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# METALLOTHIONEIN RECEPTOR EXPRESSION IN LEUKOCYTES

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# Metallothionein Receptor Expression in Leukocytes

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# ABSTRACT

Metallothionein (MT) represents a family of low molecular weight, cysteine-rich proteins that play a number of roles in cellular homeostasis. MT is synthesized as a consequence of a variety of cellular stressors, including exposure to toxic metals, increased temperature, tissue wounding, as well as inflammatory and tumorigenic agents. This protein has been found in both intracellular compartments and extracellular spaces, and its function may depend in part on its location. Extracellular MT is able to redistribute heavy metals between tissues, act as a powerful antioxidant, affect cell proliferation, and cause the suppression of T-dependent humoral immunity. The nature of the interaction of MT with the plasma cell membrane has yet to be characterized, despite many observations that there is a significant pool of extracellular MT, and that this extracellular MT will bind to leukocyte plasma membranes. In light of studies that MT can be detected on the surface of leukocytes from animals immunized in the presence of adjuvant, and that an MT specific receptor has been found on the surface of astrocytes, we have investigated the nature of the potential MT-specific surface receptor-binding site(s) on the plasma membrane of leukocytes. The identification of MT-receptors will allow for the characterization of the mechanism MT uses for immunomodulation, for the manipulation of MT in its immunomodulatory role, and for the identification of patients at higher risk for those potentially harmful immunomodulatory effects.

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# 1. INTRODUCTION

Cells often come in contact with a variety of chemical and physiological stressors that can significantly alter their cellular functioning. These stressors can affect the cells' abilities to produce appropriate and effective immune defense mechanisms, and they may also induce immune attack of self tissues. One way mammalian cells respond to stressors is by increasing their expression of a specific set of stress response proteins. It was previously presumed that these proteins served exclusively to protect cells against the adverse effects of stress. However, it is now known that stress proteins provide both protective outcomes, such as acting as molecular chaperones, as well as non-protective outcomes, such as serving as targets for autoimmune attack (1).

One such stress protein is Metallothionein (MT), which represents a family of small, 7kDa proteins with high metal content. There are four known isoforms in mammals, of which only isoforms I and II are known to be expressed in all organs and during all stages of development. MT has a unique amino acid composition, including 20 cysteine residues and no aromatic amino acids. The tripeptide sequence of cys-x-cys repeats itself several times along the chain and may serve as the primary chelation site for the seven group-2B metal ions usually bound to this protein, most often zinc, but including both toxic (e.g. Cd, Hg, etc) and other non-toxic metals (e.g. Cu). These sequences may be essential for the formation of the metal-thiolate clusters typically seen in MT (2).

MT is known to be both an intracellular protein as well as an extracellular protein. It is found in serum in increasing concentrations following stress induction (3,4), in urine (5,6), in milk (7), in prostatic fluids (8), in pancreatic ducts (9), and has been shown to be selectively released by cells in culture (10-12). It's method of secretion is not yet understood. Most extracellular proteins have a specific N-terminal signal peptide sequence that routes the protein from the ER to the Golgi where they are sorted and prepared for the exit from the cell. MT, however, lacks this signal peptide, which suggests it is secreted by nonclassical mechanisms similar to that of the proinflammatory cytokines IL-1α and IL-1β (13-15).

MT is induced by exposure to divalent heavy metal cations, oxidants, irradiation, and several acute phase cytokines. In 1995, Youn *et al* (16) confirmed that MT binds to the plasma membrane of macrophages by performing a fluorescence labeling experiment using macrophages incubated with biotinlyated MT (MT-b) followed by streptavidin-FITC staining. A similar experiment done by Borghesi *et al*, 1996 (17), showed MT to also bind to both T and B lymphocytes. This binding is thought to be the method for how MT exerts its many effects. In light of the study that cigarette smokers, who are exposed to cadmium in tobacco smoke, have elevated serum MT (18), it is suggested one such effect MT may have is that it may serve to protect cellular components by sequestering this toxic cadmium and other toxic metals (19). MT also has a definitive role as a powerful antioxidant in scavenging free radicals. There are several studies that show MT to be induced by acute-phase cytokines such as IL-1 (20), IL-6 (21), TNF-α (22), and IFN-γ (23), suggesting that MT may have a role in the inflammatory response. Thus, it is possible that the induction of MT for a long period of time may protect and/or minimize

tissue injury, and the discovery of an MT receptor would enable new experiments to be run to help to answer that question.

According to one hypothesis, the molecular structures that the immune system recognizes as foreign are not by themselves sufficient enough to initiate a strong immune response. In order to produce an effective immune response, indications of tissue damage or stress must also be present. Heat shock proteins have been suggested as one form of these danger signals because of their production during tissue damage and their capability to influence the immune system. It is suggested that MT may represent another form of danger signal due to its roles in wound healing, inflammation, and the immune response (24).

Extracellular MT has been shown to influence cell proliferation in many different ways. MT isoform II can increase neurite elongations in culture (25). In contrast, MT isoform III (also known as growth inhibitory factor, or GIF) can suppress neuronal growth elongations and survival in culture (25-27). MT isoforms I and II can stimulate lymphocyte proliferation. Interestingly, studies conducted in our lab have shown MT-mediated suppression of T-dependent humoral immunity (28, 29). This suppression is able to be blocked with a monoclonal anti-MT antibody (UC1MT). Furthermore, in the absence of exogenous MT, UC1MT can actually enhance the humoral response to T-dependent antigen challenge (30). This may suggest that extracellular MT is secreted during a normal immune challenge to control the strength of the humoral response. This phenomenon can also be seen when MT-knockout mice develop a more vigorous humoral response to T-dependent antigen challenge than wild type mice of the same strain (31).

Many of these observations suggest that there are specific molecular interactions between MT and cell surface receptors that result in the regulation of immune activity. There are a number of possible ways MT and this putative receptor might interact. MT may bind non-specifically via the formation of mixed disulfides. Alternately, MT may bind via pattern recognition receptors. Studies have shown the functionally similar heat shock proteins to bind in this manner. Another possibility is that there is a specific MT-selective receptor as has been previously described on astrocytes in the brain. In 1997, El Refaey *et al* discovered this by using an MT 1 probe fluorescently labeled with fluorescein isothiocyanate (FITC) and visualizing the MT receptors on human glial cells by confocal microscopy. They were also able to show that glutathione, cysteine, and MT isoforms I-IV, but not other low molecular weight peptides, could compete effectively with FMT to those receptor sites and could dissociate the bound FMT (32). As yet, there is no existing information describing an MT receptor on cells of the immune system.

In light of the observations that extracellular MT can bind to immune cell membranes (10), and that this event alters cell behavior, we propose an attempt to purify and characterize the putative MT receptor on immune cells. The identification of such a receptor will allow for the manipulation of MT levels as a means to reduce its undesirable immunomodulatory effects and will allow for an understanding of the role played by MT in a variety of disease processes ranging from cancer to immunodeficiency to autoimmunity. It may also facilitate manipulations of endogenous and exogenous MT as a therapeutic opportunity.

## 2. MATERIALS and METHODS

**2.1 Mice**. C57BL/6J mice were obtained from Jackson Laboratory, Bar Harbor, ME, or bred from animals obtained from there. Animals were maintained on a 12h:12h light:dark cycle, with food and water available *ad libitum*.

**2.2 Cell Lines.** C57BL/6J hybridoma cell line was cultured in complete RPMI 1640. Cells were incubated at 37°C in mixed gas (10% $CO_2$, 7% $O_2$, 83% $N_2$) in a humidified chamber. RPMI media was replenished every three days.

**2.3 Media and Reagents.** RPMI 1640 was supplemented with 10% fetal bovine serum (FBS, Hyclone, Loga, UT), sodium bicarbonate, 0.1 MM non-essential amino acids, 0.1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, and 1X Basal Medium Eagle vitamin solution (Sigma). Cyanogen-Bromide-activated sepharose beads were purchased from Sigma and stored at 4°C.

**2.4 Antibodies.** Mouse anti-Metallothionein (Murine $IgG_1$ E9) produced by Zymed was used for ELISA assay positive control. Goat-anti-mouse-IgG-AP labeled produced by Southern Biotech was used as a secondary antibody for ELISA assay. UC1MT, a purified monoclonal murine $IgG_1$ anti-MT antibody produced from in-house hybridoma cell line, was used to couple with sepharose to capture MT.

**2.5 Determination of antibody content by BCA (bicinchoninic acid) Protein Assay.** The working reagent was made using 1 part BCA reagent B with 50 parts BCA reagent A.  10 ul of E9 standard or UC1MT samples was added to each well. Then 200 ul of working reagent was added to each well of a 96-well flat-bottom plate. The plate was covered with sealer, mixed, and incubated @ 37 degrees Celsius for 30 minutes. Finally, the plate was read at 560nm.

**2.6 Enzyme linked immunosorbent assay (ELISA)**. 96-well Immunlon plates were coated with 100ul 10ug/ml MT from Rabbit Liver (Sigma-Aldrich, St. Louis, MD). Plates were incubated overnight at 4ºC. Plates were washed three times with PBS (137 mM NaCl, 2.6 mM KCL, 1.6mM $Kh_2PO_4$, 1mM $Na_2HPO_4$, pH 7.2) containing 0.05% Tween 20 and 0.2% NaN3 in an automated plate washer (Biotek, Burlington, VT). Wells were blocked with 200 ul of 2% BSA in coating buffer (15 mM $NaHCO_3$, 35 mM $Na_2CO_3$, 0.2% $NaN_3$, pH 9.6) for one hour at 37ºC. Plates were washed again three times. 100 ul/ well of both standards (using known amounts of MT) and unknowns were added to wells in triplicates. Binding buffer was used for a negative control. Mouse anti-Metallothionein (E9) was used as a positive control. Plates were then incubated one hour at 37ºC. Plates were washed again three times as before. Plates were coated with 100 ul (1:500) goat-anti-mouse-IgG-AP labeled secondary antibody produced by Southern Biotech., and again incubated one hour at 37ºC. Plates were washed again as before. Lastly, plates were coated with 100 ul/well of 1 PNPP tablet/5 ml substate buffer. Kinetic color development was determined in a $T_{max}$ ELISA microtiter plate reader (Molecular Devices, Menlo Park, CA) at 405 nm.

**2.7 Affinity Column Chromatography.** UC1MT was purified by column affinity chromatography using Protein A column to which UC1MT was linked. The columns were purchased from Pierce. The column was washed with PBS for one hour or until marker reached baseline. Approximately 50 ml of hybridoma cell supernatant was then run over column in 4ºC cold room. The bound material was eluted with 0.2M Glycine pH 2, and neutralized to pH 7 with dilute NaOH. The purified solution was stored at -20ºC until used.

**2.8 Coupling of Antibody to CNBr Activated Beads.** 0.1 gram CNBr-activated sepharose beads were weighed out and mixed with 50 ug of UC1MT in 500 ul coupling buffer (0.1 M NaHCO$_3$, 0.5 M NaCl) in a polypropylene test tube, incubated for 24 hours at 4ºC on a rotor. After incubation, samples were centrifuged and supernatant was removed. 500 ul ethanolamine (pH 8) was added and rotated 1-2 hours at 4ºC. Samples were centrifuged and supernatant was removed as previously stated. Then beads were washed two times, alternatively using pH 4 buffer ( 4.75 ml acetic acid, 2.45 g Sodium Acetate H$_2$0, 58.4 g NaCl, to 1 liter ddH$_2$0) and a pH 8 buffer (6.2 g boric acid, 58.4 g NaCl, to 1 liter with ddH$_2$0), again with centrifuging and supernatant removal. Finally, beads were washed twice in PBS and stored at 4ºC.

**2.9 Coupling of MT to Antibody-bound CNBr Activated Beads.** Antibody-bound CNBr-Activated beads were centrifuged and the supernatant was removed. 180 ul of PBS and 20 ug of MT were added to samples and allowed to mix on rotor overnight at 4ºC. After incubation, samples were washed twice with PBS, centrifuged, and supernatant was removed. Samples are were then ready for SDS-PAGE.

**2.10 Molecular Weight Determination by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.** SDS-PAGE was performed according to the procedure of Laemmli, using the BioRad Mini apparatus (Bio-Rad USA). A gel containing15% acrylamide for separating gel (1.5 M Tris-HCl pH 8.8, 10% SDS, DI water, 10% ammonium persulfate (APS), and TEMED) and a 4 % acrylamide stacking gel (0.5 M Tris-HCL pH 6.8, 10 % SDS, DI water, 10% APS, and TEMED) were used.

**2.11 Silver Stain**. At the end of electrophoresis, the gel was stained with a silver stain protocol. The gel was fixed for 25 minutes with 50% methanol-10% acetic acid. Then it was washed 3 times for 10 minutes in $dH_2O$. It was next sensitized for 30 minutes in 1 % glutaraldehyde/0.2M $NaHCO_3$. It was washed again twice for 10 minutes with $dH_2O$. Next the gel was stained for 30 minutes in 0.2% ammoniacal silver, and again washed with $dH_2O$ for 3 minutes. Last, stain was developed for 8 minutes using 0.005 % citric acid/0.009% HCHO. Development was stopped using 5% acetic acid.

# 3. RESULTS

**3.1 ELISA verification of purified UC1MT made by cell line.** C57BL/6J hybridoma cell line produces UC1MT as shown by Figure 1. The OD reflects the concentration of UC1MT in the samples
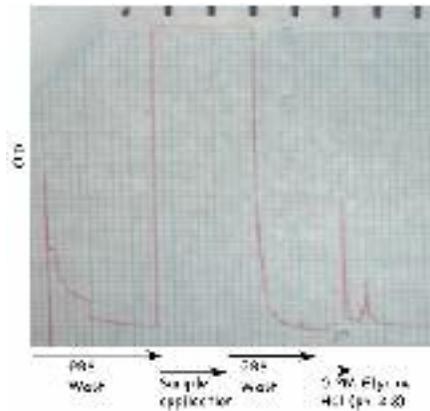
**Figure 1. UC1MT ELISA**
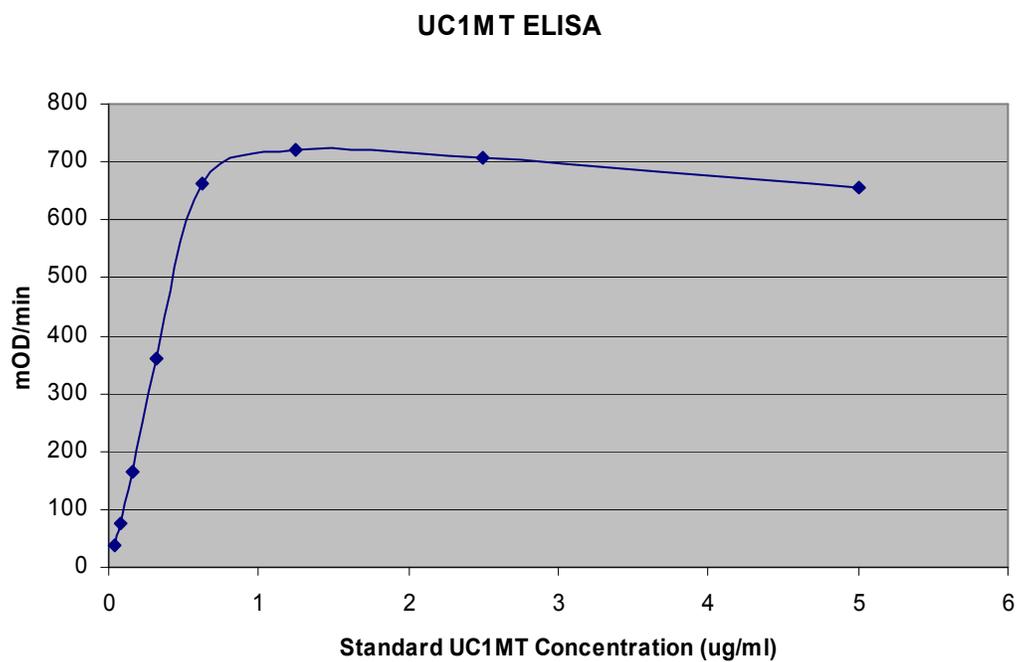


**3.2 Purified UC1MT is eluted from affinity column chromatography.** For every 50 ml of UC1MT hybridoma cell supernatant run over column, approximately 3.5 ml of eluent is obtained.

**Figure 2. Affinity column chromatography machine**



**Figure 3. Affinity column chromatography chromatogram**



**3.3 BCA Protein Assay.** UC1MT concentration determined by BCA Protein Assay to be .380 ug/ul. From the estimated column chromatography volume, the total protein yield is approximately 1.33 mg/ml.

**3.4 Comparison of UC1MT binding affinity to CNBr-activated beads, Protein A-bound beads, and to Protein G-bound beads.** The supernatant of the UC1MT samples mixed with various beads was tested by BCA Protein Assay before and after mixing. Results seen in table below show the most UC1MT binding occurs with the CNBr-activated beads; thus, these are used for rest of experiments.

**Figure 4. Chart: Comparison of bead binding affinities**

| Type of Bead | Protein in Supernatant (ug/ml) | Uncaptured UC1MT (ug/ml) | Captured UC1MT (%) |
|---|---|---|---|
| Protein A Sepharose | .380 | .220 | 42 |
| Protein G Sepharose | .380 | .194 | 49 |
| CNBr-activated Sepharose | .380 | .076 | 80 |

**3.5 Determination of appropriate UC1MT concentration to add to CNBr beads.** Based on Figure 5, 50 ug UC1MT was the decided fixed amount to add to CNBr-activated beads based on clarity of bands after silver stain without being wasteful of the antibody.

**Figure 5. SDS-PAGE and silver stain of UC1MT titration**
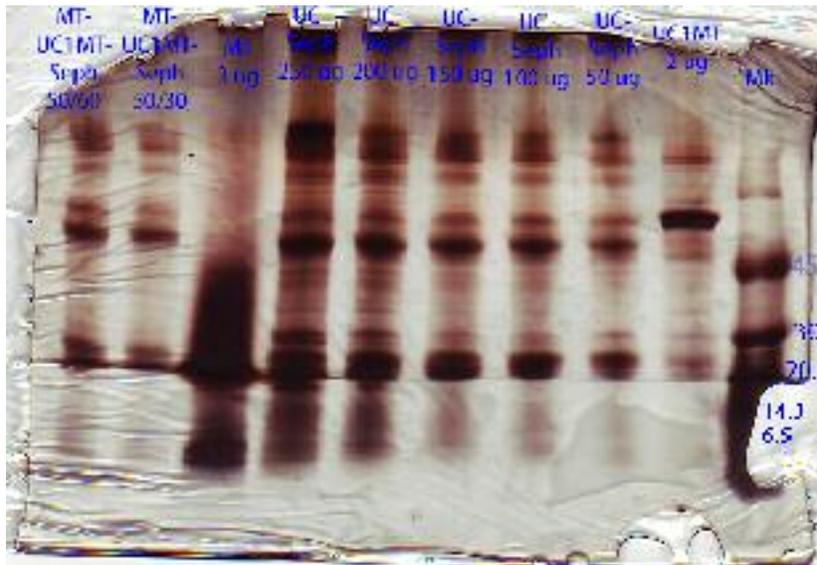
**3.6 SDS-PAGE and Silver Staining of MT bound-UC1MT-coupled CNBr-activated bead samples.** As shown in Figure 6, the purified UC1MT shows clear heavy and light chain bands located at the 50 and 25 kDa mark, respectively. The MT falls as a diffuse band at approximately 7kDa.

**Figure 6. SDS-PAGE and silver stain of samples with MT**
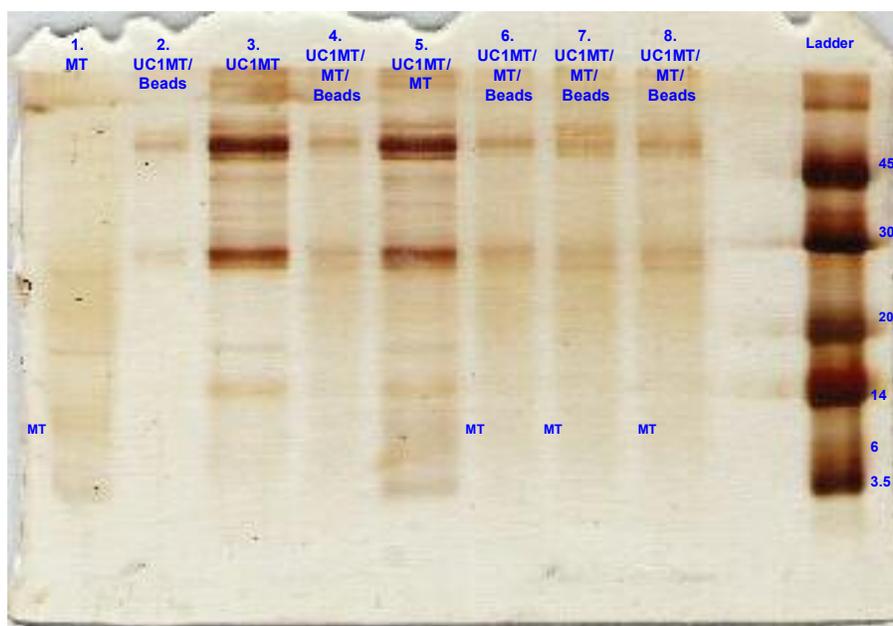


**3.7 Image J Results.** Lanes 1 and 5 are the MT positive control and show the most color intensity based on the SDS-PAGE gel shown in **3.6** above. Lanes 2 and 3 are the negative controls that only have UC1MT-coupled beads, or UC1MT alone, respectively. These two lanes show the least color intensity. Lanes 4, 6, 7, and 8 are the samples containing UC1MT-coupled bead bound to MT and show color intensities in between that of the positive and negative

controls. Hence, this is evidence that MT is binding to its antibody and running in the SDS-PAGE gel.

**Figure 7: Graph: Image J results of MT band on SDS-PAGE gel**

## Quantification of MT Band
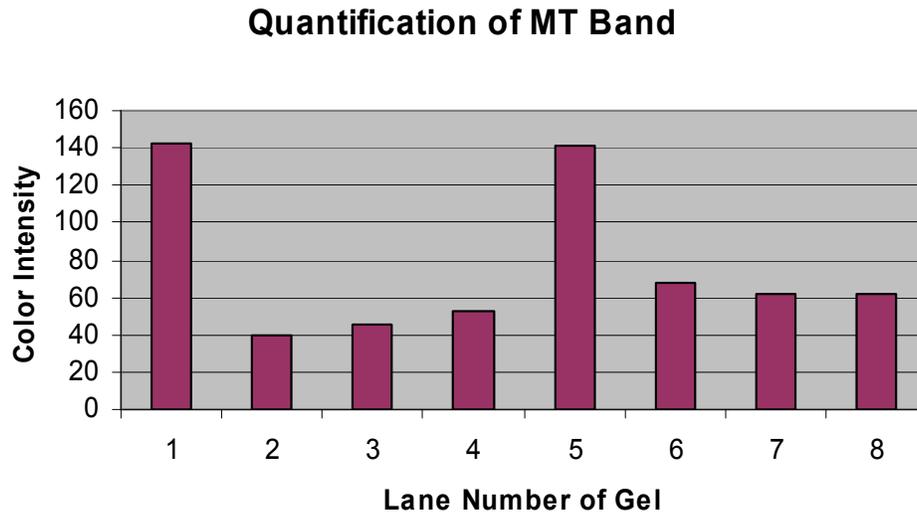
# 4. DISCUSSION

The experiments described here were designed to investigate the metallothionein receptor expression in leukocytes. Although there is still more research left to do, significant progress has been made thus far to establish the conditions for receptor purification.

It has been determined that the C57BL/6J hybridoma cell line effectively produces UC1MT, and each 50 ml of supernatant can yield approximately 1.33 mg/ml of antibody. The amount of antibody purified after each column run varies depending on when the supernatant is collected from the cells. It seems maximal antibody production of the cells, without overcrowding them, occurs approximately four days following passage.

The methods of how UC1MT binds to CNBr, Protein A, or Protein G all differ. Protein A binds to the Fc portion of most mouse IgG subtypes, including IgG$_1$ which is the subtype of UC1MT. Protein G binds to all mouse IgG subclasses, preferentially binding to the Fc portion but can also bind to the Fab region as well, giving it greater affinity for UC1MT. However, the CNBr-activated sepharose beads turned out to have the highest affinity for UC1MT, covalently coupling to the antibody's primary amines. The only drawback with using CNBr-activated sepharose is that it is not orientation specific, such that each antibody molecule may bind to the bead differently and potentially block the binding site(s) for MT.

The SDS-PAGE and silver stain assay allowed for the visualization of the molecular weights of the UC1MT bound to the sepharose and the MT bound to its antibody. The UC1MT

shows clear bands for both the heavy chain, running at 50 kDa, and the light chain, running at 25 kDa. However, there were more bands to show up in the purified UC1MT than hoped for which could make the later progress of the project more challenging. These extra bands may be the result of other non-specific proteins that are captured by the Protein A column during chromatography. The molecular weight of MT is 7 kDa, but on the gel the MT positive control falls as a diffuse band between approximately 7 and 14 kDa. This suggests that even under the reducing conditions used to run the gel, some of the MT may still become oxidized. The hydrogens of the thiols may come off and allow the sulfurs to mix with each other, forming mixed disulfides and enabling it to run as a dimer at 14 kDa. Based on the Image J results, there is evidence that a band for MT is on the gel in the lanes with MT and the UC1MT-coupled beads; however; it would be better to try to make those bands darker and more visible to the naked eye by further increasing the protein concentration.

The next step of this project will involve incubating a lymphocyte cell line (most likely T-cells) with MT, which will bind to the cell via the putative receptor, and then binding that to the UCIMT-coupled sepharose beads. This conglomerate will then be lysed and washed so all the bonds will be broken. It will then all be run in an SDS-PAGE and compared to the gels run earlier. We should be able to match up which bands correlate to UC1MT, which bands are MT, and any new bands should represent the molecular weight of the putative receptor.

After the molecular weight has been determined, the band will be cut out and run through a matrix assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometer

for amino acid sequencing. Once the amino acids are known, the sequence will be put into a sequence database and protein identification of the putative receptor should accomplished.

The final characterization of the putative MT receptor on immune cells will come with a great award. It will allow for a thorough understanding of the role played by MT in a variety of disease processes ranging from cancer to immunodeficiency to autoimmunity. We will also be able to manipulate MT levels as a means to reduce its undesirable immunomodulatory effects and may also facilitate manipulations of endogenous and exogenous MT as a therapeutic opportunity.

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