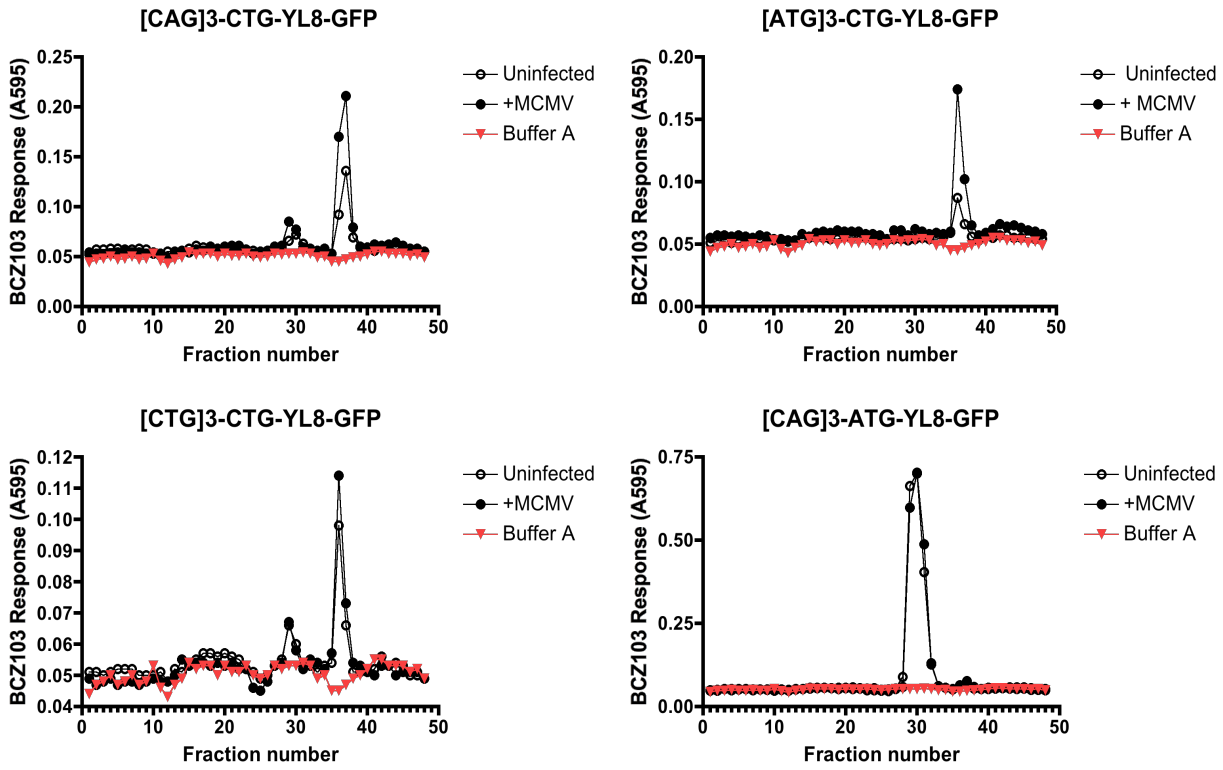


Figure 6.2: **Cryptic peptide amounts from the peptide-GFP-fusion constructs are slightly enhanced upon MCMV infection.** K89 fibroblasts were transfected with the indicated GFP-fusion constructs and then infected with MCMV. Cells were then harvested, peptides were acid-extracted and fractionated by RP-HPLC. A BCZ103 assay was then performed to detect peptide amounts.

When these constructs were transfected into Cos7 cells, both the antigenic peptide and GFP could be detected. 1492, which had ATGs upstream of the CTG initiation codon, elicited the lowest level of GFP but a significant BCZ103 response. 1493, which had CAGs upstream of the CTG initiation codon, elicited a significant BCZ103 response, stronger than that of 1492 and a potent GFP signal as well. 1495, which had CTGs upstream of the CTG initiation codon, elicited a reduced BCZ103 response compared to 1492 and 1493 but the GFP signal was as strong as that of 1493. This was an interesting result because we would have expected the GFP signal to also be as low as 1492 but it seemed like there was wobble decoding of the CTG initiation codon as Methionine. However, when MYL8 is generated

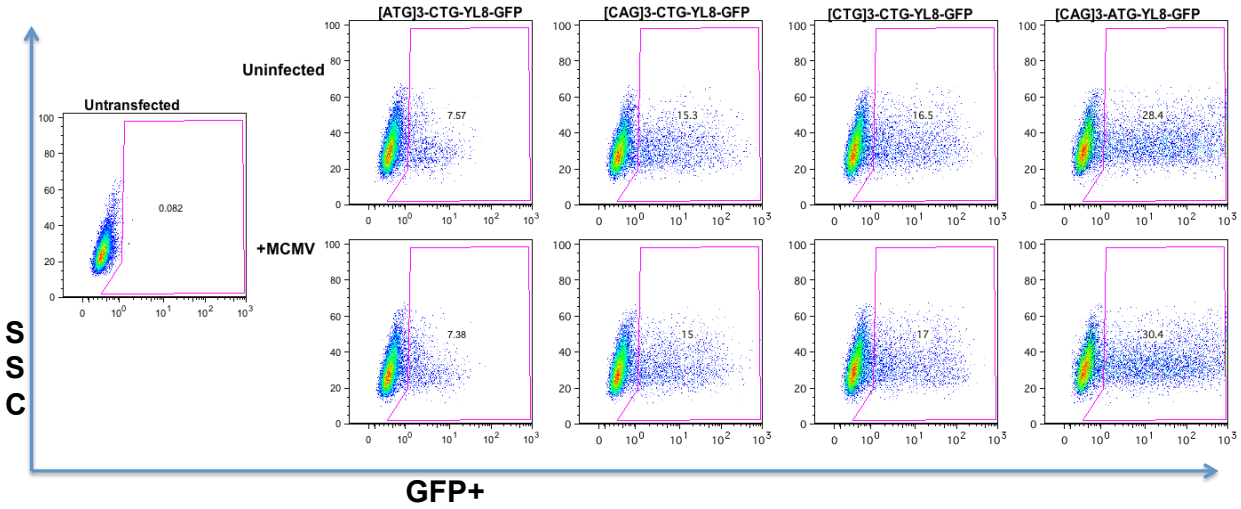


Figure 6.3: **Cryptically generated GFP from the fusion constructs is not enhanced upon MCMV infection** K89 fibroblasts were transfected with the indicated GFP-fusion constructs and then infected with MCMV. Cells were then harvested and analyzed by flow cytometry.

as the antigenic peptide, it is detected at a lower sensitivity by the BCZ103 hybridoma, explaining the low T-cell response. 1554, which has CAGs upstream of the ATG initiation codon, elicited a strong BCZ103 response and the strongest GFP signal. Even though 1554 codes for the MYL8 peptide, there is such a large amount of MYL8 peptide produced that despite the decreased sensitivity, the T-cell response is similar to that seen in 1493. Finally, 1549 generates almost no BCZ103 response and a very minimal amount of GFP is produced since the upstream ATGs inhibit initiation at the ATG codon.

These constructs were transfected into Cos7 cells and extracted by acid and fractionated by high-performance liquid chromatography (RP-HPLC). When these constructs were fractionated by RP-HPLC, different peptide amounts were detected from each construct. 1492 produced a single peak of LYL8, visible at fraction 28-30. [The positions of the peak have been determined prior to this experiment by running synthetic peptides through the column]. 1493 produced 2 peaks; one of the MYL8 peptide, which fractionates early at around fraction 24-26 and one of the LYL8 peptide. The MYL8 peak is low in amount compared to the LYL8 peptide, which shows that wobble decoding is occurring, but at a low rate. 1495 also produced 2 peaks, one of the MYL8 peptide and one of the LYL8 peptide. The LYL8 peak in this sample is, however, lower than the LYL8 peak observed when construct 1492 is run through the column, showing that CTG codons upstream of the CTG initiation codon manage to inhibit the amount of peptide produced. The 1554 construct produced a single peak of MYL8 that was strongly detected by the BCZ103 hybridoma. The 1549 construct, which has multiple ATGs upstream of the ATG initiation codon, produced a very small single peak of the MYL8 peptide since translation at the ATG start site was diminished.

These constructs were transfected into K89 cells and then infected with MCMV and

harvested for HPLC analysis. Fractionation of the peptides showed increased LYL8 peaks from construct 1492, 1493 and 1495 when the cells were infected with MCMV. However, there was no visible change in the GFP levels as quantified by the mean-fluorescence intensity. There was also no change in the MYL8 peak produced from construct 1554 and 1549 in the presence of MCMV compared to uninfected levels (Figure 6.2) and (Figure 6.3). There was also no change in their GFP expression levels.

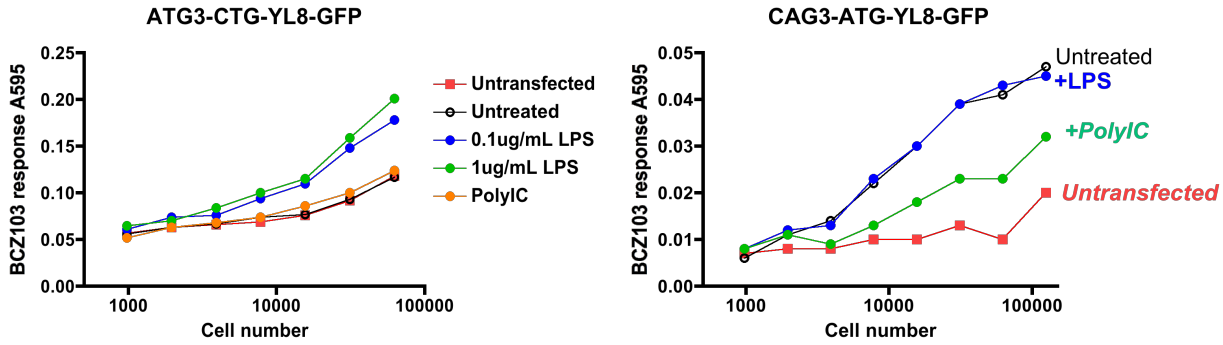


Figure 6.4: **mRNA of peptide-GFP-fusion constructs was transfected into immortalized macrophages.** Immortalized macrophages were transfected with [AUG]3-CUG-YL8-GFP and [CAG]3-AUG-YL8-GFP mRNAs and then stimulated with LPS and PolyI:C

In addition to using these cDNA constructs, mRNA was generated from each of these constructs. This mRNA was transfected into C57BL/6 immortalized macrophages and subsequently the cells were stimulated with LPS and PolyI:C. Peptide expression from 1554 was inhibited in the presence of PolyI:C and unchanged in the presence of LPS stimulation. However, LYL8 generated in a cryptic manner from 1492 was enhanced with the presence of LPS, but unchanged in the presence of PolyI:C. (Figure 6.4). However, the GFP expression from these constructs was barely detectable, possibly because of the transfection efficiency. So these cDNA constructs were transfected into HeLa-$K^b$ cells (HeLa cells stably expressing the MHC Class I molecule $K^b$), and then stimulated with LPS and PolyI:C. When the GFP expression was examined there was no change in the GFP expression from these constructs in the presence of the TLR agonists. However, peptide presentation was mildly enhanced for the cryptically generated peptides. These data suggested that the enhancements in peptide presentation, due to inflammatory stimuli, were an effect of enhancements in the antigen presentation pathway alone and not of cryptic translation of proteins. If the inflammatory stimuli led to global translation enhancements, then both conventional and cryptic peptide presentations were increased or in events when conventional peptide presentation was saturated, cryptic peptide presentation alone was detectably increased (Figure 6.5) and (Figure 6.6).

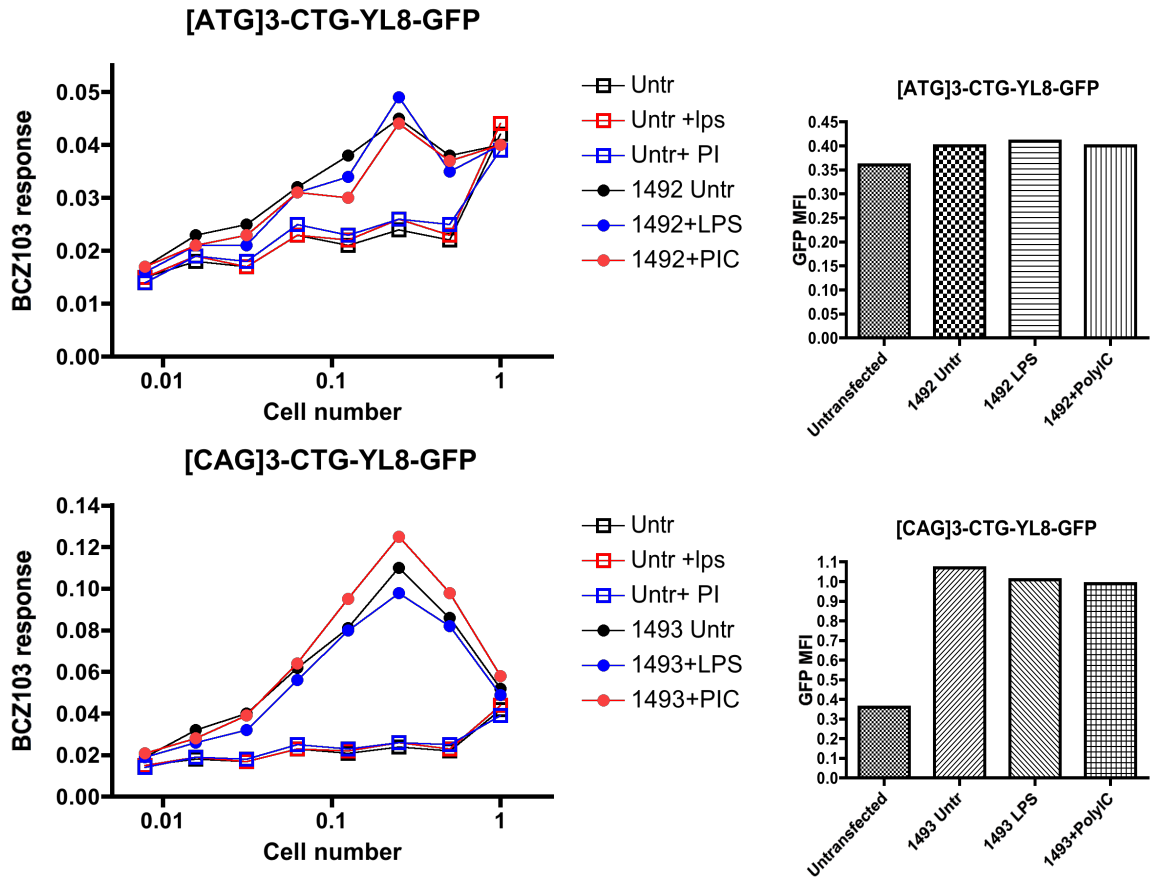


Figure 6.5: **Transfection of the cryptic peptide-GFP-fusion constructs into HeLa cells.** HeLa cells were transfected with [ATG]3-CTG-YL8-GFP and [CAG]3-CTG-YL8-GFP and then stimulated with LPS and PolyI:C. GFP expression was analyzed by flow cytometry.

## 6.2 Discussion

The model systems used in the previous chapters rely heavily on a T-cell response as a measure of cryptic translation. The WI9.LYL8 system is under the control of an MHC I promoter and many of the effects seen could have been robust due to increased transcript levels as a result of the MHC I promoter. The use of the fusion GFP scanning constructs aims to move away from that system and provides an alternate method to look at cryptic translation more directly through GFP expression. The aim would be to test if virus infection can indeed enhance cryptic translation through this system. For this purpose, stable-cell lines expressing these fusion GFP constructs will be made using a retroviral vector expressing a puromycin resistant gene (pQCXIP). The GFP constructs have been cloned into these constructs. This retroviral vector can now be transduced into primary macrophages and into fibroblast cells. This experiment will tell us if the phenomenon of enhanced cryptic

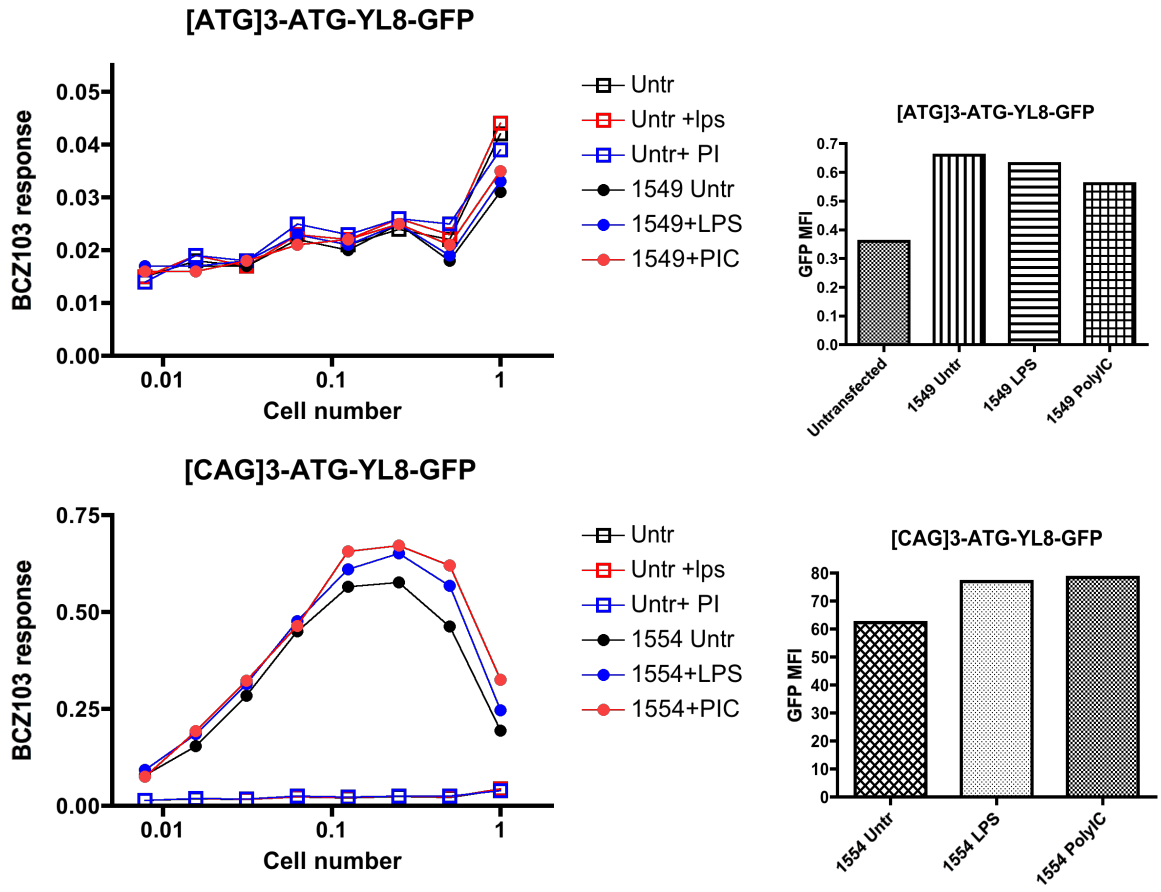


Figure 6.6: **Transfection of the conventional peptide-GFP-fusion constructs into HeLa cells.** HeLa cells were transfected with [ATG]3-ATG-YL8-GFP and [CAG]3-ATG-YL8-GFP and then stimulated with LPS and PolyI:C. GFP expression was analyzed by flow cytometry.

peptide presentation is just an antigen presentation effect or a cryptic translation-related event.

This system also helps us rationalize the mechanism by which enhanced cryptic peptide presentation is occurring. This fusion system can be used to test how proteasomes degrade proteins and how antigens arise from those degraded proteins. Since this system encodes a generic protein and an antigen, the cell can be stimulated by various proteasome inhibitors and the generation of these antigens and expression of GFP can be consequently determined. Similarly, to address the question of whether peptides are being translocated into the ER at the same rate, this system can be used to compare how the different peptides enter the ER via TAP.

# Chapter 7

# Future Directions

**Summary**

This thesis has established that the presentation of cryptically translated peptides is enhanced by inflammatory stimuli like virus infection and cytokines. This phenomenon of enhanced cryptic peptide presentation would be useful to detect viruses that inhibit host translation and presentation of antigenic peptides. In such an event, the assumption is that cryptic peptide presentation would still be enhanced and the immune system would be alerted. Another environment where enhanced cryptic peptide presentation would be useful is in a tumor microenvironment, which is rife with cytokines. Cryptic peptides arising from mutations might be enhanced in response to these inflammatory cytokines. Therefore, enhanced cryptic peptide antigen presentation could result in that cell being targeted for elimination by T-cells. This chapter aims to provide some of the therapeutic applications of cryptic antigen presentation to the immune system.

## 7.1 Developing an in-vivo model of cryptic translation to assess T-cell responses to cryptic peptides

In order to be able to use cryptic antigens in therapeutic applications like vaccines, cryptic peptide responses should be detectable in-vivo. Therefore, we attempted to develop an in-vivo model of cryptic antigen presentation using the WI9.LYL8 transgenic mouse model. In this model, the T-cell response to a self cryptic peptide would be measured and compared to the T-cell response to a conventionally translated antigenic peptide. For this purpose, C56BL/6 (wild-type) female mice would be immunized with splenocytes from the WI9.LYL8 male transgenic mice. Since neither the WI9 peptide nor the LYL8 peptide is present in female mice, T-cell responses to both of these peptides will be generated. The T-cell responses in-vivo can be measured by performing an IFN$\gamma$ assay ex-vivo, by restimulating the immunized splenocytes with WI9 or LYL8 peptide-pulsed antigen presenting cells or WI9.LYL8 transgenic splenocytes. In order to specifically measure the quantity of each T-cell popula-

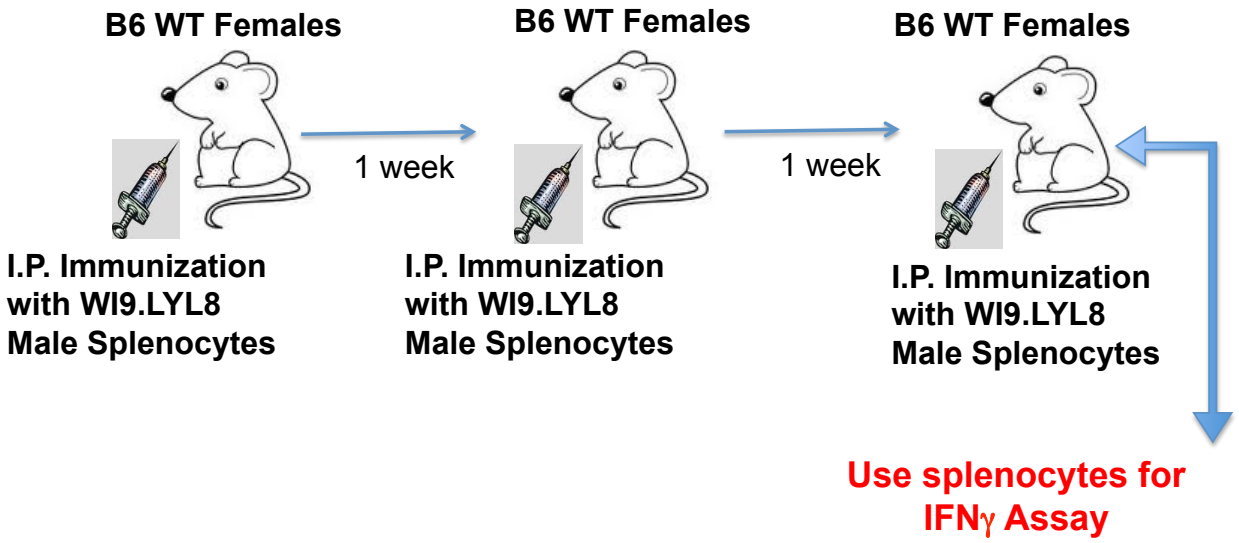


Figure 7.1: **Experimental model for measuring cryptic peptide presentation in-vivo.**

tion, tetramer staining of T-cells can be performed for the LYL8 and WI9 peptides. In this experimental set-up, the WI9.LYL8 splenocytes that are used to immunize the wild-type mice, can be treated with Bruceantin and other host translational inhibitors (which would inhibit conventional translation and enrich the expression of cryptic peptides). Alternatively, the transgenic splenocytes can also be infected with MCMV to determine if the T-cell responses against the LYL8 peptide are significantly enhanced in-vivo as well. Moreover, if there are any other endogenous cryptic peptides, whose presentation is enhanced by either Bruceantin or MCMV, the T-cell responses of wild-type T-cells (immunized with WI9.LYL8 splenocytes treated with Bruceantin or MCMV) against wild-type antigen presenting cells, treated with Bruceantin or MCMV ex-vivo, would be enhanced. This can be ascertained by an IFN$\gamma$ assay. This experiment would then make the phenomenon of enhanced cryptic peptide presentation, as a result of inflammatory stimuli, a more widepsread phenomenon. Figures (7.2) and (7.3) show data obtained from a preliminary experiment that was performed to measure the extent of cryptic translation in-vivo. Robust T-cell responses against WI9 and LYL8 were detected in wild-type mice immunized with WI9.LYL8 splenocytes. In the experiment with Bruceantin inhibition, we expected to observe a decrease in the WI9 specific T-cell responses and an overall decrease in T-cell responses of wild-type cells against Bruceantin treated splenocytes. Though we observed a mild decrease in the WI9 specific T-cell response and a slight increase in the LYL8 specific T-cell response against Bruceantin treated cells, these were not significant enough differences that we could attribute to the Bruceantin treatment.

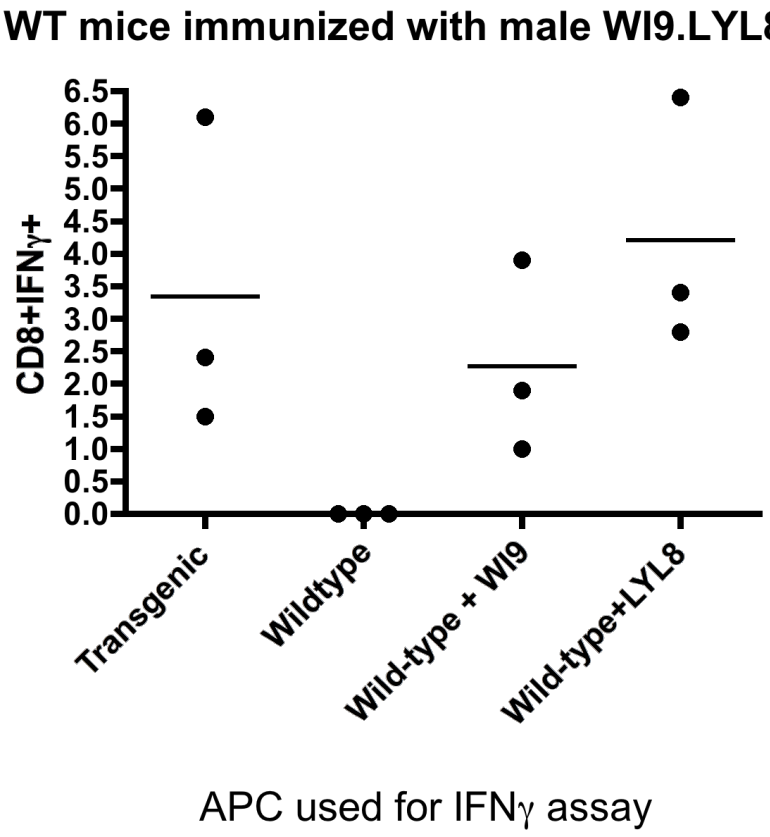


Figure 7.2: **Measuring cryptic peptide presentation in-vivo.** WI9.LYL8 male splenocytes were used to immunize C57BL/6 female mice. The immunization was performed 3 times. The female splenocytes were then harvested and re-stimulated ex-vivo with WI9.LYL8 splenocytes. The re-stimulated splenocytes were then pulsed with WI9 or LYL8 peptide and tested for IFN$\gamma$ production.

## 7.2 Developing a model of virally derived cryptic and conventional antigenic peptides

Studies in the previous chapters looked at virus infection as a way to enhance endogenously expressed cryptic peptides. However, the alternate application of enhanced cryptic peptide presentation is when a virus infection inhibits host translation and propagates the translation of its own viral proteins. It is well known that a lot of viral peptides are generated in a cryptic manner. So it would be important to determine if viral cryptic peptides are enhanced when a cell is infected with a virus, that inhibits host protein synthesis. One way to assess this would be to clone the WI9.LYL8 bicistronic gene into a viral vector like that of MCMV. Once the cells are infected, the extent of T-cell responses against the WI9 and LYL8 peptide

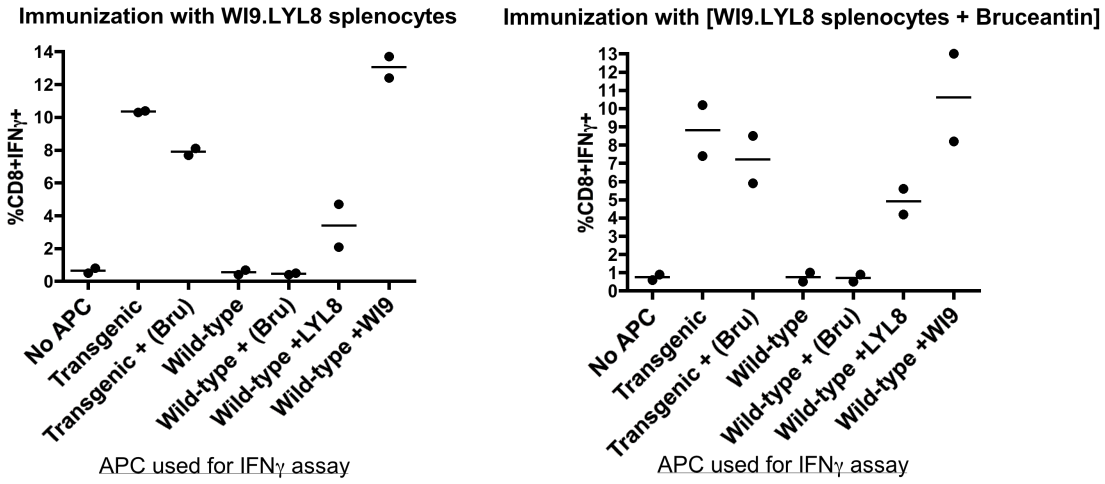


Figure 7.3: **Measuring T-cell responses to conventional and cryptic peptides.**WI9.LYL8 male splenocytes that were treated with Bruceantin or DMSO control were used to immunize C57BL/6 female mice. The immunization was performed 3 times. The female splenocytes were then harvested and re-stimulated ex-vivo with either WI9.LYL8 splenocytes treated with Bruceantin or DMSO. The re-stimulated splenocytes were then pulsed with WI9 or LYL8 peptide and tested for IFN$\gamma$ production.

can be ascertained through T-cell assays. If the virus is indeed acting to evade the immune system, there should be a mechanism in play, to prevent the presentation of these viral cryptic peptides. Moreover, when the cell is stimulated with anti-viral cytokines, the level of T-cell responses against these particular cryptic peptides can be determined. This will tell us if anti-viral cytokines are effective in enhancing presentation of viral peptides to the immune system and if there is a difference in the presentation of conventionally translated peptides versus cryptically translated peptides.

## 7.3 Developing a model to determine if enhanced cryptic peptide presentation can increase T-cell responses against tumor cells

Previous work, in Chapter 4, has shown that cytokines like TNF-$\alpha$, type I and type II IFN can enhance cryptic peptide presentation in bone-marrow derived macrophages. It is also known that these very cytokines are produced in a tumor microenvironment (Smyth et al. 2004). While these cytokines are shown to increase cryptic peptide presentation, their effects on tumorigenesis may vary. Some of these cytokines have an anti-tumor effect while others can promote carcinogenesis. For example, IFN$\gamma$ has been shown to enhance cancer immunosurveillance by upregulating MHC I and II on tumor cells and increasing

tumor-specific cytotoxic T lymphocyte (CTL) activity. However, TNF-$\alpha$ has been shown to provide survival signals to cancer cells (Smyth et al. 2004). Given these reports, we can examine how endogenous cryptic peptide presentation is regulated within tumors and in tumor microenvironments which are enriched with cytokines and antigen-presenting cells. Through these studies, we will be able to determine whether the use of cryptically translated peptides in peptide vaccines against certain cancers is feasible.

# Chapter 8

# Materials and Methods

## Mice

WI9.LYL8 transgenic mice have been described elsewhere ([66].) C57BL/6J and B10.D2 mice were purchased from Jackson Laboratory (Bar Harbor ME). Use of all mice was done with the approval of the Animal Care and Use Committee of the University of California, Berkeley.

## Cell lines

K89 ($K^b$ expressing L cells) have been described before in ([32]), $D^b$-L cells have been described before in ([24]), Cos7 cells have been described before in ([45]), BCZ103 has been described before in ([47]), 11p9Z in ([68]), 30NXZ in ([50]), HeLa-$K^b$ in ([39]). NIH3T3 and Balb3T3 cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA.)

## Generation of primary WI9.LYL8 bone-marrow macrophages

Legs were dissected from WI9.LYL8 mice, and the femur and tibia were cleaned off from the surrounding muscle tissue and cleaned in 70 % ethanol. The bones were cut and the marrows were flushed using a 24 1/2 G needle into complete RPMI with 10 % serum. The suspended marrows were then filtered through a 0.4$\mu$m mesh filter. The bone marrow cells were then re-suspended in 1mL red-blood cell lysis buffer for 1 minute and then washed with complete RPMI media. The cells were then counted and plated into sterile (non-TC treated) petri dishes at 5-6 million cells per dish in special media with 20 % FCS and 20 % MCSF (macrophage colony stimulating factor).
**Note:** MCSF-producing cells (3T3-MCSF) were obtained from the laboratory of Prof. Russell Vanceand then grown in bulk. The supernatant was then filtered and stored at -80 deg.C

## mRNA transfection (Qiagen kit)

The TransMessenger Transfection Reagent (Qiagen) was used for the transfection of mRNA generated using the Ambion kit. For each $\mu$g of mRNA, a mix of 16.5 $\mu$L of Enhancer and up to 100$\mu$L Buffer EC was made and incubated for 5 minutes at room temperature. 33$\mu$L of TransMessenger Reagent was added and incubated for 10 minutes at room temperature. 900$\mu$L of serum-free media or OPTI-MEM-I reduced serum media was then added to the mix and added on to the cells for 3-4 hours. Complete RPMI was then added to the cells with or without various Toll-like receptor ligands.
**Note:** Toll-like receptor ligands were obtained from InVivogen.

## T-cell hybridoma assays

After co-culture of antigen-presenting cells and T-cell hybridoma cells for 15-17 hours in a 96-well flat bottom plate, the plate was spun down at 2000 rpm for 2 minutes. The supernatant was then flicked off, and 100$\mu$L of CPRG (Chlorophenol red-$\beta$-D-galactopyranoside, obtained from Roche Diagnostics) solution is added to each well, after which the enzymatic reaction begins. Plate readings are taken at dual absorbance wavelengths, between 595nm and 655 nm.

## HPLC fractionation assay

NIH3T3 cells / K89 cells (150,000-250,000 cells per well of 6 well-plate) were transfected with the different x-YL8-GFP constructs and thereafter infected with MCMV at an MOI of 0.5-1.0. The cells were then trypsinized with 0.25% trypsin with 2mM EDTA and re-suspended in 10% acetic acid and boiled at 100 deg. C for 10 minutes. The boiled suspension of cells was then spun down at 10,000 rpm for 15 minutes. The supernatant was then transferred to a 10kDa Millipore filter (Ambion) and spun down at 13,000 rpm for 45 minutes. The flow-through was then injected into a C18 column and separated by reverse-phase HPLC. A program with a ratio of 80 % Buffer A (0.1% Tri-fluoro acetic acid in water) and 20 % Buffer B (0.1 % Tri-fluoro acetic acid in acetonitrile) was used. 3-drop fractions were collected in a flat-bottom 96-well plate. The plates were dried by spinning overnight in a vacuum-trap based plate dryer. On the following day, 50,000 K89 cells and 100,000 BCZ103 hybridoma were added to each well of the 96 well plate. The amount of peptide was quantified by the Lac Z assay.

WI9.LYL8 splenocytes treated with LPS (1mg/mL) or sterile water for 6 hours and prepared for injection into the HPLC column in the same way as described above. 2 spleens were used for each condition. WI9.LYL8 bone marrow derived macrophages (50 million cells per condition) were infected with MCMV at a MOI of 0.5-1.0 for 6 hours and harvested and prepared for HPLC analysis as described above.

## Cell Proliferation Assay

For this assay the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) from Promega was used. While preparing for the T-cell assay, an equal aliquot of cells, which was used for the T-cell assay, was used to test for cell viability and proliferation. 100$\mu$L of PMS reagent was added to 2mLs of MTS solution. 20$\mu$L of this mixture was added to each well of the titrated cells. The cells were incubated at 37 deg. C for 1 hour and then the plates were read at an absorbance wavelength of 490nm (single wavelength).

## Virus infection with Mouse Cytomegalovirus (MCMV)

MCMV was obtained from the laboratory of Dr. Laurent Coscoy (University of California, Berkeley). 1 million bone marrow macrophages were plated into each well of a 6 well plate. The next day, the media on the cells was removed and stored in a 50mL tube as conditioned media. 1 mL of viral supernatant (at MOI of 0.5-1.0) or complete RPMI media was added to the cells for 2 hours. This was then removed and replaced with 1 mL of fresh media and 1mL of the conditioned media. After 6 hours, the macrophages were harvested with cold PBS with 1mM EDTA, and scraped off the plate using cell-lifters. Cells were re-suspended in complete RPMI and a T-cell assay was set up.

## Virus infection with Influenza

Influenza virus (Strains WSN33 and PR8) was obtained from the laboratory of Dr. Laurent Coscoy (University of Califonia, Berkeley). 1million bone marrow macrophages were plated into each well of a 6 well plate. Cells were infected at an MOI of 1.0 for either 6 hours or 24 hous. Cells were then harvested and a T-cell assay was set up.

## Virus preparation

1 million NIH3T3 cells were plated into a T75 flask on the previous night in 10 mLs of complete DMEM media. Media on the cells was replaced with DMEM complete media + MCMV virus at an MOI of 0.1, in a total volume of 4 mLs, for 2 hours. 6 mLs of complete DMEM was added thereafter, and the cells were observed for 4 days. Once a beaded formation of cells began to form, the supernatant from the cells was harvested the next day.
**Note:** Harvesting virus: Supernatant from the cells was spun down at 1200 rpm for 5 minutes. Supernatant was then filtered through a 0.45$\mu$m filter and stored at -80 deg. C.

## Plaque assay

150,000 BALB 3T3 cells were plated into each well of a 6-well plate. Serial dilutions of virus were made in complete DMEM media. Serial dilutions of 1:10, 1:100, 1:1000, 1:10,000 were

made by adding 300$\mu$L of virus into 2.7 mLs of complete DMEM. 1mL of the dilution was added into each well, and was incubated for 2 hours. After 2 hours, the supernatant was aspirated, and a mixture of Low-melting agarose, MEM + serum, antibiotic, and 40% glucose was added to the cells. On the fifth day, plaques were fixed by adding 10% formaldehyde and visualized by removing agarose and adding crystal violet.

## Peptide titration assay

LYL8 or WI9 peptide of 1mM stock in DMSO or water was used. From this stock a working stock of 50uM was made. For the experiment, the titration was begun at 200nM and then a 1:3 dilution of peptide was made in PBS. 50,000 K89 or $D^bL$ cells were then added as antigen presenting cells to each well. 100000 of the BCZ103 or 11p9Z hybridoma was then added to each well, and incubated over night. CPRG substrate was added the next day.

## Flow cytometry analysis

Surface expression of MHC Class I was analyzed by flow cytometry. B6WT primary macrophages that have been treated with virus or TLR Ligands were re-suspended in FACS buffer (PBS +5% FCS and 1mM EDTA). Primary macrophages were treated with FcBlock at a 1:200 concentration in a final volume of 50$\mu$L. Anti-$K^b$ antibody(AF6-88.5) or anti-$D^b$-antibody(KH95) was added at a 1:100 concentration for 30 minutes and then analyzed on the FC-500 machine.

## TNF intracellular staining assay

Brefeldin-A (Golgi Plug) was added to the treatment condition after 2 hours of treatment initiation, and left in solution for 4-6 hours. Cells were then harvested in FACS buffer and stained for surface MHC Class I expression. Primary macrophages were treated with FcBlock at a 1:200 concentration in a final volume of 50$\mu$L for 30 minutes. Cells were then fixed and permeabilized using CytoFix/CytoPerm solution (100$\mu$L per well) for 20 minutes. Cells were then washed with 1X Perm/wash buffer and incubated with anti-TNF antibody (anti-mouse TNF-$\alpha$-PE, Clone: MP6-XT22, eBiosciences) or an Isotype Control antibody (Isotype-PE conjugated, Rat IgG1, Clone: eBRG1, eBiosciences) at a concentration of 1:100 in a final volume of 50$\mu$L for 30 minutes-1hour. Cells were then analyzed on the FC-500.

## Co-culture assays with B10.D2 cells

Primary macrophages were prepared from B10.D2 mice, which have the MHC-$H2^D$ haplotype. B10.D2 macrophages were first infected with MCMV for 6 hours. Cells were then harvested, washed, and titrated into a 96-well plate or split into a 96-well plate. B6WT primary macrophages were then added to these cells for 6 hours. The supernatant was removed

and 100,000 BCZ103 cells were added to the mixture of B10.D2 cells and BCZ103 cells, and a T-cell assay was performed

## Generation of fusion GFP constructs

The GFP coding sequence was cloned out using a primer that contained an XbaI restriction site in the forward primer and a preceding handle sequence. The reverse primer contained an Hpa I restriction site and a preceding handle sequence. The cloned out GFP sequence was then subcloned into the various scanning pcDNA1 constructs, into the XbaI and HpaI restriction sites.

**Forward:**
5 GT ACG CTC TAG ATA GTC AGC AAG GGC GAG GAG CTG **Reverse:**
5 GTA CGC GTT AAC TTA CTT GTA CAG CTC GTC CAT GCC

Sequences of scanning constructs:

**ATG3-CTG-YL8** TAATGGGTCGACCATGCACCATGGTAGTCGACCCTGACCTTCAAC-TACCGGAATCTCG

**CAG3-CTG-YL8** TACAGGGTCGACCCAGCACCCAGGTAGTCGACCCTGACCTTCAAC-TACCGGAATCTCG

**CTG3-CTG-YL8** TACTGGGTCGACCCTGCACCCTGGTAGTCGACCCTGACCTTCAAC-TACCGGAATCTCG

**ATG3-ATG-YL8** TAATGGGTCGACCATGCACCATGGTAGTCGACCATGACCTTCAAC-TACCGGAATCTCG

**CAG3-ATG-YL8** TACAGGGTCGACCCAGCACCCAGGTAGTCGACCATGACCTTCAAC-TACCGGAATCTCG

## UV-inactivated virus

MCMV-GFP (obtained from the laboratory of Dr. Laurent Coscoy) was exposed to UV light inside a sterile hood for 30 minutes. Cells were infected with this supernatant and GFP expression was then tested in these cells by flow cytometry. Supernatant that was exposed to UV did not exhibit any GFP expression in the cells, whilst wild-type MCMV-GFP exhibited GFP production in the cells. Inactivation was also tested by a plaque assay. UV-inactivated MCMV did not produce any plaques in the diluted samples.

## TNF-blocking experiments

Primary macrophages were infected with MCMV or left untreated and treated with anti-TNF$\alpha$ antibody (Mouse TNF-$\alpha$ Antibody, Monoclonal Rat IgG1, Clone# MP6-XT22, R&D Systems.) or an Isotype control antibody (Rat IgG1 Isotype Control, Monoclonal Rat IgG1, Clone # 43414, R&D Systems) for 6 hours. Cells were harvested and T-cell assay was performed. During the incubation with T-cells, anti-TNF$\alpha$ or Isotype antibody was included.

## IFNAR-blocking experiments

Primary macrophages were infected with MCMV or left uninfected and they were cultured with an anti-IFNAR-1 antibody (LEAF Purified anti-mouse IFNAR-1, Clone MAR1-5A3, BioLegend.) or Isotype control (LEAF Purified Mouse IgG1 $\kappa$ Isotype Control, Clone MG1-45, BioLegend.) for 6 hours. Cells were harvested and a T-cell assay was performed. The antibody was included during the incubation with T-cells.

## Retroviral transduction experiments

WI9.LYL8 cDNA was subcloned into the MSCV retroviral vector. Phoenix cells were transfected with MSCV-WI9.LYL8 and vsv-g. 48 -72 hours after transfection, the supernatant was harvested and along with polybrene (500X) was added onto Day 2 bone-marrow macrophages from B6WT mice and spun down at 3000g for 2 hours. The supernatant was then removed and regular complete media was added. This was repeated 2-3 times. Macrophages were then harvested on Day 5 or Day 6 and were infected with MCMV virus and a T-cell assay was performed.

## Immunization of mice

1. **Preparation of WI9.LYL8 splenocytes:** WI9.LYL8 male mice were euthanized and the splenocytes were cultured ex-vivo in RPMI with 10% FCS (Hyclone) and treated either with DMSO or 25uM-50uM Bruceantin for 3 hours. The splenocytes were then washed with PBS and re-suspended in 1 mL of PBS, to be used for immunization.

2. **Immunization:** C57BL6/J (B6) Female mice were immunized intraperitoneally with 2,000,000 WI9.LYL8 male splenocytes, that were irradiated at 14,000 rads and re-suspended in 100uL of PBS. This immunization was performed 3 times, 1 week between each immunization. 1 week after the third immunization, the B6 mice were euthanized and the spleens were cultured ex-vivo in RPMI with 10% FCS (Hyclone). The splenocytes were restimulated ex-vivo with WI9.LYL8 splenocytes once. After 1 week, the splenocytes were pulsed with WI9 or the LYL8 peptide at 100nM-500$\mu$M of peptide concentration and analyzed for IFN$\gamma$ production by intracellular staining assay for IFN$\gamma$.

## Northern Blot Analysis

RNA was isolated using Trizol (Invitrogen) and resolved on a 1 % formaldehyde gel (Ambion) and transferred onto a Hybond-XL membrane (GE Healthcare). Th blot was probed with tRNA probes that were labeled with T4 polynucleotide kinase (NEB) and [$\gamma$32P] ATP (3000 Ci/mmol; Perkin Elmer) at 56 deg. C in hybridization buffer (6X SSC, 0.1% SDS and 4X Denhardt's solution). [The recipe for SSC 20X is 175.3g NaCl, 88.2g sodium citrate, water to 1L, pH to 7.0 with 1M HCl]. Blots were washed successively with 3X SSC, 0.1% SDS

and 1.5X SSC, 0.1% SDS. The blots were then exposed for 60 minutes to a PhosphoImager screen and analyzed using a Storm PhosphoImager (Molecular Dynamics). Probes used Leu-tRNA-CAG and Met-tRNA.

## Treatment with EGCG and Resveratrol inhibitors

EGCG(E4143-50MG) and Resveratrol (R5010-100MG) were obtained from Sigma-Aldrich.

## Luciferase assays with L929-ISRE cells

Supernatant from uninfected and infected cells were added to L929-ISRE cells (obtained from Dr. Astar Winoto's laboratory, UC Berkeley). These cells were then lysed using the reagents of the Promega Luciferase assay. Luciferin reagent was then added to the lysed cells and the absorbance was measured.

## Use of PI3-kinase inhibitors

WI9.LYL8 macrophages were infected with MCMV and then treated with Ly294002 (10$\mu$M) or Wortmannin (2$\mu$M) for a period of 6 hours. Inhibitors were obtained from the Coscoy and Winoto labs. The cells were then harvested and a T-cell assay was performed.

## RT-PCR of WI9.LYL8

RNA was extracted from macrophages using the Trizol reagent. The RNA was treated with DNase I using the Promega reagents. cDNA was then prepared from this RNA using the Invitrogen kits and the reverse transcriptase enzyme. This cDNA was diluted and used in the RT-PCR reaction using the primers indicated below. Primers used for RT-PCR
**Forward:**
C G T G G T C G A C T A G A T G T G G A
**Reverse:**
C A G G A T C C T A G A G A T T A C G A T A G T T G A

## Antibodies and Cytokines

- Antibodies used for Western Blotting
    - EIF2A polyclonal antibody, Source:Rabbit, Isotype: IgG, Catalog No: 11233-1-AP, ProteinTech
    - Mouse(monoclonal) Anti-eIF-2-$\alpha$ Unconjugated, Isotype: IgG1(mouse), Catalog No: AHO0802, Invitrogen.

- Cytokines used to stimulate cells: IFNy (Recombinant Mouse Interferon-gamma) obtained from GIBCO and re-suspended in PBS at 100units/$\mu$L. TNF-$\alpha$ and IL-10 were obtained from ProSpec.

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