Nitric Oxide Signal Stimulated by the Calreticulin and Shared Epitope Interaction

Margaret Hannah
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Department of Molecular, Cellular and Developmental Biology
University of Michigan
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Sponsor: Laura J. Olsen, Ph. D
Readers:
Dr. Joseph Holoshitz, MD
Dr. Matt Chapman, Ph. D

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BACKGROUND

Generally speaking, Rheumatoid Arthritis is an autoimmune disease that normally presents itself during adulthood, within the ages of 30-50 years old[11]. Currently, it affects more than 1.3 million people within the United States alone, with 70% of Rheumatoid Arthritis patients being female[11]. The disease results in chronic, systemic inflammation in which the immune system is attacking healthy tissues. It typically affects small synovial joints such as those in the hands and feet but also has the ability to impact shoulders, hips and other tissues and organs[12]. Clinically, it is described using the following criteria: the presence of four or more of the following factors for a duration of no less than six weeks "1. morning stiffness in and around joints lasting at least 1 hour before maximal improvement; 2) soft tissue swelling (arthritis) of 3 or more joint areas observed by a physician; 3) swelling (arthritis) of the proximal interphalangeal, metacarpophalangeal, or wrist joints; 4) symmetric swelling (arthritis); 5) rheumatoid nodules; 6) the presence of rheumatoid factor; and 7) radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints[1].

Symptoms include pain, swelling and stiffness of the affected joints with the pain tending to be worse after being sedentary for long periods of time[12]. Rheumatoid nodules also can form around the affected areas within the joints[12]. In addition to symptoms directly impacting the affected area, loss of energy, low fever, weight loss, and a shortage of red blood cells are also symptoms that present themselves in rheumatoid arthritis patients[12]. Currently, there is no cure for Rheumatoid Arthritis

because much is still unknown about the disease mechanism and there are multiple factors that have been proposed to contribute to the disease. However, many treatments are available to sufferers. Patients are generally started on DMARDs (disease-modifying antirheumatic drugs) that act to slow the progression of the disease[13]. Methotrexate, Leflunomide, Hydroxychloroquine and Sulfasalzine are just a few of the DMARDs that are commonly prescribed to patients[13]. In addition to DMARDs, individuals may also be advised to take anti-inflammatory drugs as needed in order to alleviate pain caused by and in addition to the swelling[13]. No single treatment will work for every patient and thus doctors and patients must work closely together in order to create a customized, individual plan on a patient-by-patient basis.

It is important to know that Rheumatoid Arthritis patients do run the risk of death attributed to the disease[10]. In 1987 a long-term study was preformed on Rheumatoid Arthritis patients. The researchers observed an increase in patient comfort levels and a decrease in bone degradation for first ten years but observed a decline in patient comfort and bone mass levels the following years. By twenty years, the researchers had found 35% of their patients to be dead with it often being due to the disease[10]. Often times this detail escapes the minds of the general public when faced with the term Rheumatoid Arthritis because the focus is on treatment of pain and swelling in the joints but there is a high risk of mortality and disability associated with the disease[11].

Immunologically speaking, Rheumatoid Arthritis is considered by many a type IV hypersensitivity autoimmune disease. Type IV hypersensitivities are often referred to as a delayed-type hypersensivity because they are cell-mediated, as opposed to antibody-mediated, and thus take time to develop. In the immunological response, the antigen is present on major histocompatibility complex (MHC) type II-coded proteins that are expressed on macrophage cell surfaces. The MHC, called HLA in humans is a large gene gamily located on the short arm of chromosome number 6. In a normal response, antigen presenting cells (APC) such as macrophages, dendritic cells, and B cells identify and phagocytose pathogens and infected cells that are displaying non-self antigens. Once the foreign particle or damaged cells are digested, they are displayed on the surface MHC class II thereby activating additional cells of the immune system, producing cytokines, activating complement pathways, and other mechanisms in order to eliminate foreign particles. In the case of Rheumatoid Arthritis, the APCs of the body are believed to display a self-antigen on their MHC class II and elicit an inappropriate immune response.

With antigen bound to MHC II, macrophages are stimulated to produce IL-12 in order to stimulate CD 4+ helper T cell's proliferation and recruitment, thus intensifying the immune response. During the interaction with the antigen, CD 4+ helper T cells type I (T_h1) are stimulated and produce cytokines such as interluekin-2 and interferon gamma. Interluekin-2 stimulates the proliferation, differentiation, and growth of neighboring T cells. Interferon gamma is responsible for the activation of macrophages, and is critical for immunological responses to

intracellular bacteria, viruses, and tumor cells. Interferon gamma in atypical quantities has been proposed to lead to auto inflammatory and autoimmune diseases such as Rheumatoid Arthritis. The symptoms caused by Rheumatoid Arthritis are thus due to the swelling that the inappropriate immunological response mediates.

ABSTRACT

Overproduction of nitric oxide causes oxidative stress in joint tissues. Under this stress, synovial cells are more prone to pro-inflammatory cytokine stimulation, which could result in the development of Rheumatoid Arthritis. This laboratory has discovered that a HLA class II-encoded sequence called the 'shared epitope' is functioning as a signal transduction ligand that activates nitric oxide production in cells. Previous studies have shown that a synthetic linear peptide expressing the shared epitope sequence can induce the production of nitric oxide in cells. They have begun testing different concentrations of cyclic peptides (derived from the shared epitope sequence) in order to check if cyclic peptides maintain the same ability to trigger nitric oxide signaling as the linear counterpart; cyclic peptides have longer half-lives than linear peptides. For detecting nitric oxide response in cells, DAF-2 DA dye is loaded into M1 cells at 37^0C for 1 hour; then cells are treated with different concentrations of cyclic peptides. Linear peptides are used as positive control, and no peptide treatment as the negative control. Nitric oxide levels are continually monitored by a fluorescence micro-plate reader during an eight hour time period. The results obtained from this experiment will be used to design a cyclic peptide to stimulate the signaling cascade that leads to the increase of nitric oxide in cells. Eventually, these data will help us to further understand the genetic causes of Rheumatoid Arthritis.

It has been previously proposed by scientists in this group that calreticulin (CRT) plays an important role in Rheumatoid Arthritis due to its ability to transduce

shared epitope activated signaling within the cell[2]. In order to assess the levels to which calreticulin enhances signaling within the cell, DAF-2DA dye is loaded into MEF K42 cells at 37^0C for 1 hour; then cells are treated with different concentrations of calreticulin, as well as 65-79*0401 (shared epitope) and 65-79*0402 (control) peptide. The cells treated with no calreticulin as well as 65-79*0401, 65-79*0402, and no peptide are used as negative control as well as the cells treated with varying concentrations of calreticulin and 100 ng/ul 65-79*0401 and 65-79*0402. A fluorescence microplate reader recorded the nitric oxide levels produced by the cells over an eight hour time period. The results from this experiment will be used in order to asses the effect calreticulin has on shared epitope signaling cascades in cells, which will be used to further understand the genetic causes of Rheumatoid Arthritis.

INTRODUCTION

Individuals carrying shared epitope positive genes have been found to be more prone to Rheumatoid Arthritis and other autoimmune diseases in comparison to individuals lacking the shared epitope sequence[3]. When speaking about the shared epitope (SE), we specifically refer to the MHC II HLA-DRB1 alleles that code a five-amino acid sequence motif in positions 70–74 of the HLA-DRβ chain: QKRAA, QRRAA, or RRRAA[8]. The disease in shared epitope-positive patients begins earlier and is more erosive than in shared epitope-negative individuals[3]. Furthermore, there is evidence supporting gene-dose effect, whereby the severity of bone destruction in Rheumatoid Arthritis correlates positively with the number of shared epitope-coding HLA-DRB1 alleles[7].

Dr. Holoshitz's lab has recently found that the SE functions as an allele-specific signal transducing ligand that activates a nitric oxide (NO) mediated pro-oxidative cell signaling, as well as an innate immune signaling pathway in other cells. The data collected from Denise De Almedia, Song Ling, Xiujun Pi, Anne Hartmann-Scruggs, Paul Pumpenst and Joseph Holoshitz have shown that regardless if the shared epitope is expressed in its native conformation on the cell surface, as a cell-free HLA-DR, inserted into large recombinant proteins, or as short synthetic peptides it activates nitric oxide mediated signaling[4]. This enforces the ligand-binding theory in which the shared epitope acts as a ligand to bind to a cellular receptor that, once activated, induces nitric oxide signaling within cells[7]. The receptor to which the shared epitope sequence has been found to bind is calreticulin.

Calreticulin (CRT) is a multifunctional calcium-binding protein originally defined as an endoplasmic reticulum molecular chaperone. Intracellularly, calreticulin plays an important role in the transport of incorrectly folded proteins out of the ER so that they may be degraded, instead of being further transported[2]. However, it has more recently been shown that calreticulin is also expressed extracellularly and attaches to the surface of many cells where it is involved in signal transduction events associated with innate immunity, cell adhesion, and apoptosis[2]. Calreticulin plays an important role in a cell's pro or anti-inflammatory response due to its role in the elimination of apoptotic cells[4]. During apoptosis, a second set of signals is released by apoptotic cells that cause the appropriate inflammatory response within the cell. It has previously been postulated that defective clearance of apoptotic cells has been a cause of autoantibody production. Apoptotic cells release danger signals and provide self-antigens for aberrant presentation by dendritic cells[4]. Calreticulin plays a pivotal role in the junction between tolerance and autoimmunity due to its effects on the clearance of apoptotic cellular products and cells[4].

With respect to calreticulin's role in the ligand-binding theory of Rheumatoid Arthritis, the signaling pathway that occurs is mediated via the interaction between the shared epitope sequence and an extracellular surface receptor, which is proposed to be calreticulin. This protein serves as the signal-transducing receptor present on the surface of many cells. Importantly, calreticulin is expressed on dendritic cells, which are believed to play a role in the pathogenesis of Rheumatoid

Arthritis[4]. The shared epitope binding site on calreticulin has previously been mapped to the amino acid residues 217-223 in the P-domain[2]. Upon interaction with dendritic cells, the shared epitope activates immune regulatory events that have been shown to increase secretion of Il-6 and Il-23 as well as to facilitate generation of Th17 cells; a subset of cells that play a role in autoimmunity[2]. Il-6 and Il-6 receptors have been found in high concentrations in rheumatoid arthritis patients within the synovial fluid and lead to the formation of Osteoclast like cell formations[9]. Osteoclasts are the cells that are primarily responsible for the degradation of bone and join tissue. With an increase in Il-6 caused by the shared epitope and calreticulin interaction, higher bone degradation has been observed due to the increase in Osteoclast like cells, thus leading to the characteristic bone degradation present in Rheumatoid Arthritis patients[9].

In addition to the increased cytokine secretion, with the shared epitope bound to calreticulin high levels of nitric oxide are observed. When nitric oxide levels are high within the cell it leads to an increase in oxidative damage due to its antagonistic effects upon cAMP production, cAMP has been shown to have oxidative damage protection effects. High nitric oxide levels have also been shown to inhibit adenosine mediated anti-oxidative pathways[8]. Inhibition of anti-oxidative and repair pathways, increased cytokine secretion, Th17 cell generation, in addition to nitric oxide being a reactive species itself and causing oxidative damage leads to the inflammatory effects and symptoms observed in individuals with Rheumatoid Arthritis.

In this study, we characterized a novel small shared epitope-mimetic compound, *c*(HS4-4), containing the shared epitope primary sequence motif QKRAA, which was then synthesized using a backbone cyclization method. The experiment involving *c*(HS4-4) was employed to see if cell nitric oxide signaling could be induced with the cyclic peptide containing the shared epitope sequence, *c*(HS4-4), and to the same extent as a synthesized linear peptide containing the sequence,65-79*0401, is capable of inducing signaling. In this experiment the linear peptide 65-79*0401 was used and compared against differing concentrations of the cyclic form in order to assess its potency and candidacy for the continuation of its use in further experiments involving the shared epitope sequence.

The experiments involving calreticulin were designed in order to investigate the nitric oxide signaling response induced in cells when treated with calreticulin as well as the linear shared epitope sequence, and a linear sequence counterpart that was epitope negative, 65-79*0402. In comparing the differences between nitric oxide produced by cells with no treatment of calreticulin, calreticulin and 65-79*0402, and calreticulin and 65-79*0402, this experiment was employed in order to test the effects various calreticulin levels had on the nitric oxide signaling production of cells. As well as, to further the evidence that calreticulin acts as the extracellular surface receptor for shared epitope binding and signal production, the ligand-binding hypothesis.

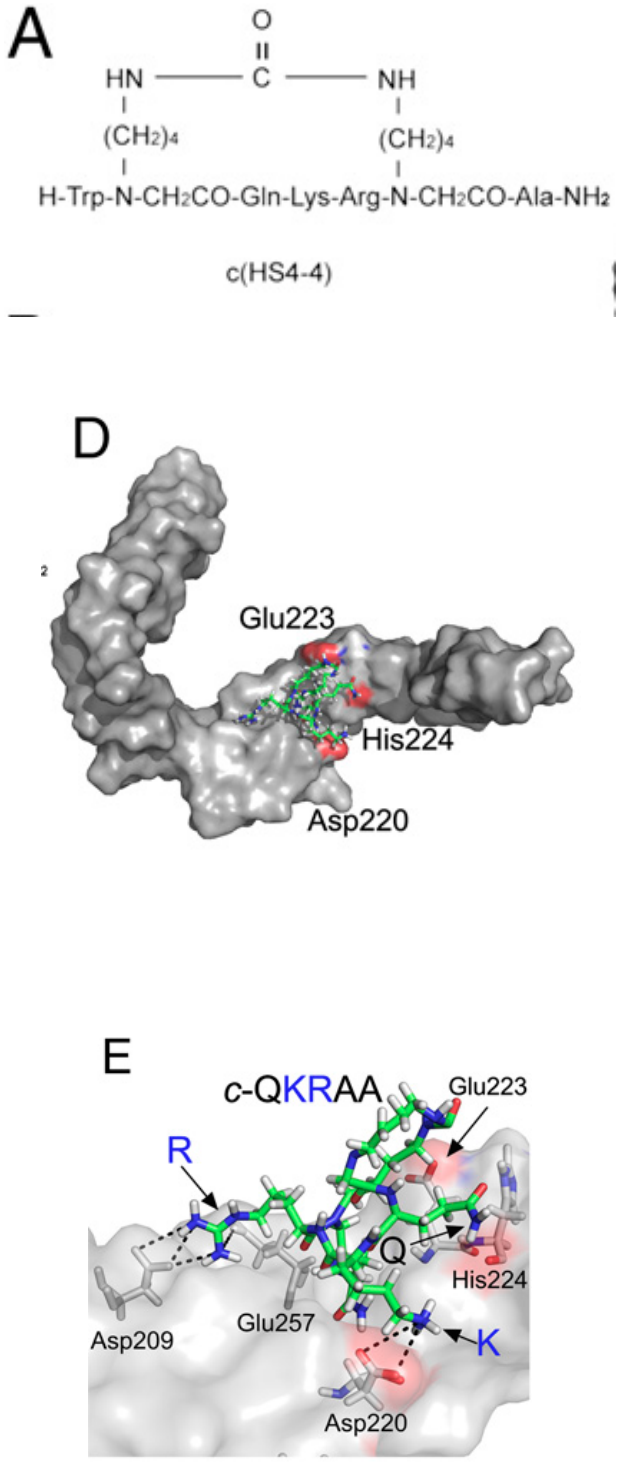


Figure 1[7]. A. Shows the chemical formula for c(HS4-4). The chemical structure of c(HS4-4), a backbone cyclic peptide analog containing the shared epitope consensus motif QKRAA, was designed to induce a stable active conformation. A backbone

cyclization methodology was used to keep the functional groups of the side chain residues intact. This feature ensures that all the functional groups in a peptide sequence are available for biologic activity[7]. Figure 1.D shows the cyclic peptide, c(HS4-4) bound to the P domain of calreticulin with the key docking amino acids highlighted. A low-power docking image of the c(HS4-4) compound (identified here in its sequence structure cQKRAA and shown in green) superimposed onto the previously identified SE binding site on the calreticulin P-domain (gray surface). Calreticulin amino acid residues previously found to play critical shared epitope ligand-binding roles (7) are highlighted in red[7]. E. A high-power view of c(HS4-4)–calreticulin molecular interactions[7].

MATERIALS AND METHODS

c(HS4-4)

Human Fibroblast M1 cell line was provided by Dr. Haseltine. The methods used to isolate, and purify this cell line can be found by referring to Experimental Cell Research 1.51 (1984) 40&420. Synthetic peptides corresponding to position 65–79 on the HLA-DRβ– chain, coded by the shared epitope-positive allele DRB1*0401 (sequence: 65-KDLLEQKRAAVDTYC-79), were synthesized and purified (>90%) as we previously described[5]. The peptide 70–74*0401 (carrying the 70-QKRAA-74 sequence) was synthesized by the University of Michigan (Ann Arbor, MI) peptide synthesis core. Peptide 0401 is expressed as 65-79*0401; 0402 as 65-79*0402. Both of which are custom synthesized by Bio-World Inc. The cyclic QKRAA, expressed as *c*(HS4-4), used in these experiments was used from Dr. Gibson's lab. It is a backbone cyclic mimetic peptide, synthesized as explained previously[7].

Nitric Oxide (NO) Assay:

3×10^4 human fibroblasts were seeded in each well of 96-well plates one day before experiment in order to allow the cells to attach to the surface. The supernatant was removed and cells were washed with Phenol-red free DMEM buffer once. Cells were first loaded with 20 μM of the fluorescent NO probe 4,5-diaminofluorescein diacetate (DAF-2DA) in the dark at 37°C for 1 hour. After dye loading, the supernatant was removed and the cells were washed once. Each well in the 96- well plate received 50 μl of Phenol-red free medium and 50 μl of either more medium to act as control wells, the linear peptide, or the cyclic peptide in varying

concentrations (0.1,1, 10,100,1000 μM). NO signal was recorded by using a Fusion αHT system at an excitation wavelength of 488 nm and emission wavelength of 515 nm.

Reactive Oxygen Species (ROS) Assay:

ROS production was quantified similarly, with the exception that cells were loaded with 10μM CM-H2DCFDA for 30 minutes.

CALRETICULIN

In order to test the effects of calreticulin on MEF-K42 cells, a plasmid containing the human calreticulin gene was obtained from Dr. Raghavan's lab. We then used GeneRunner in order to sequence the plasmid. We sequenced the plasmid in order to ensure there were no mutants introduced (See Figure 1). Once the plasmid was sequenced and it was confirmed that there was no mutation in the human calreticulin gene, we moved on to transforming the plasmid containing human calreticulin into *E. coli* in order to express the calreticulin protein in larger amounts. The *E. coli* strain used was BL21(DE3). This particular strain was selected to be transformed because it contains no ampicillin resistance and is deficient in the proteases Lon and OmpT. Our plasmid carries a gene that confers ampicillin resistance, therefore allowing only *E. coli* colonies that have been successfully transformed with the plasmid to grow on the agar plate containing ampicillin. Cells from colonies on the agar plate were seeded in 500 mL of LB medium. We began to induce calreticulin protein expression by adding Isopropyl β-D-1-

thiogalactopyranoside (IPTG) to the medium. In order to purify the calreticulin protein from the *E*. *coli,* Lyszyme was used to break the cell wall. We then employed three freeze-thaw cycles to break the cell membrane. We used centrifuge in order to separate the soluble protein from the debris of lysed cell solution. Our protein of interest was in the supernatant. We incubated the supernatant with a Nickel NTA column because there is a His6 tag at the N-terminus of Human calreticulin. Therefore, only His6-calreticulin will bind to the nickel on the Nickel NTA column and the other protein will be washed away. In the final steps of purification, imidazole was used to remove His6-calreticulinfrom the Nickel NTA column. In order to assess the purity of the collected calreticulin, the sample was ran on an SDS-PAGE gel to confirm > 90% purity. Protein concentration was determined by using a BCA protein assay kit.

NO Assay:

3×10^4 MEF-K42 cells and the corresponding concentrations of calreticulin (4, 1.5, 0.5, 0.2, 0.1, or 0 ng/μl) were loaded into each well of 96-well plates one day before the experiment was ran in order to allow the cells to attach to the surface and to allow for the calreticulin to interact with the cells. The supernatant was removed and cells were washed with Phenol-red free DMEM buffer. Cells were first loaded with 20 μM of the fluorescent NO probe 4,5-diaminofluorescein diacetate (DAF-2DA) and then incubated in the dark at 37°C for 1 hour. After dye loading, the supernant was removed and the cells were once again washed. Each well in the 96-well plate received 50 μl of Phenol-red free medium and 50 μl of either more

medium to act as control wells, medium as well as 0402 or 0402, just calreticulin, calreticulin at varying concentrations and 0401 (100ng/ul), or calreticulin at varying concentrations and 0402 (100 ng/ul). The 96-well plate was designed and set up as followed in Figure 3. NO signal was recorded by using a Fusion αHT system at an excitation wavelength of 488 nm and emission wavelength of 515 nm.

CRT DNA SEQUENCE

gcggcgtccg tccgtactgc agagccgctg ccggagggtc gttttaaagg gcccgcgcgt tgccgccccc tcggcccgcc atgctgctat ccgtgccgct gctgctcggc ctcctcggcc tggccgtcgc cgagcctgcc gtctacttca aggagcagtt tctggacgga gacgggtgga cttcccgctg gatcgaatcc aaacacaagt cagattttgg caaattcgtt ctcagttccg gcaagttcta cggtgacgag gagaaagata aaggtttgca gacaagccag gatgcacgct tttatgctct gtcggccagt ttcgagcctt tcagcaacaa aggccagacg ctggtggtgc agttcacggt gaaacatgag cagaacatcg actgtggggg cggctatgtg aagctgtttc ctaatagttt ggaccagaca gacatgcacg gagactcaga atacaacatc atgtttggtc ccgacatctg tggccctggc accaagaagg ttcatgtcat cttcaactac aagggcaaga acgtgctgat caacaaggac atccgttgca aggatgatga gtttacacac ctgtacacac tgattgtgcg gccagacaac acctatgagg tgaagattga caacagccag gtggagtccg gctccttgga agacgattgg gacttcctgc cacccaagaa gataaaggat cctgatgctt caaaaccgga agactgggat gagcgggcca agatcgatga tcccacagac tccaagcctg aggactggga caagcccgag catatccctg accctgatgc taagaagccc gaggactggg atgaagagat ggacggagag tgggaacccc cagtgattca gaaccctgag tacaagggtg agtggaagcc ccggcagatc gacaacccag attacaaggg cacttggatc cacccagaaa ttgacaaccc cgagtattct cccgatccca gtatctatgc ctatgataac tttggcgtgc tgggcctgga cctctggcag gtcaagtctg gcaccatctt tgacaacttc ctcatcacca acgatgaggc atacgctgag gagtttggca acgagacgtg gggcgtaaca aaggcagcag agaaacaaat gaaggacaaa caggacgagg agcagaggct taaggaggag gaagaagaca agaaacgcaa agaggaggag gaggcagagg acaaggagga tgatgaggac aaagatgagg atgaggagga tgaggaggac aaggaggaag atgaggagga agatgtcccc ggccaggcca aggacgagct gtagagaggc ctgcctccag ggctggactg aggcctgagc gctcctgccg cagagctggc cgcgccaaat aatgtctctg tgagactcga gaactttcat ttttttccag gctggttcgg atttggggtg gattttggtt ttgttcccct cctccactct cccccacccc ctccccgccc ttttttttt ttttttttaa actggtattt tatctttgat tctccttcag ccctcacccc tggttctcat ctttcttgat caacatcttt tcttgcctct gtccccttct ctcatctctt agctcccctc caacctgggg ggcagtggtg tggagaagcc acaggcctga gatttcatct gctctccttc ctggagccca gaggagggca gcagaagggg gtggtgtctc caacccccca gcactgagga agaacggggc tcttctcatt tcaccctcc ctttctcccc tgcccccagg actgggccac ttctgggtgg ggcagtgggt cccagattgg ctcacactga gaatgtaaga actacaaaca aaatttctat taaattaaat tttgtgtctc caaaaaaaaa aaaaaaaaa

CRT AMINO ACID SEQUENCE

GVRPYCRAAAGGSF.RARALPPPRPAMLLSVPLLLGLLGLAVAEPAVYFKEQFLDGDGWT
SRWIESKHKSDFGKFVLSSGKFYGDEEKDKGLQTSQDARFYALSASFEPFSNKGQTLVVQ
FTVKHEQNIDCGGGYVKLFPNSLDQTDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKN
VLINKDIRCKDDEFTHLYTLIVRPDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAS
KPEDWDERAKIDDPTDSKPEDWDKPEHIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGE
WKPRQIDNPDYKGTWIHPEIDNPEYSPDPSIYAYDNFGVLGLDLWQVKSGTIFDNFLITN
DEAYAEEFGNETWGVTKAAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAEDKEDDEDKDED
EEDEEDKEEDEEEDVPGQAKDEL.RGLPPGLD.GLSAPAAELAAPNNVSVRLENFHFFPG
WFGFGVDFGFVPLLHSPPPPPRPFFFFFLNWYFIFDSPSALTPGSHLS.STSFLASVPFS
HLLAPLQPGGQWCGEATGLRFHLLSFLEPRGGQQKGVVSPTPQH.GRTGLFSFHPSLSPL
PPGLGHFWVGQWVPDWLTLRM.ELQTKFLLN.ILCLQKKKKK

T7 PLASMID DNA SEQUENCE

TGAGNAATTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCACCATCATCATCAT
NATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTACTTCCAATCCAATGCTGTCGCCGAGCCTGCCGTC
TACTTCAAGGAGCAGTTTCTGGACGGAGACGGGTGGACTTCCCGCTGGATCGAATCCAAACACAAGTCAGAT
TTTGGCAAATTCGTTCTCAGTTCCGGCAAGTTCTACGGTGACGAGGAGAAAGATAAAGGTTTGCAGACAAG
CCAGGATGCACGCTTTTATGCTCTGTCGGCCAGTTTCGAGCCTTTCAGCAACAAAGGCCAGACGCTGGTGGT
GCAGTTCACGGTGAAACATGAGCAGAACATCGACTGTGGGGGCGGCTATGTGAAGCTGTTTCCTAATAGTT
TGGACCAGACAGACATGCACGGAGACTCAGAATACAACATCATGTTTGGTCCCGACATCTGTGGCCCTGGCA
CCAAGAAGGTTCATGTCATCTTCAACTACAAGGGCAAGAACGTGCTGATCAACAAGGACATCCGTTGCAAGG
ATGATGAGTTTACACACCTGTACACACTGATTGTGCGGCCAGACAACACCTATGAGGTGAAGATTGACAAC
AGCCAGGTGGAGTCCGGCTCCTTGGAAGACGATTGGGACTTCCTGCCACCCAAGAAGATAAAGGATCCTGAT
GCTTCAAAACCGGAAGACTGGGATGAGCGGGCCAAGATCGATGATCCCACAGACTCCAAGCCTGAGGACTGG
GACAAGCCCGAGCATATCCCTGACCCTGATGCTAAGAAGCCCGAGGACTGGGATGAAGAGATGGACGGAGA
GTGNGAACCCCCCAGTGATTCANAACCCTGAGTACAAGGGTNGAGTGGAAGCCCCGGCAGATCGACAACCN
AAATTACAAGGGCNACTTGGATCCACCCAGAAATGANCAACCCCGAGTTATTTCTCCCNNATCCCCAGTAA
CTATGCCTATGATAACCTTTGGCGTGCTGGGNCCTGGAACCTNTGGCAGGTCAAGTNTGGNAACCATNCTT
GAAAACTTTCCNCATTCCCCAACGAATGAGGCCTNACCCCTGANGGATTTTTGCCANCAANCCGGGGGNCG
TTNACAAAGGNCCNCCAANAAACCAANGNANGGNNAAAACGGAACCANGGACCAAAGTTTTTAGGANGAA
GAAAAAACCA

PROTIEN ENCODED IN T7 PLASMID SEQUENCE

XNSL.NNFV.L.EGDIHMHHHHHXSSGVDLGTENLYFQSNAVAEPAVYFKEQFLDGDGWT
SRWIESKHKSDFGKFVLSSGKFYGDEEKDKGLQTSQDARFYALSASFEPFSNKGQTLVVQ
FTVKHEQNIDCGGGYVKLFPNSLDQTDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKN
VLINKDIRCKDDEFTHLYTLIVRPDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAS
KPEDWDERAKIDDPTDSKPEDWDKPEHIPDPDAKKPEDWDEEMDGEXEPPSDSXP.VQGX
SGSPGRSTTXITRXTWIHPEMXNPELFLXXPQ.LCL..PLACWXLEPXAGQXWXPXLKTF
XIPQRMRPXP.XIFAXXPGXVXKXXPXNQXXXKTEPXTKVFRXKKKP

Protein Sequence
TEV protease cutting Site: ENLYFQ (G/S)
Histidine Tags

Figure 2. The calreticulin DNA sequence was found using GeneBlast, as was the amino acid sequence. GeneBlast was also used to find the sequence T7 plasmid sequence. Sequencing of these two components was critical in order to sequence our entire plasmid in order to ensure it was pure prior to transformation and protein amplification. Also while sequencing the plasmid we needed to ensure the presence of Histidine Tags, which are what was used to bind to the protein to the Nickel beads during purification. The TEV protease cutting site was necessary in order to remove the histidine tags after purification.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		4 ng/ul CRT Control	4 ng/ul CRT Control	4 ng/ul CRT 100 ng/ul 0401	4 ng/ul CRT 100 ng/ul 0401	4 ng/ul CRT 100 ng/ul 0401	4 ng/ul CRT 100 ng/ul 0402	4 ng/ul CRT 100 ng/ul 0402	4 ng/ul CRT 100 ng/ul 0402	4 ng/ul CRT Control	4 ng/ul CRT Control	
C		1.5 ng/ul CRT Control	1.5 ng/ul CRT Control	1.5 ng/ul CRT 100 ng/ul 0401	1.5 ng/ul CRT 100 ng/ul 0401	1.5 ng/ul CRT 100 ng/ul 0401	1.5 ng/ul CRT 100 ng/ul 0402	1.5 ng/ul CRT 100 ng/ul 0402	1.5 ng/ul CRT 100 ng/ul 0402	1.5 ng/ul CRT Control	1.5 ng/ul CRT Control	
D		0.5 ng/ul CRT Control	0.5 ng/ul CRT Control	0.5 ng/ul CRT 100 ng/ul 0401	0.5 ng/ul CRT 100 ng/ul 0401	0.5 ng/ul CRT 100 ng/ul 0401	0.5 ng/ul CRT 100 ng/ul 0402	0.5 ng/ul CRT 100 ng/ul 0402	0.5 ng/ul CRT 100 ng/ul 0402	0.5 ng/ul CRT Control	0.5 ng/ul CRT Control	
E		0.2 ng/ul CRT Control	0.2 ng/ul CRT Control	0.2 ng/ul CRT 100 ng/ul 0401	0.2 ng/ul CRT 100 ng/ul 0401	0.2 ng/ul CRT 100 ng/ul 0401	0.2 ng/ul CRT 100 ng/ul 0402	0.2 ng/ul CRT 100 ng/ul 0402	0.2 ng/ul CRT 100 ng/ul 0402	0.2 ng/ul CRT Control	0.2 ng/ul CRT Control	
F		0.1 ng/ul CRT Control	0.1 ng/ul CRT Control	0.1 ng/ul CRT 100 ng/ul 0401	0.1 ng/ul CRT 100 ng/ul 0401	0.1 ng/ul CRT 100 ng/ul 0401	0.1 ng/ul CRT 100 ng/ul 0402	0.1 ng/ul CRT 100 ng/ul 0402	0.1 ng/ul CRT 100 ng/ul 0402	0.1 ng/ul CRT Control	0.1 ng/ul CRT Control	
G		Control	Control	100 ng/ul 0401	100 ng/ul 0401	100 ng/ul 0401	100 ng/ul 0402	100 ng/ul 0402	100 ng/ul 0402	Control	Control	
H												

Figure 3. The 96 well plate was designed to test a multitude of different conditions within a single read. For each read various levels of CRT were tested (4ng/ul, 1.5, 0.5, 0.2, 0.1, and 0) in order to see at which concentration the strongest response was recorded from the cells. Along with the various levels of CRT tested, the effects of CRT along with 65-79*0401 and 65-79*0402 were assessed.

RESULTS

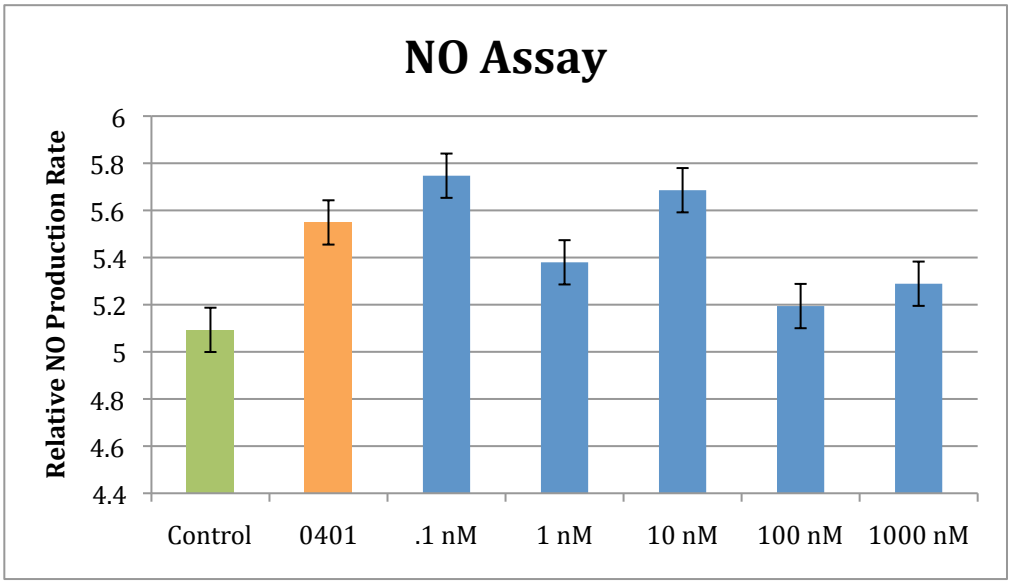
c(HS4-4)

Analysis of the resulting NO and ROS assays showed that cells treated with 57μM of 65-79*0401 and the cyclic peptide c(HS4-4) had higher relative NO production rates compared to the control cells. This was seen by Fusion αHT machine recording higher NO for cells treated with 65-79*0401 and c(HS4-4) compared to the control cells. The highest NO production rates were found at concentrations of 0.1 nM and 10nM of cyclic peptide c(HS4-4). Thus, further confirming that cells treated with cyclic and linear counterpart peptides produce more NO as opposed to cells without. The cyclic peptide in concentrations of 0.1 and 10 nM induced greater NO production in the M1 cells than the linear 57 μM 65-79*0401 peptide. While cells treated with concentrations of 1, 100, and 1000 nM had relative NO production rates lower than those of cells treated with 57 μM 65-79*0401.

The results obtained in this experiment further acted as evidence of the ligand-binding hypothesis because when the shared epitope is present it has been proposed to bind to a cell surface receptor. Once bound to the cell surface receptor (calreticulin), a signaling cascade has been proposed to occur through still unknown mechanisms in order to produce higher concentrations of NO[5]. With the increased levels of NO, higher rates and severity of oxidative damage has been shown to occur within cells due to blockage of oxidative damage repair pathways and an increase in reactive oxygen species[5]. The results from this experiment were published in The Journal of Immunology, "A Small

Shared Epitope–Mimetic Compound Potently Accelerates Osteoclast-Mediated Bone Damage in Autoimmune Arthritis."

A.



B.

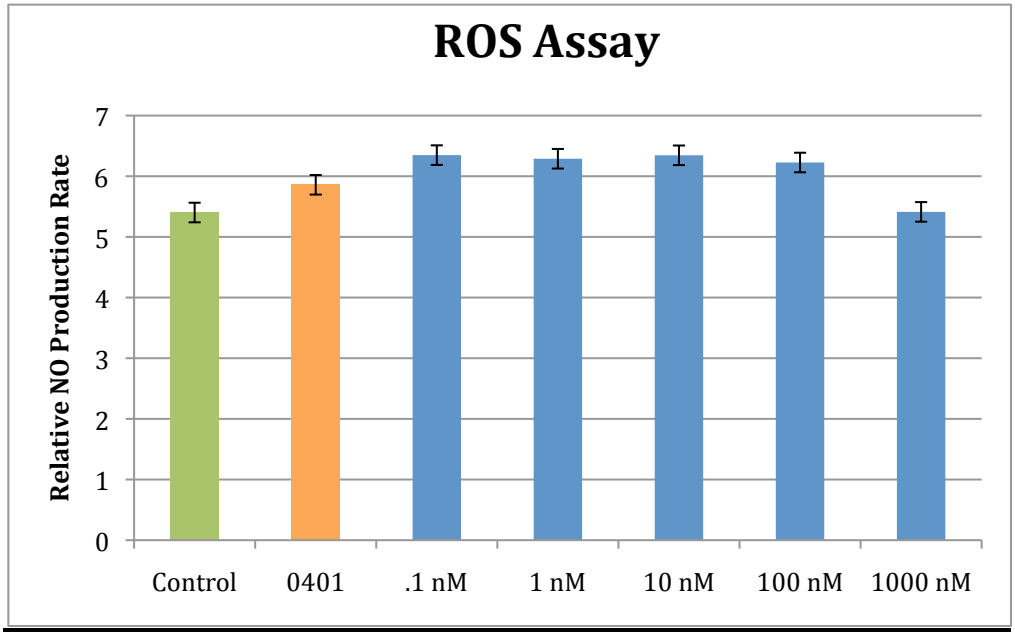
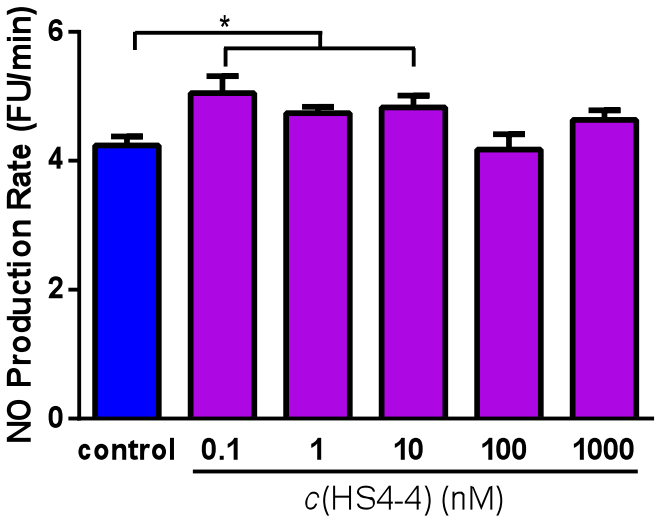


Figure 4. Figures 4A and 4B show the results of the experiments containing c(HS4-4) and 65-79*0401. The data was collected every five minutes and the NO production was

measured for the time spans in which a linear trend was observed. Both NO and ROS assays show that 65-79*0401 produces a higher NO output as opposed to control cells, while the cyclic peptide concentrations of 0.1 and 10 nM produced the highest NO output.

A.



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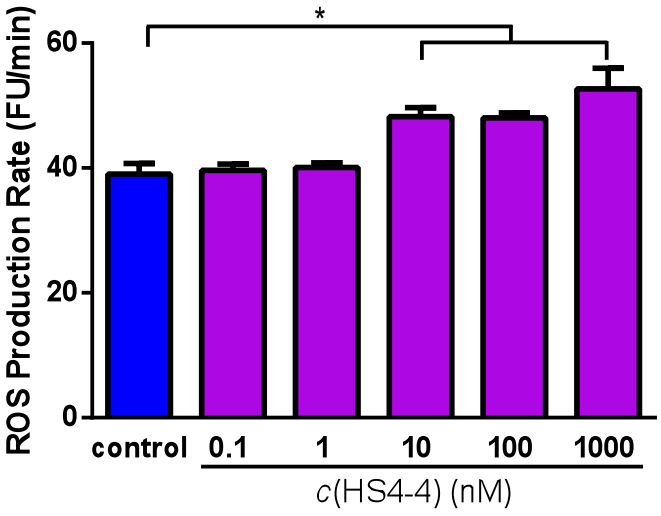

C.

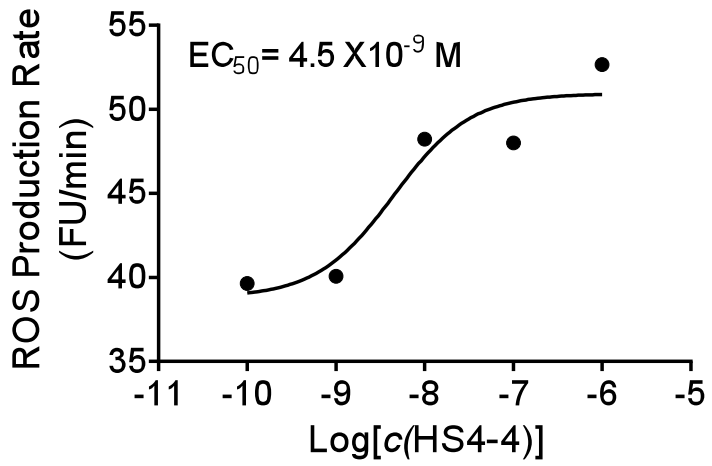

Figure 4C: RAW 264.7 pre-OC cells (33104 per well) were plated in flat-bottom 96-well plates in the presence of various concentrations of c(HS4-4), and ROS production was measured over time[7]. The above NOS and ROS assays pictured above compare the relative NO production rates recorded from the M1 cells over a nine hour period treated with medium to serve as a control, 57 μM 65-79*0401, and differing concentrations of c(HS4-4). The above bar and line graphs were obtained from my publication, "A Small Shared Epitope–Mimetic Compound Potently Accelerates Osteoclast-Mediated Bone Damage in Autoimmune Arthritis" that appeared in The Journal of Immunology.

CALRETICULIN

The experiments involving calreticulin are in the preliminary stages. Our lab was excited to finally identify the cellular receptor and thus began our work right away using calreticulin for our experiments. In doing so, we were required to collect high amounts of purified calreticulin. Thus far, early data collected has shown that when calreticulin knock-out MEF-K42 cells are loaded with calreticulin, we can observe a change in the nitric oxide signaling response. This can be seen by comparing the control group to all other concentrations of calreticulin in Figure 6 A and B. In doing so, one can see that cells that contained no calreticulin had higher levels of nitric oxide production compared to those at calreticulin concentrations of 4 ng/ul and 1.5 ng/ul. However, at concentrations lower than 1.5 ng/ul it was interesting to see a strong increase in nitric oxide production in cells treated with the shared epitope. Our lad had expected to see an increase in nitric oxide production as calreticulin levels increased to the point of saturation and is in the process of collaborating and designing experiments in order to test why we observed the opposite phenomenon.

It can also be seen from the collected data that the shared epitope positive peptide, 65-79*0401, induces a higher nitric oxide signaling response as opposed to control cells and the shared epitope negative peptide, 65-79*0402. From concentrations of 1.5 ng/ul to 0 ng/ul it can clearly be seen that the shared epitope effected the amount of nitric oxide produced. At the lower levels of calreticulin, this effect is further amplified due to the calreticulin concentration dependency. Likewise, the

highest nitric oxide production rates occurred within MEF-K42 cells for which they were treated with 0.5-0.1 ng/ul hCRT, in addition to 65-79*0401.

As previously mentioned above further experiments must be carried out in order to understand why we observed lower levels of nitric oxide production in cells treated with 4 and 1.5 ng/ul hCRT and 65-79*0401, as opposed to cells that contained no calreticulin. We are aware of the flaws in the ligand-binding hypothesis that these data may propose and we are working on resolving this gap of knowledge.

A.

	AVG	SD	SE
4 hCRT Control	1.811588889	0.46047016	0.162800786
4 hCRT 0401	1.794925212	0.562265651	0.229543991
4 hCRT 0402	1.814533015	0.563003236	0.229845109
1.5 hCRT Control	1.811588889	0.190969108	0.067517776
1.5 hCRT 0401	1.986957433	0.150204959	0.061320918
1.5 hCRT 0402	1.828591505	0.287155781	0.117230857
0.5 hCRT Control	1.811588889	0.25226859	0.089190415
0.5 hCRT 0401	2.402223118	0.327381065	0.13365276
0.5 hCRT 0402	2.049073635	0.408269472	0.166675314
0.2 hCRT Control	1.811588889	0.49756981	0.124392453
0.2 hCRT 0401	2.416052668	0.542929716	0.156730309
0.2 hCRT 0402	2.146868452	0.397624697	0.114784363
0.1 hCRT Control	1.811588889	0.454146886	0.113536721
0.1 hCRT 0401	2.89193216	0.783408427	0.226150533
0.1 hCRT 0402	2.224702099	0.386085286	0.111453222
0 hCRT Control	1.811588889	0.494663458	0.123665864
0 hCRT 0401	2.203824534	0.804734157	0.268244719
0 hCRT 0402	2.188686729	0.666927877	0.201086321

B.

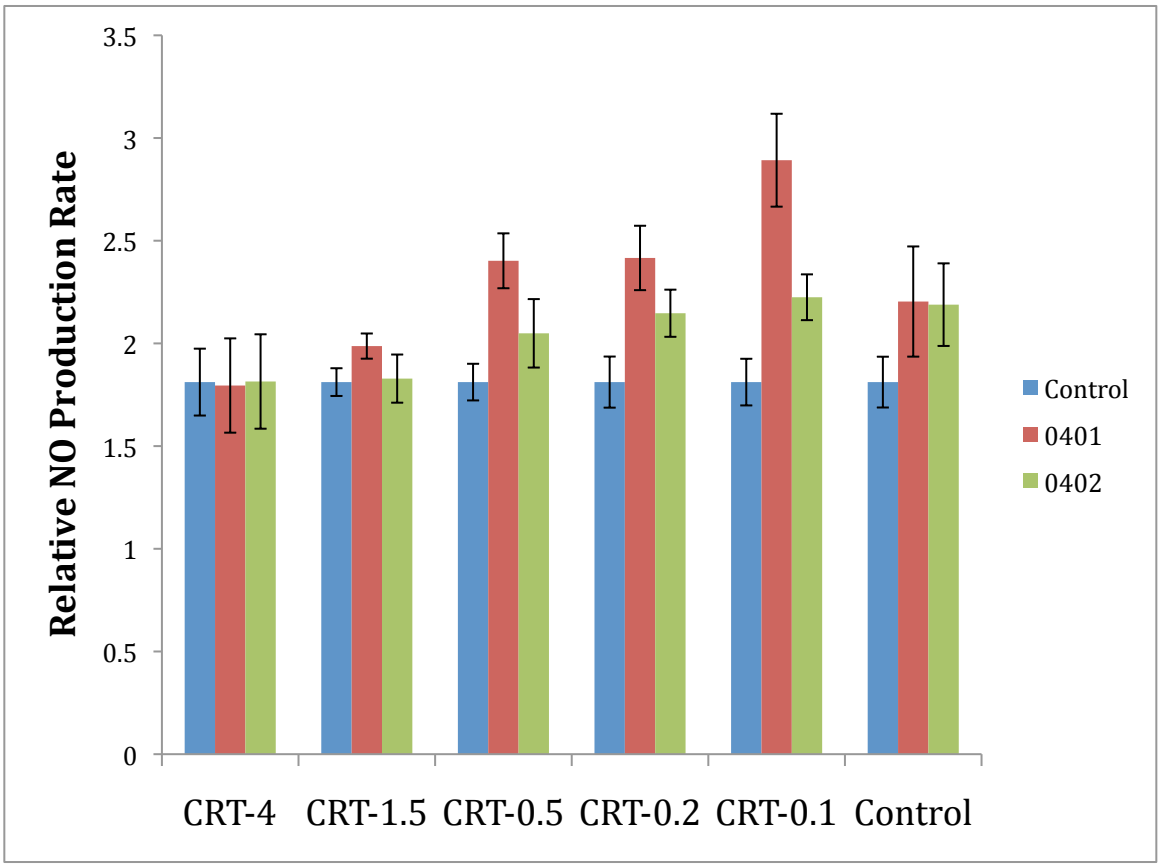

Figure 6. Figure 6 A displays the various production rates of nitric oxide for each calreticulin concentration and it's corresponding 0401, 0402, and control peptide subsets. The averages were calculated over a time span for which the data was found to be increasing linearly (thus data received from the ending times in which the cells were dying were not recorded and accounted for). This data was collected from averaging four experiments' data. Figure 6B displays the relative NO production rates for the various concentrations of calreticulin tested along with 65-79*0401 and 65-79*0402 in MEF-K42 cells. This graph is derived from the data presented in table Figure 5. A. Error bars were added to the graphs in order to show the variation among samples that were used to create the data.

DISCUSSION

The shared epitope sequence is the single most significant genetic risk factor for RA, but the mechanistic basis of its effect is unknown. Of interest, the shared epitope not only confers a higher risk for Rheumatoid Arthritis but also increases the likelihood of developing a more severe version of the disease[7]. Furthermore, there is evidence supporting gene-dose effect, whereby the severity of bone destruction in Rheumatoid Arthritis positively correlates with the number of shared epitope-coding HLA-DRB1 alleles[7].

Prior to my work in lab, it had previously been identified that the shared epitope sequence acts as a ligand for cell surface calreticulin that, once activated, induces nitric oxide mediated pro-oxidative signaling. It has been proposed that the shared epitope has the ability to bind to the extracellular surface receptor, calreticulin, which causes receptor activation[8]. The activated receptor is then able to increase PKG levels through still unknown mechanisms, which then lead to an increase in nitric oxide. High nitric oxide levels within the cells have been shown to increase cellular susceptibility to oxidative damage by blocking the adenosine mediated anti-oxidative pathway, as well as acting as an antagonist to cAMP production pathways[8]. With decreased levels of cAMP it has been observed the cells show a significant decrease in oxidative damage protection[8].

Previous work had demonstrated strong support for this ligand-binding hypothesis for the effects of shared epitope-Rheumatoid Arthritis; however, this previous work

was done using linear shared-epitope sequences. Linear peptides in solution were capable of taking on random conformations and were subject to a high level of degradation[7]. Therefore, the work for determining whether a backboned cyclic compound containing the shared epitope sequence could be created and act at a potency level close to that of our linear peptides became a necessity in order to further support the ligand-binding hypothesis. When creating the backboned cyclic peptide, the lab was careful to create it keeping the primary amino acid sequence intact. This allowed the shared epitope sequence to remain in its native alpha helix secondary structure and protect itself against biodegradation.

The beginning of this experimental process started with working on the cyclic, c(HS4-4) versus linear, 65-79*0401, peptides and the potency at which they are capable of stimulating nitric oxide signaling in M1 cells. The data collected from NO and ROS assays supports that peptide 65-79*0401 induces nitric oxide production in human M1 fibroblasts cells. The data also shows that the cyclic peptide is fully capable of producing relative NO production in rates exceeding those of the linear peptide 65-79*0401. 10 nM of the cyclic peptide has a more potent ability to stimulate NO signaling than the 57 μM linear peptide, indicating that the cyclic peptide is 5000 folds more potent than the linear peptide. It should be noted that the cyclic peptide has a more stable structure and longer half-life than the linear peptide thus making it a better candidate for our continued research. The knowledge gained from these experiments is currently being used in studies with

human dendritic cells in order to fully understand the signaling cascade that occurs in shared epitope positive individuals.

The work with calreticulin allowed us to assess its purity within the sample plasmid we were given by Dr. Raghavan's lab. Once determining its purity, we perfected ways in which to transform *E.coli* and to purify the cellular lysates for our desired sample by exploiting the use of histidine tags. The histidine tags were then removed by TEV protease before their use in cells. Once the sample was properly modified, we treated MEF-K42 cells with a variety of concentrations and found the most optimal range to be from 4 ng/ul to 0 ng/ul. This range is being used to continue our research in exposing calreticulin-deficient cells to calreticulin.

Results from the experiments showed that the linear peptide 65-79*0401 produced the highest nitric oxide in comparison to the control peptide 65-79*0402-treated cells. This demonstrates the importance of the shared epitope in nitric oxide cell signaling. In addition, the highest nitric oxide production levels were found to occur when cells were treated with 0.5 ng/ul – 0.1 ng/ul hCRT and 65-79*0401. This demonstrates that calreticulin plays a critical role in increasing nitric oxide signaling levels when the shared epitope sequence is present. These data strongly support the role that the interaction between the shared epitope and calreticulin together play in nitric oxide signaling. This study provides evidence of shared epitope-calreticulin interaction and suggests that therapeutic targeting of this pathway should be considered when designing drugs in order to combat Rheumatoid Arthritis

symptoms in individuals who carry the shared epitope positive MHC II HLA-DRB1 alleles.

FUTURE WORK AND DIRECTIONS

The data collected from the experiment involving c(HS4-4) showed that it is capable of producing nitric oxide signaling in cells exceeding those induced by 65-79*0401. This has allowed our lab to further our work on inducing cell signaling by using this cyclic peptide as opposed to the linear peptide. This has allowed us to design experiments in which the signaling pathway can be amplified to a stronger level, allowing us to see the effects of treating cells with different peptides that may only have a weak effect. It has also allowed us to induce a signaling cascade that is more stable due to the nature of the cyclic peptide as opposed to linear.

Work involving the calreticulin, began at a very basic stage. This work has allowed us to refine the procedure in which to transform cells and obtain purified human calreticulin. It has also allowed us to create a protocol and a stock solution to be used for future experiments. More work is still necessary with regards to the human calreticulin. Experiments involving human calreticulin in order to see how the concentration affects nitric oxide signaling must continue to be performed involving MEF-K42 cell, which is a calreticulin knock-out cell line, as well as M1 cell. In addition to the necessity of these experiments, a direct, sound relationship between concentration of calreticulin and nitric oxide must be provided in order to further our understanding of the signaling cascade that occurs to produce nitric oxide. Once the signaling pathway is determined, the information will be used to further study the events that occur in the shared-epitope positive cells that lead to Rheumatoid Arthritis.

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