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Molecular Mechanism and Drug Development of Tumor Immunotherapy Targets

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1 Cancer immunotherapy

Cancer immunotherapy is a way of treating cancer by improving, restoring, or activating the patient's natural immune system to attack cancer cells using different agents. Therapies aim to reduce using off-target cancer treatments such as chemotherapy that kills cells indifferently. With the field rapidly advancing, the more and more immunotherapy drugs are being developed and approved by the FDA. For example, one of the first approved drugs is the recombinant versions of the cytokine interferon- α in treating hairy cell leukemia. More recently, the CAR (chimeric antigen receptor) for the CD19 B lymphocyte molecules has been approved for treating refractory pre-B cell acute lymphoblastic leukemia. Nowadays, there are more than 12 immunotherapies approved and available for treatment and even more in clinical trials. However, many problems still remain with immunotherapy drugs. Firstly, patients with the same type of cancer don't always respond the same to the same drug. Some may respond positively while others may not respond at all, and this makes treatment responses hard to predict, so efforts are put into making patient-specific drugs to improve positive response rates. Also, the mechanism by which immunotherapy drugs work (up regulating immune responses) can have side effects such as autoimmune diseases, nonspecific inflammation, cytokine release syndrome, and vascular leak syndrome. Other side effects may be that the target to the cancer exists on some healthy cells too, and the immune system will attack all cells with its target regardless of its identity. Most importantly, the effectiveness of immunotherapies on solid tumors is restricted by delivery system barriers and thus is only capable of treating hematological cancers. In response, better delivery systems are being developed such as nanoparticles, scaffolds, implants etc[1]. There are also many different classes of immunotherapies, and checkpoint inhibitors and engineered T cells will be investigated in detail[2].

1.1 Checkpoint inhibitors

One of the most thoroughly researched immunotherapy is checkpoint inhibitors. Checkpoint inhibitors work by inhibiting immune systems from attacking healthy cells thus ensuring appropriate immune responses. One of the most common checkpoint inhibitor strategy is PD-1(programmed-death 1)/PD-L1 blockade. PD-1 is a part of the CD28 family, and it is identified as the receptor for B7H1 – also called PD-L1 as it is a ligand for PD-1. It is only found on activated T cells because only when responding to an inflammation, immune cells express PD-1 to help them to recognize abnormal or cancerous cells. However, the engagement of PD-L1 and PD-1 results in T cell dysfunction, and as one of the evading mechanisms many cancerous cells express PD-L1. When PD-L1 expressing tumor cells engage with PD-1 T cells, the T cells may undergo apoptosis, exhaustion, anergy etc. Since normally without engagement with PD-L1, the T cells will mediate lysis, PD-L1 protects and shields cancerous cells from being destroyed by immune cells. One way to stop the interaction between PD-L1 expressing cancerous cells and PD-1 expressing T cells is to use mABs that target either PD-1 or PD-L1 can restore T cell function such as $CD8^+$ mediated

tumor cell lysis. Another example of checkpoint inhibitory method is CTLA4. The interaction between CTLA4 and its ligands CD80 and CD86 inhibit the immune response and similar to PD-1, by blocking this interaction T cells can remain active. There are roughly 5 PD-1/PD-L1 and 1 CTLA4 inhibiting drugs are approved and are shown to have an overall higher survival rate compared to methods like chemotherapy. Some limitations to this approach are that it administers side effects to many organs, and that the responsiveness varies between individuals because different types of cancer have different types of immune suppression system due to their different environment. These limitations are still being addressed by methods such as developing new delivery systems[3].

1.2 Adoptive T cell transfer (ACT)

This method relies on collecting T cells from patient's blood and then engineering them express a certain type of receptor that would bind to a specific antigen on tumor cells. These engineered T cells are then transferred back to the patients with long lasting effects. 3 types of ACT methods in development is tumor infiltrating lymphocytes (TILs), T cell receptor (TCR) T cells, and chimeric antigen receptor (CAR) T cells. TILs have been successful at treating metastatic melanoma because it can target neoantigens in melanoma. TCRs respond to intracellular tumor antigens by major histocompatibility complexes. Activation of the T cell happens when TCR recognizes peptides non-covalently bonded to MHC on tumor cells. One of the first time TCR was clinically used was target towards metastatic melanoma. The TCR targeted towards melanocytic differentiation antigen present on the tumor cells, however they were also present on normal melanocytes in the eyes and skin. This became a problem because T cells would attack both tumor cells as well as healthy cells. Other limitations to this approach rose because it is dependent on MHC expression, identity, and co-stimulation. Lastly, unlike TCRs, CARs are MHC independent – this is advantageous because many tumor cells no longer express MHC. One of the first target of CARs is CD19 – a molecule often found on B cell leukemias and lymphomas, however it is also on healthy B cells and CARs may cause B cell aplasia. Other limitations to ACTs are that both CARs and TCRs can result in cytokine release syndrome and neurotoxicity. They are also very expensive, and they are not capable of penetrating solid tumors[4].

2 Construction of in vitro recombinant expression of potential target-related proteins in tumor therapy

Previously, the method of constructing a protein would be to use restriction enzymes to isolate the fragment of DNA that would code for the target protein, and use polymerase chain reaction to amplify the products. Then, recombinant DNA would be associated with a carrier such as E. coli and after protein synthesis the target protein could be isolated from the other components of the cell using ways such as chromatography. The reason why this method is no longer prevalent in use is because different species of carriers, for example E. coli, would have different preferences in codon when coding for an amino acid, and thus the protein may not always be expressed correctly or at all. Nowadays, different companies have the ability to construct proteins based on its amino acid sequence and where restriction enzymes are targeted towards. To find the amino acid sequences of a protein, the first thing to do would be to read through previous reports and see if others have expressed and constructed this protein already. Websites like Uniprot, PDB, and Pubmed are places to start. To demonstrate the process of researching, the protein CD80 will be used.

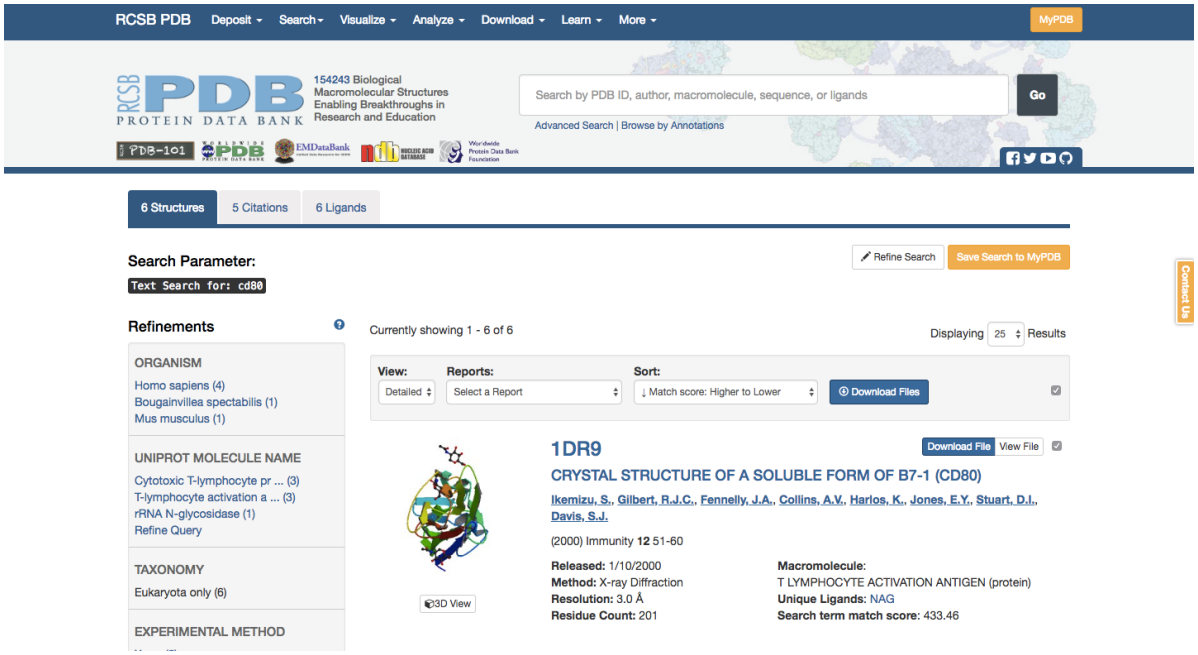
2.1 Summary of CD80

CD80, or cluster of differentiation 80, is a B7 type I membrane protein in the immunoglobulin superfamily - it has an extracellular immunoglobulin constant like domain and a variable like domain for receptor binding. It is associated with the regulation of both the innate and adaptive immune system. It is the receptor to CD28 and CTLA-4 on T-cell membranes. The binding of the protein to its receptor results in various important functions of the cell such as autoregulation, intercellular association, and regulation of cellular disassociation. CD80 also allows quick interaction between communicating cells due to its chemical properties, and results in important costimulatory signals in the immunological synapse between immune cells. Such interactions with the immune cells allow CD80 to play an important role in the regulation of the differentiation, activation and proliferation of T cells and B cells, and secretion and expression of various cytokines and antibodies in immune responses. It can be found in various immune cells such as dendritic cells, monocytes, B cells etc. Through observation,

the up-regulation of CD80 is associated with several autoimmune disease, while the down regulation has been shown to help the spread of the HIV virus. Since CD80 plays an intricate role in the immune system, it can be used to find ways to treat diseases. For example, it is being investigated of uses of treating autoimmune diseases by inducing the down regulation of CD80. It is also being studied in cancer immunotherapy to artificially express CD80 on cancer cells and induce the up regulation to trigger apoptosis or induce natural killer cell mediated death.

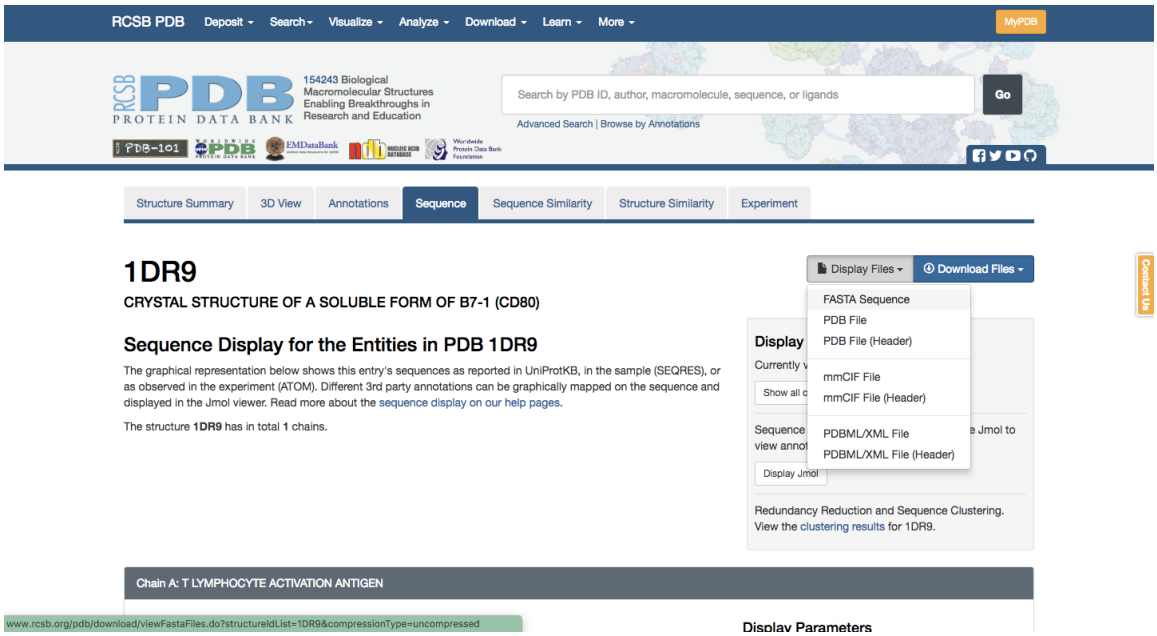
2.2 Steps in browsing for the information and sequence of CD80

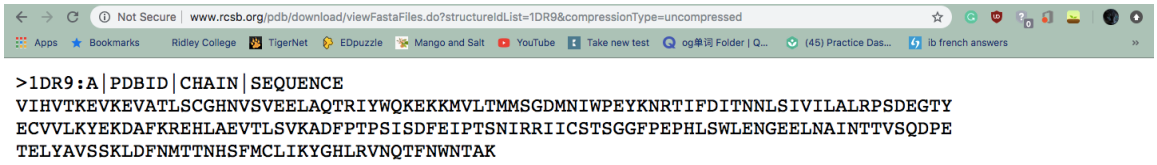
1.To start, go to http://www.rcsb.org/. It is a protein data bank where every protein to be worked with has entries on its information. Enter CD80 in search bar and specify for the desired species and refine search in any parameters if needed. Browse and choose a report that is suitable for the purpose of the search.



2.For CD80, the 3D structure has been discovered and information on its characteristics and structure can be found immediately after going into is structure summary.

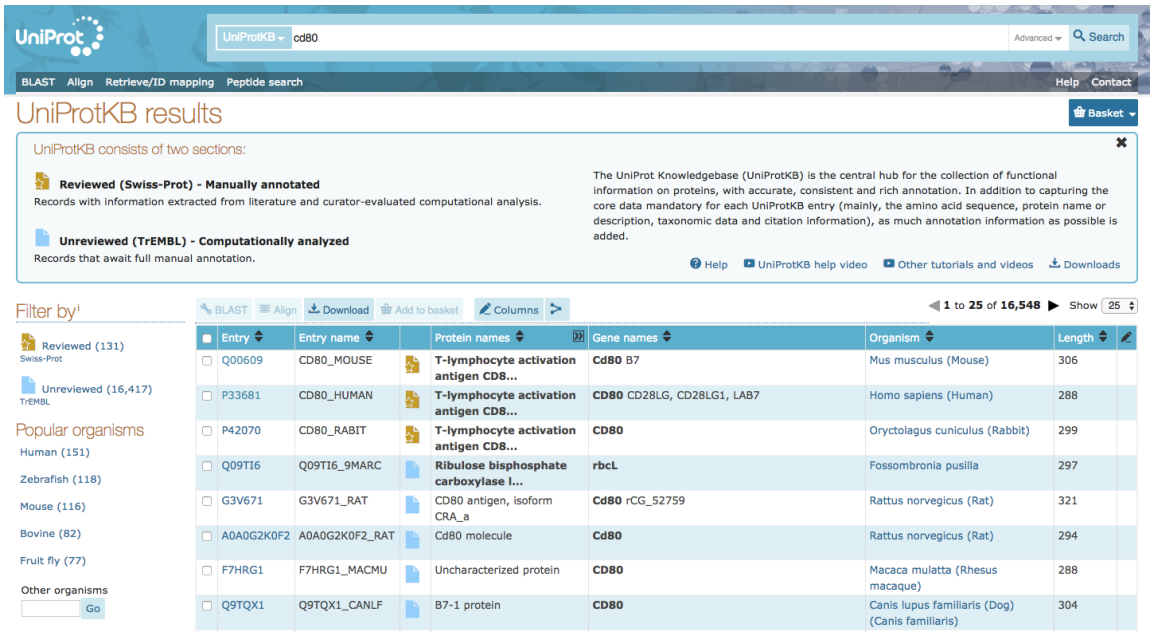
3.To find the amino acid sequence of the protein, go to tab “sequence” at the top and choose to display or download FASTA sequence. The abbreviation of the specific sequence will be shown on an independent page.





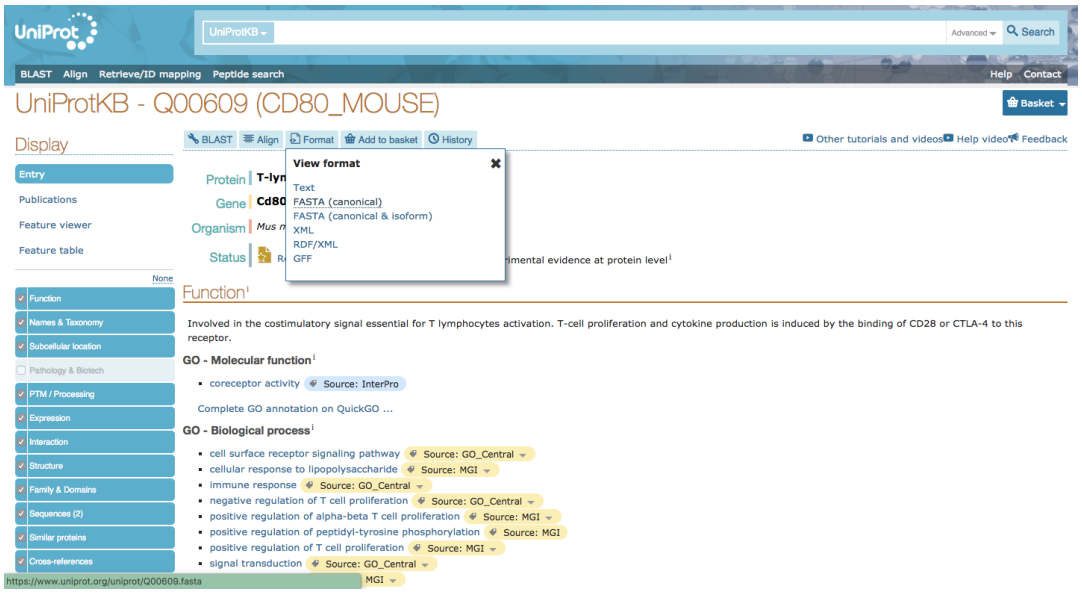
Each report could have used different domains of the protein specific to their topic of investigation, so even for the same protein, the FASTA sequence would be different for different reports. In order to see the specific positions and description of the shown sequence, you could go to the referenced published article. A convenient alternative option would be to use the website https://www.uniprot.org/uniprot/.

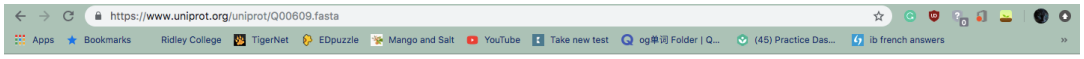
4.Similar to PDB, Uniprot also shows a summary of your target protein based on specific reports. However, Uniprot also tells you exactly on what position the shown sequence is found on the entire amino acid sequence, and also where the domains are relative to the cell membrane.



Uniprot also allows refined search by species and has the BLAST function to search for similarities in sequence or structure between proteins. Choose an appropriate entry to investigate.

5.To see the abbreviated sequence, choose the "format" tab and choose view format in "FASTA (canonical)"



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>sp|Q00609|CD80_MOUSE T-lymphocyte activation antigen CD80 OS=Mus musculus OX=10090 GN=Cd80 PE=1 SV=1
MACNCQLMQDTPLLKFPCPRLILLFVLLIRLSQVSSDVDEQLSKSVKDKVLLPCRYNSPH
EDESEDRIYWQKHDKVVLSVIAGKLKVWPEYKNRTLYDNTTYSLIILGLVLSDRGTYSCV
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NGRELPGINTTISQDPESELYTISSQLDFNTTRNHTIKCLIKYGDAHVSEDFTWEKPPED
PPDSKNTLVLFGAGFGAVITVVVIVVIIKCFCKHRSCFRRNEASRETNNSLTFGPEEALA
EQTVFL
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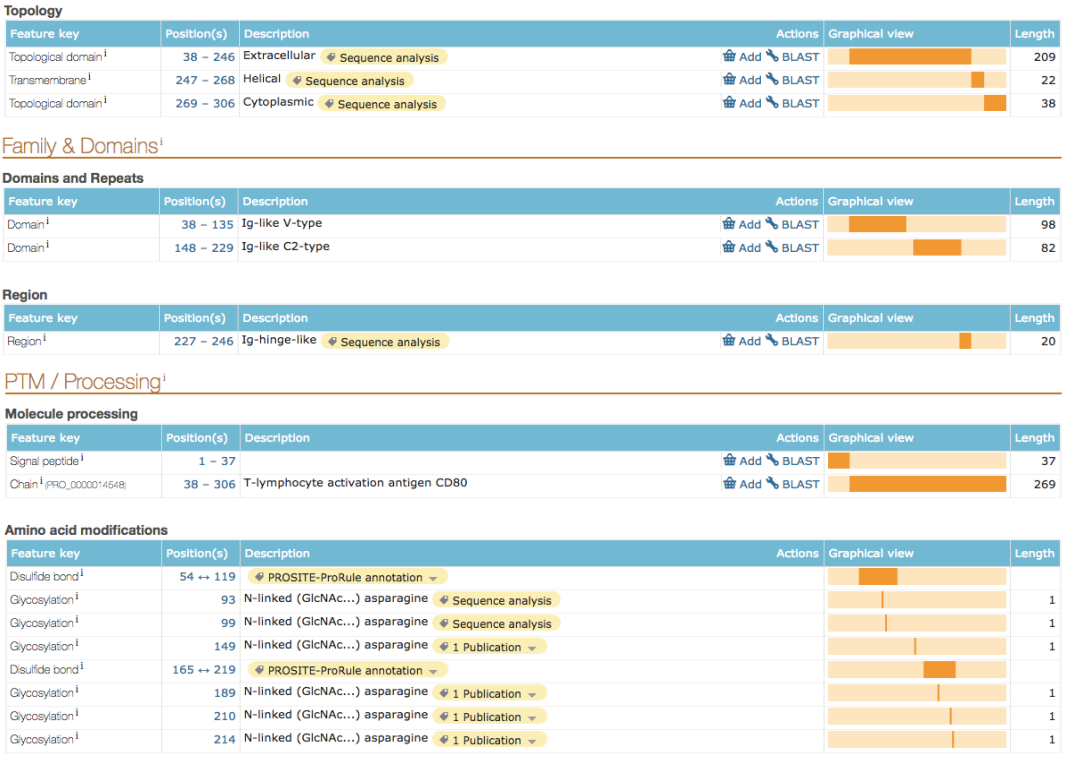
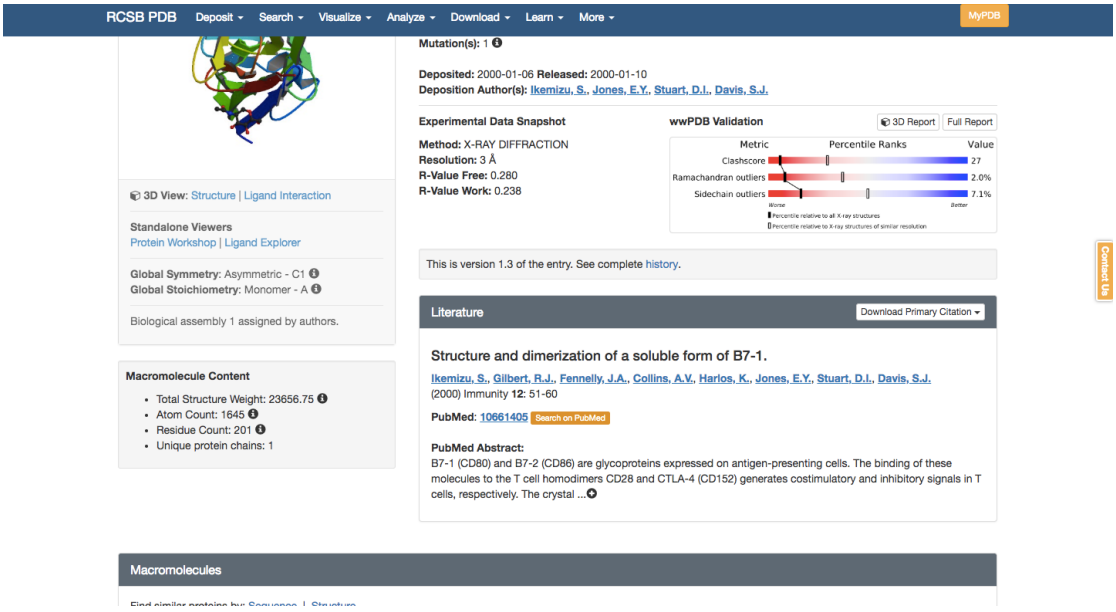
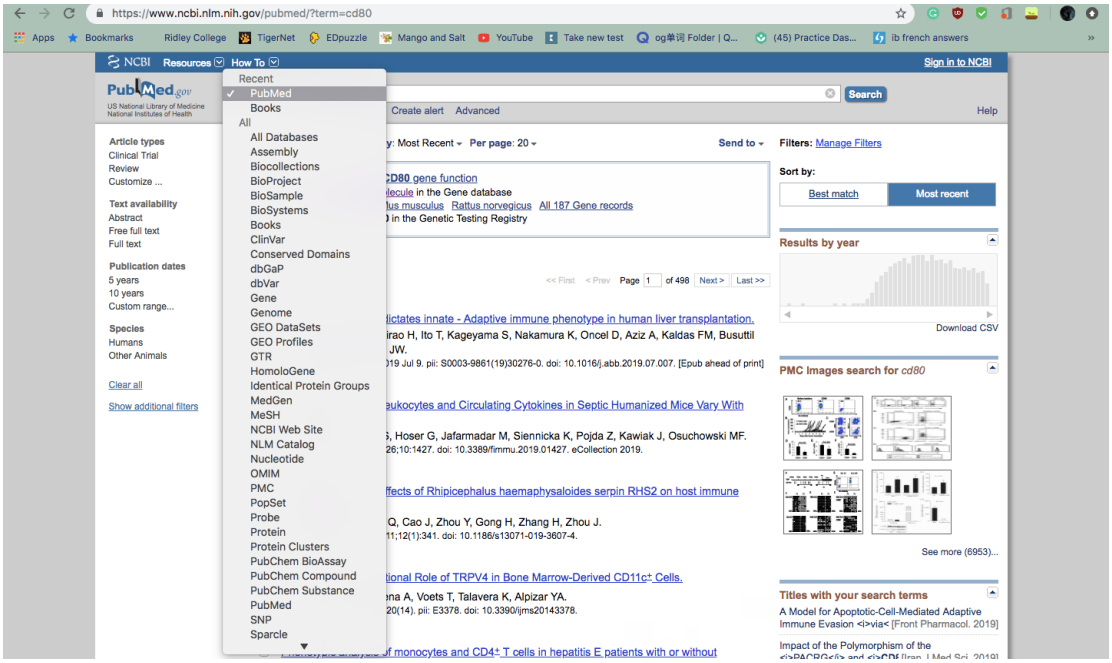
6.To see the detailed description of the protein, go back to the entry and further down the page, there will be tables describing the protein's topology, domains etc.



7.To view the original published reports for in depth analysis or reference, both PDB and Uniprot are linked to Pubmed and may give the serial number in finding the report on Pubmed. The website for Pubmed is https://www.ncbi.nlm.nih.gov/pubmed/, and it provides functions like different and specific search engines for your topic of investigation such as protein, nucleotide, and gene.





3 Refolding of prokaryotic expressed recombinant proteins

When recombinant proteins are expressed by prokaryotes such as E. coli, during rapid amplification the peptide may not have enough time to be folded or be correctly folded. For the protein to have its desired properties and function, the tertiary and quaternary structure is vital, so in order for the recombinant proteins to have the function that would be normally possessed by proteins generated naturally, they have to be refolded. The methods used in refolding may not generated exact replicas of natural proteins, and it would be hard to determine if they match, but generally the proteins after refolding would have the desired function, such as binding to a receptor, for the research purpose. Whether the refolding is success would be based such criteria. Below is the procedure to refold the MHC complex. It consists of preparation of the refolding buffer, and adding the light and heavy chain

to the diluted refolding solution.

3.1 Procedure of refolding the MHC complex

3.1.1 Materials for preparing refolding buffer (200ml):

100 mM Tris-HCl pH8.0.
400 mM L-arginine
2 mM EDTA-Na
Resulting solution should be cooled to 4°C, and then add the following agents:
5 mM glutathione [GSH]
0.5mML-Glutathione oxidized [GSSG].
At 4°C, the solution should be stirred for 10-20 minutes before adding the inclusion bodies and peptides.

3.1.2 Inject MHC heavy chain, light chain (β2m) to the refolding solution

Order of reagents added to the refolding solution:
a.Light chain (β2m); b. peptide; c. heavy chain (HC).
Molar ratio of β2m: HC=1:1.
1)Injection of light chain
Using a needle from 1mL syringe, inject light chain close to the rotating stir bar to obtain fast and efficient dilution. β2m refolds relatively easy and remains stable in the absence of HC.
2)Injection of peptides
After the β2m has been dissolved in refolding solution, add 2.5 mg of peptides to DMSO and inject into the refolding solution quickly. Slowly stir for 10-20min before adding the HC.
3)Injection of HC:
Using a needle from 1mL syringe, inject HC close to the rotating stir bar. HC is very unstable, thus the order of adding each component is important. Place refolding at 4°C for 8-10h before adding the second aliquot of HC. After waiting period, add the remaining HC in the same manner as above, and incubate for another 8-10 hours.

4 Protein purification

The resulting solution of refolded protein would be very dilute, and thus it has to be purified through methods like ultrafiltration and gel filtration chromatography. Gel filtration chromatography relies on the size, shape, and mass of the protein to separate and purify the protein solution. The filling in the filtering column is a specific inert permeable structure such as proteoglycan. They act as a molecular sieve where smaller molecules can enter the inside compartment with a longer time to flow down into collecting tubes while larger molecules are separated on the outside and can flow to collecting tubes quicker. The strengths of this method are that the gel/filling used is inert, without charge, and low affinity so it can be conducted in a wide range of temperatures and conditions. It is especially useful in purifying large molecules. Continuing from refolding the protein, the following is the procedure of purifying the refolded protein.

4.1 Procedure of protein purification

First, ultrafiltration in a pressurized chamber with 10 000 MMCO membrane is used for concentrating the refolding proteins. Add exchange buffer (20mM Tris-HCl, 50mM NaCl, pH8.0) to the chamber and concentrate to a final volume of 30-50ml.
1)Transfer resulting refolding solution to a centrifuge tube, spin at 12000rpm/min (15min; 4oC) to remove precipitates
2)Carefully transfer supernatant and further concentrate to a final concentration of ~1ml.
3)12000rpm, 10-20min. Transfer the supernatant to a sterile tube and purify the protein with gel filtration
4)Collect the MHC complex peak and concentrate to a final volume of <700ul.

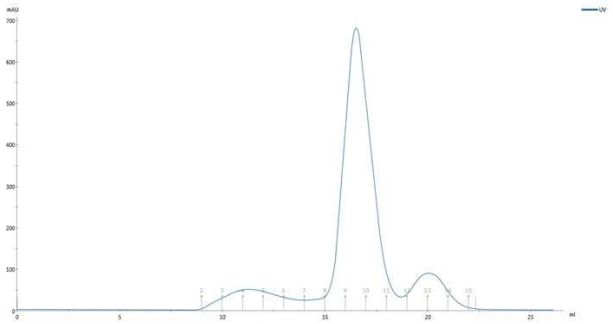
4.1.1 Reagents

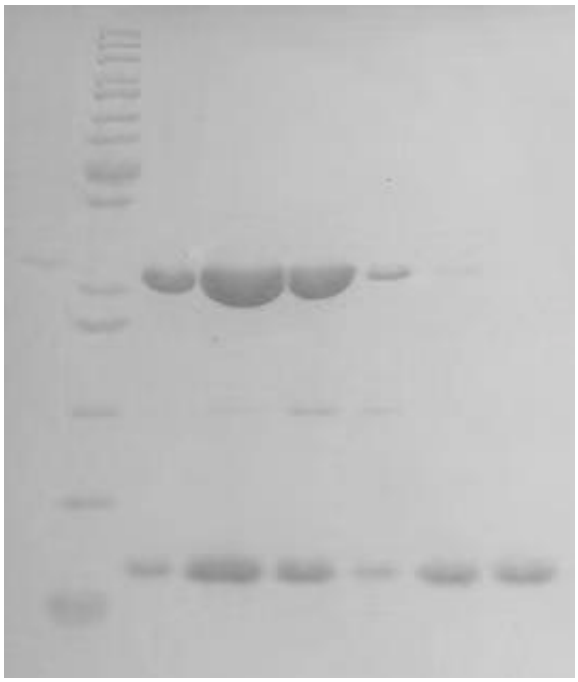
1.1×PBS, pH7.4	1000ml
NaCl	8g
KCl	0.2g
Na2HPO4 .12H2O	3.63g
KH2PO4	0.24g

2. Washing buffer
0.5% Triton-100
50mM Tris pH8.0 (Stock solution 1M)
300mM NaCl (Stock solution 5M)
10mM EDTA (Stock solution 0.5M)
10mM DTT (Stock solution 1M, add before use)
3. Resuspension buffer
50mM Tris pH8.0
100mM NaCl
10mM EDTA
10mM DTT (add before use)
4. Dissolution buffer
6M Gua-HCl(or 8M Urea)
10% Glycerol
50mM Tris pH8.0
100mM NaCl

10mM EDTA

The following is the chart generated by the gel filtration chromatography. The closer to the peak, the more concentrated the purified protein. Therefore according to the chart, the MHC complex peaks at collecting tubes 8, 9, and 10. This information is also validated by testing the concentration of the protein in a fixed volume of solution. Shown in the image following the chart, the 9th collecting tube has the most protein.





5 MHC tetramer

The soluble form of single MHC molecules have low affinity to T cell receptors and stop bonding quickly. However, multiple molecules of MHC can bind to multiple specific receptors on T cells slowing down the process of dissociation. Using this principal, the concept and method of building MHC tetramers is developed. The 15 amino acid long bio A substrate (BSP) is added to the carboxyl end of HLA-A2 heavy chain to form a fusion protein. When mixed together with specific antigenic short peptides and folded into the correct conformation, the tetramer becomes a pMHC complex. Through this method, not only is the dissociation slowed but detection of bonding is amplified as single monomers bonding is harder to detect than 4 monomers bonding together. Biotin tags can also be labeled onto the peptide leaving fluorescein labeled streptavidin binds. This is useful because when the tetramers are associated with TCR, it can be detected quantitively through flow cytometry. Below is the method to prepare biotinylated MHC tetramers.

5.1 Preparation of biotinylated MHC protein

MHCs used for biotinylation are constructed with a biotinylation site tag on the C-terminus of the heavy chain. Because the biotin-tag were easy to be cleaved, the addition of protease inhibitors were critical for the efficiency of biotinylation.

5.1.1 Protease inhibitors for 200ml refolding solution:

2ml PMSF (Stock 100mM),
100μL pepstatin (stock 2mg/ml),
100μL leupeptin (stock 2mg/ml)).

Adding protease inhibitors during buffer exchange and MHC complex sampling or concentration is also preferred.

5.1.1.1 Biotinylation reaction

Material and Reagents used in biotinylation:

BirA enzyme: 3mg/ml.
Solution A: 0.5M bicine, pH8.3
Solution B: 100mM ATP, 100mM MgOAc, 200mM biotin.
Extra d-biotin (500μM biotin)

Calculate the yield of the MHC complex (1-2mg/ml) from a 200ml refolding solution. To form a 1ml reaction volume, add:

100μL solution A
100μL solution B
100μL extra d-biotin
20μL BirA enzyme (60μg)
0.5μL pepstatin
0.5μL leupeptin

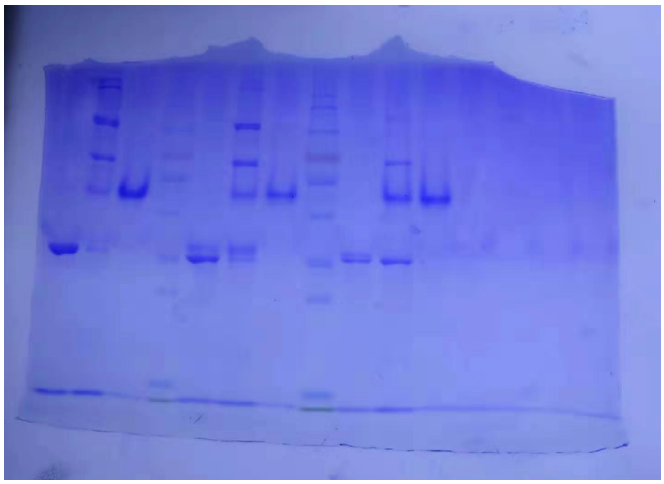
Note: do not add any EDTA to the biotinylation reaction.

Incubate overnight at 4oC.

5.2 SDS-PAGE

In order to test the efficiency of the biotinylation,

SDS-PAGE can be used. SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) is a variant of gel electrophoresis where charged molecules in a solution is separated based on their molecular masses in an electric field. It uses sodium dodecyl sulfate (SDS) to identify and isolate proteins (1.4 grams of SDS binds to 1 gram of protein – 1 SDS molecule per 2 amino acids), and it is often used to separate proteins with molecular mass between 5-250 kDA. The SDS covered protein travels through a medium/matrix usually made of polyacrylamide-based discontinuous gel. As a constant electric field is applied, the proteins travel towards the anode with different speeds based on their mass. Specific to the MHC tetramers, the biotinylated MHC complex will have a higher mass than single monomers unbonded, and thus a difference would be seen on the medium. Below is the result of biotinylated MHC shift assay.



5.3 Analysis

The column 4 and 8 shown above are the markers, and the diagram shows 3 samples being tested with columns 1, 2, 3 as one group, 5, 6, 7 as the second group, and 9, 10, 11 as the last. Columns 1, 5, 9 has only MHC added, columns 2, 6, 10 has the MHC with streptavidin while columns 3, 7, and 11 has only streptavidin. So to compare the efficiency of generating MHC streptavidin complexes between the 3 groups columns 2, 6, 10 would be compared with each other. When MHC streptavidin complexes form, they increase in molecular and thus would shift to a higher position than the single monomers. Therefore, to compare efficiency of the previous procedure the amount of protein that shifts should be compared. Looking at the diagram above, sample 1 and 2 demonstrates good amounts of shifting indicating there is high efficiency in producing the tetramers. Sample 3, however, shows inadequate shifting meaning the complexes produced are not of high quality (i.e. instead of 4 MHC bonded together maybe only 2 attached).

6 Heat shock bacterial transformation

Discovered first by Griffith from his experiment with Streptococcus pneumoniae, bacterial cells have the ability to take up free extracellular genetic material from the environment through a process called transformation. Transformation does not require live donor cells, but persistent DNA in the environment. When the extracellular DNA is taken up, if it's different from the recipient's bacterial DNA it may be degraded by nuclease, while if it's similar to the bacterial DNA it can be integrated into the chromosome or coexist as a plasmid. This ability has allowed bacteria species to increase genetic variation, which would help them to survive in a rapidly changing environment favoring different traits. However, not all types of cells have the ability to undergo transformation, and those with the ability are called competent cells. The natural competency vary across genera, and there are various factor influencing it. Competent cells can also be acquired through methods like chemical induction with heat shock and electroporation. Since bacteria replicates easily and rapidly, transformation can be used to amplify the desired DNA, proteins, enzymes etc. Below is the materials and procedure for a typical transformation experiment.

Materials

LB growth medium without antibiotic, plate with antibiotic, competent bacteria, shaker, laminar flow bench, glass cell spreader

6.1 Procedure for transformation experiment

1.Add 2µl of plasmid (or 10µl of ligation product) to EP tube with 100µl of competent bacteria, ice bath for 30min

2.Heat shock mixture in water bath at 42˚C for 90s

Note: The rapid change in temperature result in a higher membrane permeability (protein channels and other membrane pores expand upon high temperature). This allows the extracellular plasmids to enter the cell. However, if shocked for too long, the heat will denature proteins in the cell and lead to cell death so 90s is optimum.

3.Immediately after heat shock, place EP tube on ice for 2 minutes

Note: This helps membrane pores to close locking DNA molecules inside.

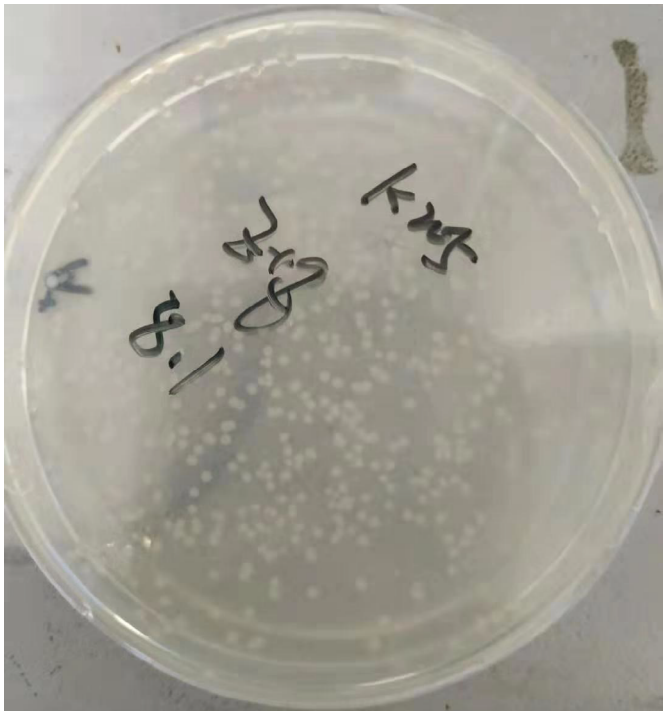
4.Add 800µl of LB growth medium with mixture EP tube to conical flask. If plasmids are transformed, shake at 200 rpm for 15-20 min at 37 °C. If ligation products are transformed, shake at same conditions but for 45 minutes to 1 hour.

Note: Bacteria cells are recovering in the growth medium while being shook.

5.If plasmids were transformed, coat 100 µl of mixture onto plate with antibiotics; if ligation product is transformed, centrifuge at 3000 rpm for 2 min, discard 700-800 µl of the medium, resuspend the rest, and plate 100 µl.

6.Observe the growth of the plate after 12-16h

The following is the result of bacterial growth on petri dish.



Plasmid Extraction

After transforming the bacteria through the previous method, the target plasmid DNA need to be extracted and purified from the bacteria. To collect the plasmid DNA, the cell has to be lysed first and then purified. There are multiples ways developed to lyse the cell, but the most common one is alkaline lysis. To begin, the bacteria in growth medium is isolated by centrifugation and the precipitate is collected. It is then suspended in solution with RNase to denature RNA as well as avoid bacteria clumps which would make the following steps harder. The bacteria in solution then given a detergent and a base such as sodium hydroxide. The detergent dissolves the bacterial cell membrane, and the increase in alkalinity denatures the DNA and proteins. The increase in alkalinity is equivalent to increasing amount of hydroxide ions, and due to their negative charge they pull off positive hydrogen ions on proteins and chromosomal DNA. This disturbs the hydrogen bonding holding the structure together and therefore irreversibly denatures DNA and proteins. The plasmids are also denatured, but the two strands of helix remain together. Upon restoration of neutral pH, the plasmids will reanneal. The irreversibly denatured DNA and protein will aggregate and through collecting the supernatant after centrifugation, the plasmid can be extracted. To explicitly purify the plasmid, ethanol is added to the solution causing plasmid DNA to precipitate out of solution. The soluble fraction contains other biomolecules and is removed.

Procedure

1.Column balancing: Add 2.5 mL of buffer BL into absorption column (add absorption column to 50 mL collecting tube), centrifuge for 2 minutes at 8,000 rpm. Discard waste fluid in collecting tube and add absorption column back to collecting tube

2.Centrifuge100 mL of overnight bacterial solution at room temperature, 8,000 rpm, for 3 min. Discard supernatant.

3.Add RNase A to solution P1, and add bacteria precipitate into 8 mL of solution P1 with RNase A. Make sure to stir fully to avoid bacteria clumps.

4.Add 8 mL of P2 into solution. Gently flip/rotate container 6-8 times to avoid contamination of target DNA. Set in room temperature for 5 min.

5.Add 8 mL of P4 into solution. Gently flip/rotate container 6-8 times to mix thoroughly. White precipitate should form and set at room temperature for 10 min. Centrifuge for 5-10 min at 8,000 rpm. Collect supernatant and filter with CS1. Collect filtrate in 50 mL collecting tube.

6.Add 0.3 times the volume of isopropanol alcohol, mix thoroughly, and filter with absorption column CP6 in 50 mL collecting tube. Centrifuge for 2 minutes at 8,000 rpm. Repeat this step twice to filter all solution.

7.Add 10 mL of washing solution PW into absorption column CP6, centrifuge for 2 minutes at 8,000 rpm. Repeat this step and wash out filtrate with 1-2 washing solution TB and centrifugation at 8,000 rpm for 2 min. Store at -20˚C.

7 Summary

Overall, I gained a deeper insight into the field of cancer immunotherapy such as its history, key principles and mechanism, and current advancements. I also focused on the specific scientific methods used in laboratories such as expressing proteins, SDS-PAGE, transformation, protein refolding etc. I learned the procedures and materials in doing such experiments, but most importantly their underlying principles. By learning the principles behind the protocols, it is possible to enhance them and do experiments quicker and with higher efficiency. Lastly, a very important aspect was the underlying rules in working in a lab such as putting things back after use, cleansing apparatus for the next user, and properly labelling things. Even though these are small details, they are still very important and even the smallest mistake can result in hazard. To conclude, this experience has provided me a valuable opportunity to work in a laboratory environment, and learn to a greater depth topics surrounding molecular biology.

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