The Presence of Cysteine-mediated Dimerization in the Transmembrane Domain of Tumor necrosis factor 1 (TNFR1)

Project Summary

Tumor necrosis factor receptor 1 (TNFR1), its ligand (lymphotoxin-α), as well as their interaction and downstream signaling process are well studied due to their proposed involvement with the inflammation response of the body. It has been shown that the pre-ligand receptors tend to form dimer complexes at PLAD - their distal end in the extracellular domain (ECD), and the liganded receptors tend to form trimer complexes with the ligand timer at their second and third cysteine rich domain also in the ECD. Both interactions (resulting in a hexagonal network) are proposed to happen simultaneously thanks to the conformational changes in and near the transmembrane domain (TMD) of the receptor. The objectives of this research project are to investigate whether there is a cysteine-mediated dimer in the TMD and whether this dimerization is ligand-dependent. If there is a disulfide-linked dimer in the TMD, disruption of this bond could potentially affect hexagonal network of the receptors and the interactions between the receptor and its ligand and/ or the downstream protein recruitment as well as signaling. This knowledge could be used to create therapeutic approach to treat diseases related to inflammation response through TNFR family. To reach the mentioned objectives, site-directed mutagenesis was used to mutate the cysteine in the TMD into alanine (C223A mutation). The wild-type and mutant plasmid were transfected into HEK293. SDS-PAGE and Western Blot were then done to study the presence of the disulfide-bond as well as its dependence and/or influence on ligand-binding. It was hypothesized that the cysteine-mediated dimer would be present in the wildtype, but not the mutant (due to the mutation of the cysteine residue); and this dimerization would be independent of ligand binding. Unfortunately, the results of the conducted experiments in this research project failed to verify the presence of the disulfide bond in the TMD due to the possible dimerization of the receptor at the PLAD. However, the results were able to demonstrate that regardless of the location of the dimer, the dimerization was ligand-independent.

Background

Tumor necrosis factor (TNF) is a cytokine known for its cytotoxicity to certain types of tumor, as well as its participation in inflammation response of the body. Since its discovery in the 1960s, TNF superfamily- with 19 ligands and 29 receptors- has been studied to create therapeutic interventions for inflammation-related diseases such as rheumatoid arthritis (1) (2), psoriasis, and Crohn's disease (3) (4). Additionally, there have also been researches on the participation of TNF in obesity (5), cardiovascular pathophysiology (6), as well as AIDS pathogenesis (7).

Two distinct and well-characterized soluble ligands from this superfamily are TNF-α and TNF-β (also known as lymphotoxin-α or LT-α), respectively produced by macrophages and lymphocytes (8). These ligands bring about their biologic effects by binding to the

transmembrane TNF receptor 1 (TNFR1- p55) and TNF receptor 2 (TNFR2- p75) (9). Between the two receptors, TNFR1 has been studied more extensively than TNFR2 due to its pharmaceutical values, and hence, is chosen for the current study.

The construct of TNFR1 comprises of an extracellular domain (ECD), a transmembrane domain (TMD), and a cytoplasmic death domain (DD). The N-terminal ECD contains four cysteine-rich domains (CRD), each of which includes six cysteines forming three disulfide bonds stabilizing this region of the protein (10), (11). Closest to the membrane is CRD4, followed by CRD3, CRD2 and lastly, CRD1. While the ligand contacts are shown to appear mainly at CRD2 and CRD3 (12), the pre-ligand assembly domain (PLAD) responsible for the ligand-independent complex assembly of TNFR1 monomers lies in the CRD1 (13). The TMD of the receptor contains an alpha-helix structure with a cysteine residue at position 223- which may be capable of dimerizing with the cysteine from neighboring TNFR1. The C-terminal DD is crucial for initiation of death signaling upon ligand binding.

Regarding the ligand-dependent oligomerization, TNFR1 is suggested to form homo-trimeric complex upon binding with its trimeric ligand assembly at CR2 and CRD3 per their X-ray crystal structure (14), as well as reaction stoichiometry (15). This ligand-activated trimerization is widely believed to result in the recruitment of other proteins, and the initiation of the down-stream signaling process (16). On the other hand, regarding the ligand-independent interaction at the PLAD, initially, this complex was identified as trimeric; however, further studies suggest that this is rather a dimeric complex (17), (18). Additionally, deletion of the CRD1, including the PLAD, not only interrupts the association of monomers, but also reduces the receptor's responsiveness to its ligand (13). However, the receptor-ligand and receptor-receptor interactions are not contradicting; rather, together, they suggest a formation of a high molecular weight, hexagonal lattice network (19).

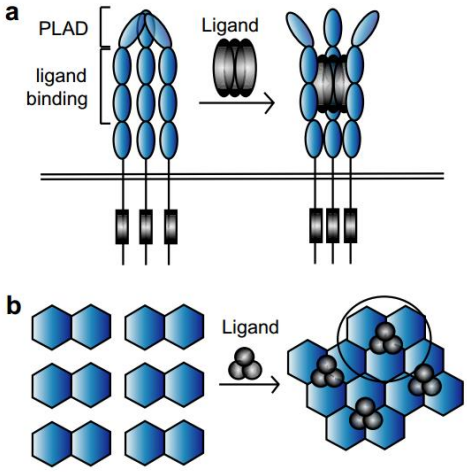

Figure 1. Proposed TNFR1 network. (a) Trimeric ligand-dependent interaction with the trimeric pre-assembled ligand. (b)On the left is the dimeric ligand-independent receptor-receptor interaction. On the right is the hexagonal lattice network formed as a result of both types of interaction. (19)

Further simulation of this network shows that the without conformational alternation, the complex would form a spiraling structure into the membrane. Hence, it is proposed that some changes in the conformation of the ligand-receptor complex are essential for the network to reside planar with the membrane. A study using normal mode analysis suggests that the ligand-binding trimers need to rotate and separate at the site close to the membrane, as well as pivot about the plane in between the two ligand-receptor complexes (20). Such orientational change is predicted to results in structural changes in the TMD and DD, as well as pose some questions about the signaling initiation of the receptor-ligand complex. A study on p75 neurotrophin receptor (p75 NTR), also a member of the TNF receptor superfamily, observes that the receptor has the capability to form disulfide-linked dimer through cysteine residue 257 in the TMD, and this dimerization is a prerequisite for further recruitment and signaling upon binding to neurotrophin- one of its ligands. A conformational change is also observed in the study; the receptor dimer assumes a scissor-like motion about the cysteine hinge separating the cytosolic domain and exposing the structure for further recruitment of adapter proteins (21).

When the receptor is activated by its ligand, the inhibitory silencer of the death domain (SODD) is released from the cytosolic DD of the receptor (22). This exposes the binding site for TNFR associated death domain (TRADD), which then recruits TNFR associated factor 2 (TRAF2) (23) and receptor interacting protein (RIP) (24). TRAF2, in turn, binds to the IκB kinase (IKK), which is activated by RIP (25) and phosphorylates the inhibitor of κBα (IκBα). When IκBα undergoes ubiquitination and degradation, the nuclear factor κB (NF-κB) transcription factors are freed and translocate into the genome to signal inflammatory responses.

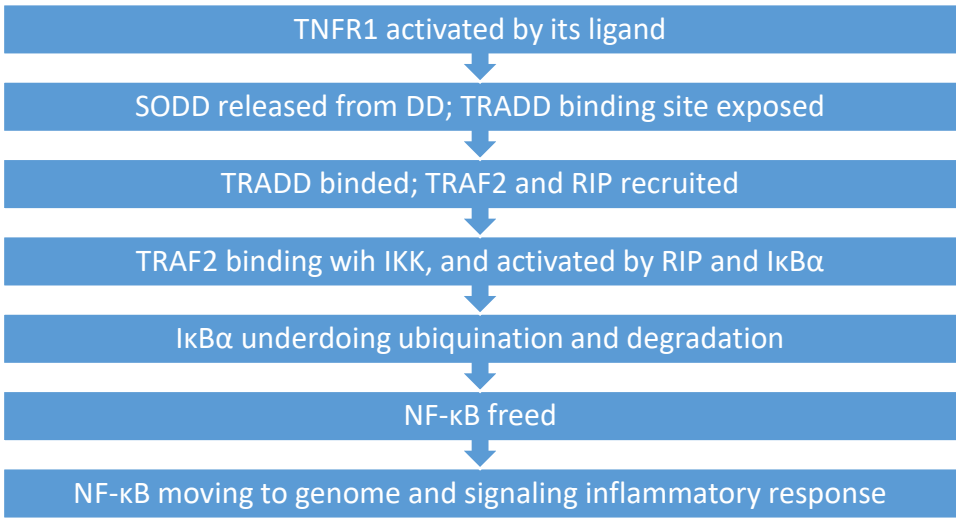

Figure 2. Process of signaling inflammatory response through TNFR1.

Objectives

The objective of this research project was to utilize molecular biology techniques (site-directed mutagenesis, Western Blot, bacterial protein over express, and mammalian cell culture techniques, etc.) to study the possibility of a disulfide bond in the transmembrane domain (TMD) of the TNFR1 and its influence on the dimerization of the receptor at this domain as well as the functionality of the receptor. This research seeks to answer the following two questions: One, is there any disulfide -linked dimer in the transmembrane domain of TNFR1? Two, if yes, is the formation of this dimer is ligand-dependent?

Materials and methods

This research project was composed of four sub-projects: One, mutation genesis to create the C223A mutant gene; Two, bacterial protein overexpression and purification to produce LT-α (a ligand of TNFR1); Three, HEK293 mammalian cell culture and transfection; Four, SDS-PAGE and Western Blot to observe the interaction between the receptors, as well as that between the receptor and the ligand. The full-length wild-type TNFR1 plasmid, the glycerol stock of LT-α plasmid in BL21(DE3), the HEK293 cell line, and primary and secondary antibody for immunoblotting were courtesy of Sachs Research Group.

The C223A mutated gene was synthesized by using Site-directed Mutagenesis protocol as shown in the instruction manual of the QuickChange® Site-Directed Mutagenesis Kit by Stratagene. The protocol included three steps: mutant synthesis by thermal cycling, Dpn I digestion of non-mutated DNA template, and transformation of the desired template into XL1-Blue cells. The mutagenic primers for thermal cycling were designed by Nagamani Vunnam. The transformation step was done with LB Broth -made with LB Broth (Lennox) powder from Sigma-Aldrich- instead of NZY$^+$ Borth as directed in the manual. Carbenicillin (100ug/ mL) was used as the anti-biotics in the LB agar plate. Colony was picked the next day, grown in LB broth with carbenicillin shaking at 225rpm and 37°C. DNA was then purified using HiSpeed Plasmid Kit (Quiagen) accordingly with product manual. Concentration and purity of plasmid samples were determined by absorbance method. Samples were aliquoted and stored at -20°C for further application.

The ligand LT-α was made through bacterial protein overexpression and purification technique. Colonies of plasmid was made by plate streaking with LT-α glycerol stock on agar plate containing carbenicillin (100ug/ mL). Next day, one colony was inoculated in 50mL LB broth in shaker at 225rpm and 37° overnight. Then, 10mL of previous day culture was diluted in 1L of LB broth containing carbenicillin, and placed in shaker with condition similar to before for 3-4 hours. When the optical density of culture at 600nm reached 0.5 -0.8, 1mM IPTG was added to induce protein production. Culture continued to be shaken for three hours. After that, culture was centrifuge at 3700rpm and 4°C for 25 minutes. The supernatant after centrifugation was discarded, and the pallet was suspended in 1X TBS buffer. The mixture was then sonicated thoroughly on ice and centrifuged at 13000rpm and 4°C for 40 minutes. The supernatant was collected and filtered through filter syringe with 25um filter. The resulted supernatant was then run through a prepared Flag-Tag bead protein fusion column three times until column was saturated. The column was washed with TBS until dripped solution reach pH 8.0. The protein in the column was eluted with 0.1M Glycine and neutralized with 1M Tris. The collected protein was concentrated by using the Amicon Centrifugal Filter unit. BCA assay and SDS-PAGE were conducted with the final product to find protein concentration and confirm protein purity.

HEK293 cell line was started from cryopreservation. The cells were grown in high glucose DMEM medium (Gibco) with 10%FBS, and incubated at 37°C with 5% CO_2. Cells were transfected using Lipofectamine 3000 Reagent (Invitrogen) accordingly with product manual. For 6-well plate, 10ug of DNA was used for each well. Additional treatment with ligand and/or H_2O_2 (for Western Blot) was done by adding a ligand-PBS mixture and/or H_2O_2 to cell for 30 minutes before harvesting.

Harvested HEK293 cells were lysed in native lysis buffer with 10% protease inhibitor, incubated on ice for 30 minutes, and centrifuged at 15000rpm and 4°C for 45 minutes. The supernatants were collected. BCA assay was used to find protein concentration of lysates. To prepare for SDS-PAGE, lysates were diluted in water to desired concentration and mixed with 2X Laemmli buffer (BioRad). 5% BME was added to the sample(s) if a reducing agent was desired. Samples were denatured by boiling for 2-3 minute and let cool. The samples were then loaded into the wells of 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad) and run in 1X Tris/Glycine/SDS Electrophoresis buffer (BioRad) for 10 minutes at 100V and then 30 minutes at 200V. Once electrophoresis was done, the gel was used for Western Blot. Transferation of protein from gel to membrane was done in cold room at 100V for 2.5 hours. Then, immunoblotting of membrane with primary and secondary antibodies was done overnight. After that, a digital image of the membrane was captured through the fluorescent detection by Li-Cor Western Blot Detection System.

First, a SDS-PAGE and Western Blot experiment was run with HEK293/WT/TNFR1 untransfected and transfected cell at different lysate volume (protein amount) to identify the presence of disulfide-linked dimer as well as optimal protein amount to use for subsequent experiments. Second, a SDS-PAGE and Western Blot experiment was run with the TNFR1/WT and TNFR1/C223A with and without ligand to compare the interaction between receptor-receptor and receptor-ligand of the two types. Third, the second experiment was repeated with and without reducing agent and H_2O_2 to observe the disulfide-linked dimer in each case. Last, a SDS-PAGE and Western Blot experiment was run with the TNFR1/WT and TNFR1/C223A at various concentration of ligand to determine to optimal amount of ligand to use for further experiments and/ or in case of repeating the third experiment.

Results

Mutagenesis results were confirmed by DNA sequencing and are shown in Figure 3 and 4. Mutation point was bolded and underlined. Western Blot results TNFR1/WT and TNFR1/C223A with and without ligand and/or H_2O_2 and reducing agent (BME) are shown in Figure 5-8. The condition of each well was noted on the top of the gel.

MGLSTVPDLLLPLVLLELLVGIYPSGVIGLVPHLGDREKRDSVCPQGKYIHPQNNSICCT
KCHKGTYLYNDCPGPGQDTDCRECESGSFTASENHLRHCLSCSKCRKEMGQVEISSC
TVDRDTVCGCRKNQYRHYWSENLFQCFNCSLCLNGTVHLSCQEKQNTVCTCHAGFFL
RENECVSCSNCKKSLECTKLCLPQIENVKGTEDSGTTVLLPLVIFFGL**C**L

Figure 3. Amino acid sequence *of WT TNFR1 up to the 24th residue. Cysteine residue at position 223 is bolded and underlined.*

MGLSTVPDLLLPLVLLELLVGIYPSGVIGLVPHLGDREKRDSVCPQGKYIHPQNNSICCT
KCHKGTYLYNDCPGPGQDTDCRECESGSFTASENHLRHCLSCSKCRKEMGQVEISSC
TVDRDTVCGCRKNQYRHYWSENLFQCFNCSLCLNGTVHLSCQEKQNTVCTCHAGFFL
RENECVSCSNCKKSLECTKLCLPQIENVKGTEDSGTTVLLPLVIFFGL**A**L

Figure 4. Amino acid sequence of mutated C223A TNFR1 up to the 24th residue. Alanine residue at position 223 is bolded and underlined.

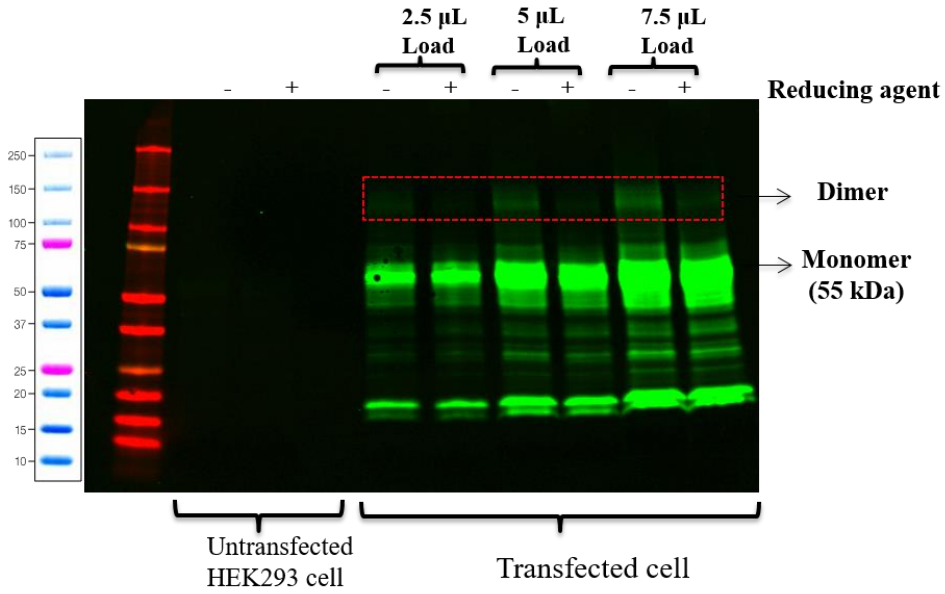

Figure 3. Western Blot result of TNFR1/WT to detect disulfide-linked dimer in absence of ligand.

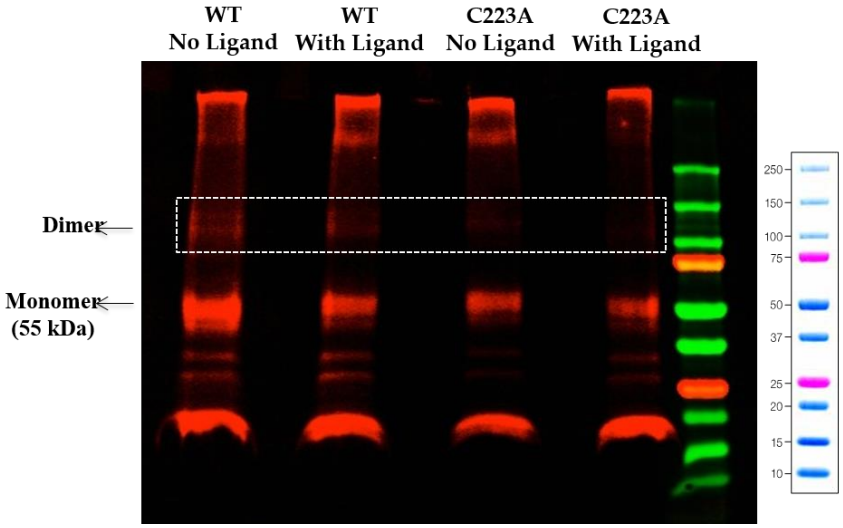

Figure 4. Western Blot result of TNFR1/WT and TNFR1/C223A to observe disulfide-linked dimer under presence and absence of ligand.

It could be observed from Figure 3 that there were no band in the lane of untransfected cell. Also, there was dimer band in the wells of TNFR1/WT transfected cell.

From the brightness of the band, it was determined that the optimal volume of lysate was 5uL (corresponding to 50ug of protein). Additionally, under presence of reducing agent, the dimer band was dissolved. From Figure 4, it was noted that there were dimer band in the lane of both the WT and the mutant. However, the dimer band from the mutant's lanes were very faint, compared to the WT. Also, there were protein aggregates in the wells at the top of the gel. In both figures, the dimer bands were consistently fainter that the monomer bands.

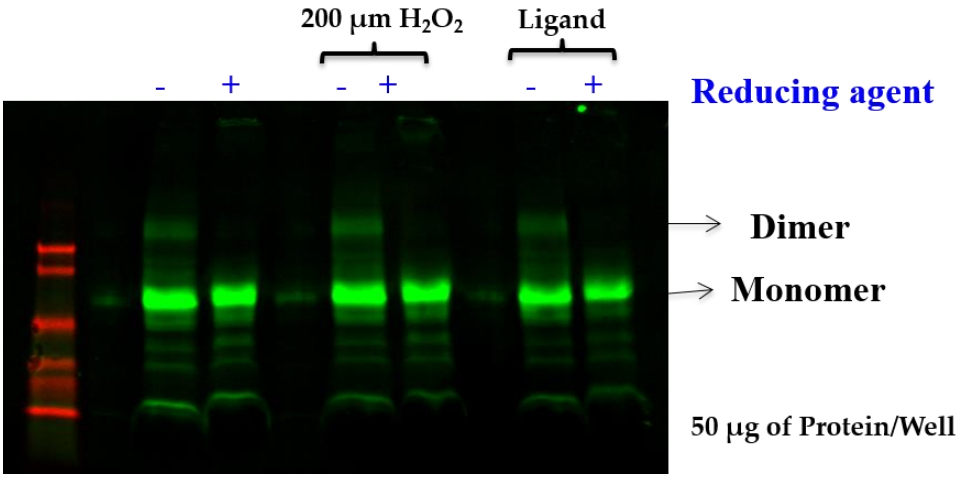

Figure 5. Western Blot result of TNFR1/WT to observe disulfide-linked dimer under presence and absence of ligand and hydrogen peroxide.

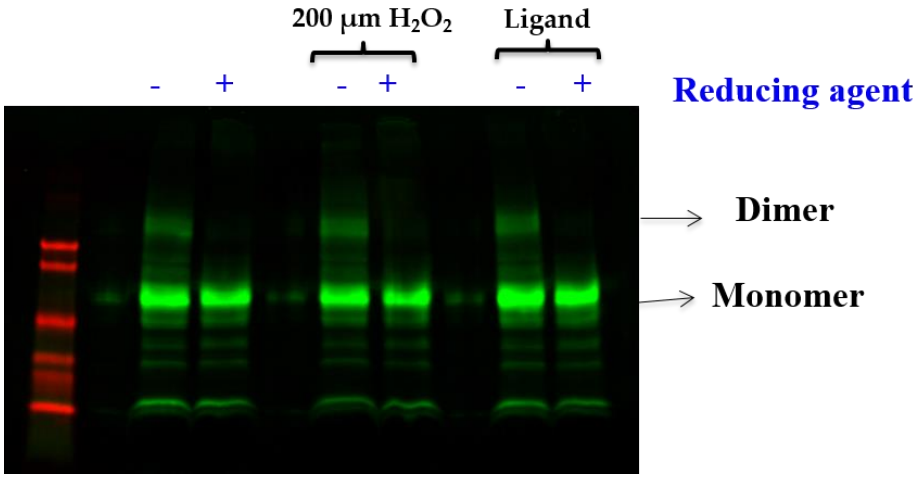

Figure 6. Western Blot result of TNFR1/C223A to observe disulfide-linked dimer under presence and absence of ligand and hydrogen peroxide.

It was seen from Figure 5 and 6 that there were dimer band in both the WT and the mutant, and in lanes with reducing agent, the dimer bands disappeared. Additionally, it was noticed that all the lanes were very similar, even the ones with addition of ligand and/or

H_2O_2 in both the WT or the mutant. The dimers continued to be fainter than the monomer bands.

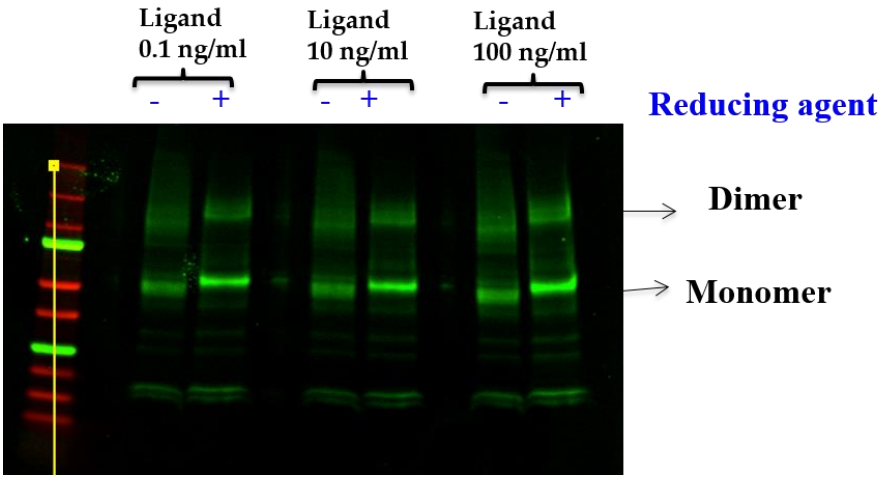

Figure 7. Western Blot result of TNFR1/WT with various concentration of ligand.

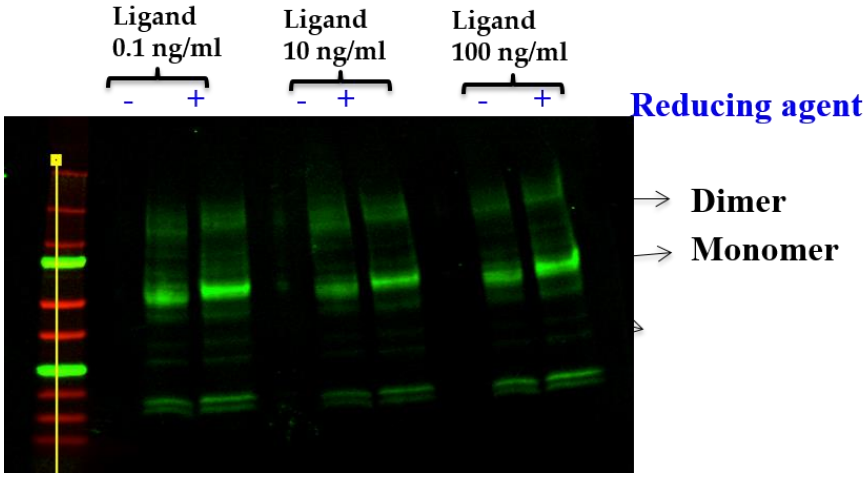

Figure 5. Western Blot result of TNFR1/C223A with various concentration of ligand.

From Figure 7 and 8, it was observed that under presence of the reducing agent, the dimer bands were not resolved, and the monomer band of these lanes was slightly brighter than that of the lanes without the reducing agent. Furthermore, it was noted that increasing concentration of ligand did not change the size of either the dimer or the monomer bands.

Discussion

In this research project, the TMD cysteine at position 233 is mutated into alanine (C233A mutation) due to their similarity in chemical structure. Like cysteine, the alanine residue also has a two-carbon backbone with an amino (-NH2) and a carboxylic (-COOH) functional group attached to the α-carbon. However, unlike cysteine, it does not have a thiol (-SH) functional group. Hence, by replacing cysteine with alanine, the changes to the protein's structure, conformation, and functionality are supposed to be less significant, compared to those resulting from replacement of cysteine with other amino acid. Nevertheless, the effect

of polarity change (cysteine is polar, while alanine is non-polar and hydrophobic) was not accounted for in this experiment.

Regarding the first and second experiment, Figure 3 showed that the transfection protocol in HEK293 cells was successful, since there were no bands in the lane of untransfected cells. Also, the presence of the dimer band in the lane of transfected cells, and its absence under the effect of the reducing agent confirmed the existence of disulfide-linked dimer in TNFR1/WT. Similarly, figure 4 showed that disulfide-linked dimer was also present in the mutant, though may be in smaller amount, compared to the WT. However, the above inference did not consider the protein aggregates inside the wells. This aggregation could be due to the gel was not run immediately after the samples were loaded or the lysis process was not completely thorough. Moreover, addition of ligand did not seem to affect dimerization, which was expected in the hypothesis because ligand binding mainly happened in the CRD2 and CRD3. Additionally, as seen in Figure 3 and 4, the dimer bands were significantly fainter that the monomer bands. This was not expected considering that the pre-liganded receptor tend to dimerize at the PLAD. It was suspected that the cytosol of the cell might have acted as reducing agent and disrupted the dimer bond during the lysis process. Furthermore, since the full-length plasmid was used to create these sample, it was undetermined whether these disulfide-linked dimers was due to the receptor-receptor dimerization at the PLAD or the cysteine-mediated dimer in the TMD. Further experiment should be done with TNFR1 plasmid whose PLAD region was mutated or deleted to identify whether dimer was formed at the TMD.

Regarding the third experiment, to make up for the reducing effect of the cytosol found in the previous experiments, H_2O_2, an oxidizing agent, was added to reduce/ counteract this effect. However, from Figure 5 and 6, it could be seen that this addition did not resolved the issue. Other repetitions of this experiment with higher concentration of H_2O_2 (result not shown above) also did not increase dimerization. Additionally, also seen here, addition of ligand did not affect dimerization of the receptor due to the proposed reason. Also, even though the amount of dimer seen in WT and the mutant were approximately similar, it was inconclusive as to whether the mutation affect ligand-independent dimerization of the receptor due to the presence of PLAD in the receptor, as mentioned above.

Regarding the fourth experiment, the result was very unexpected and contradicting to that of the third experiment. The dimer band was present even with the presence of reducing agent, which suggested that the dimer did not respond to the reducing agent, for both the WT and ligand. One possibility for such phenomenon was that the samples may have been prepared a long time before the SDS-PAGE and/or that the samples may have been stored at inappropriate condition, which caused the reducing agent to be oxidized and/or denatured. Since the disulfide-bond in protein is constantly formed and dissociated, after the reducing agent was compromised, the S-S bond may have formed when the samples were loaded into the gel. Future experiment, if repeated, should consider this possibility to avoid this result.

Conclusion and future direction

In conclusion, while this research project failed to determine the presence of the cysteine-mediated dimer in the TMD of TNFR1, it suggested that if such dimerization existed, the formation of the dimer would be ligand-independent. In addition, the experiment also

showed that whole cell lysis resulted in a low ratio of dimer to monomer. With these conclusions, it demonstrated that more experiments were necessary to answer the objective questions, considering the pitfalls observed in this project.

As discussed above, future experiment to study this dimerization in TMD should consider mutating or deleting the PLAD in the ECD to ensure that the dimerization seen in Western Blot belongs in the TMD, while also bear in mind that deletion of the PLAD may affect the ligand sensitivity of the receptor. Additionally, further experiments should investigate a protocol that could separate the cytosol and the membrane, such as this procedure suggested by Xiaoyong Liu and François Fagotto (26), or using Mem-PER™ Plus Membrane Protein Extraction Kit (ThermoFisher). Additionally, it would be essential to ensure that the lysis buffer (for separation of cytosol and membrane) did not contain too much detergents, which may dissolve the membrane-bound protein. A buffer containing reducing agents (Tris, EDTA, BME etc.) but not detergent may be desired. After sonication and centrifugation of the suspension, the pellet containing the membrane and membrane-bound protein could be extracted using RIPA buffer with appropriate incubation time. Finally, further experiment to study the influence of the cysteine-mediated dimer in the TMD on the network formation and functionality of the TNFR1 should also be considered, since changes in the TMD could alter the conformation of the protein in TMD, which could disrupt the hexagonal lattice and/or affect the recruitment of downstream protein and hence, the functionality of the receptor.

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