Developing a color-coded platform for detection of antigen-specific T-cells

SUMMARY

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by

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Table of Contents

List of Illustrations:

List of tables:

Summary

[based and adapted from Final Report and Master-Thesis: *Developing a color-coded platform for detection of antigen-specific T-cells* by Henna Ahmad]

The goal of this research was to create a multi color-coded MHC-platform that can detect antigen-specific T cells with a higher sensitivity. In recent years, fluorescent labeled major histocompatibility complex tetramers have emerged as a significant and widely utilized technique for the study of T cells via flow cytometry. For the detection of specific biomarkers in a blood sample that have a high binding affinity, classical tetramers, which are biotinylated and streptavidin-tetramerized peptide MHC, maintain stable binding to multiple TCR. For T cell receptors, various antigens have different levels of affinity. Therefore, the Rashidian laboratory strives to be able to identify as many of them as possible. This MHC-tetramer is produced by following the Tetramer assay. First MHC heavy chains expressing a bacterial BirA-recognition site are synthesized. Then MHC heavy chains with a BirA-recognition site for bacteria are made. The next step is to fold the MHC heavy chain with 2-microglobulin and a synthetic peptide. The BirA enzyme is then added to biotinylate the complex, adding one biotin molecule to each MHC monomer. Four MHC monomers are combined to form a tetramer with streptavidin molecule, which has four biotin binding sites. (1) For Flow cytometer analysis, this streptavidin molecule is conjugated to a fluorochrome.(2) The most effective method for locating particular biomarkers with a high affinity for binding in a blood sample is using tetramers. This can be used to make treatments work better.(3)To address the low-affinity T cell receptor problem, the plan is to develop a multimer platform. So, we put two tetramers together to make the octamer a larger platform. First it was important to produce the different tetramers with the different subunits, in this project we used modified streptavidin H2-SA-LPLP-LAETG and H8-SA-LPESG. After producing these inclusion bodies, they were refolded in a ratio to 1:7. The histidine-tagged protein purification strategy is used to separate the homoteramers and heterotetramers from the mixture of refolded products. The method known as immobilized-metal affinity chromatography, or IMAC, has been around for a long time and is well established. It is common knowledge that transition metal ions, such as Cu2+, Co2+, Ni2+, and Zn2+, have a strong attraction to the amino acid histidine. Although this is not the only possibility, it is most likely that the metal ion will bind to histidine residues n and n+2. The His-tag structure is

adaptable, so a binding pattern involving the histidine residues n+1, n+3, or n+4 is also possible. His-tagged proteins are frequently purified using this technique. After the proteins are bound to the IMAC column, they can be removed using a solution that contains imidazole. The protein will detach because imidazole will compete with histidine for binding nickel on the two accessible valencies. The tetramer mixture was separated on the IMAC using an optimised protocol for gradient imidazole.(4) Because H2LPLP-SA-LAETG homotetramers do not bind to the IMAC with high affinity, they are primarily found in the wash containing 60 mM imidazole. If eluted with 140 mM imidazole,a specific heterotetramer made up of one subunit H8-SA-LPESG and three subunits H2LPLP-SA-LAETG. By taking a sample and heating it, this was confirmed by SDS-PAGE, because the upper band was lighter than the lower band. The next gradient is 180 mM imidazole, which reveals two equal bands, which correspond to the heterotetramer composed of two subunits of each protein. The heterotetramer with three H8-SA-LPESG subunits and one H2LPLP-SA-LAETG, is washed out with 200 mM imidazole. The homotetrmaer with just H8-SA-LPESG subunits is received with just 250 mM imidazole. In conclusion, the homotetramers of H2LPLP-SA-LAETG with very low affinity for the IMAC can be washed away with 20 to 50 mM imidazole, whereas the tetramers with more than one H8-SA-Flag-LPESG subunit bind more strongly to the column and are eluted at higher imidazole concentrations. With the different Tetramers and subunits, we used the orthogonality of eSrtA(2A-9) and eSrtA(4S9) to make two site-specific changes on the same protein that has two different motifs. Proteins were made to have the sortase recognition motif (like LAETG or LPESG) where changes are wanted. The cognate sortase makes a cut between the threonine and glycine residues of the protein after it has been recognized, allowing a chosen substrate to attach. With the different motifs, different fluorophores, and antibodies or also in our case other tetramer were attached. There are numerous antigen-specific T-cells, so the ability to simultaneously locate multiple antigen-specific T-cells using microscopy and flow cytometer is used. On the other hand, due to the way that technology operates, it is not possible to locate all the cell markers at the same time. During the flow cytometer experiments, there were always some complications, but after attempting the procedure and making some adjustments to it, the desired outcomes were attained. To wrap up the project, the idea was to make a library of tetramer with all possible combinations of 3 colors and then add MHC I and run the samples on Flow

cytometer, as proof of concept. The library of the platforms is shown in Table 1 and Table 2 and illustrated in Figure 1.

Table 1 library table of the 6 tetramers with the corresponding fluorophores

Tetramer	Fluorophores
4 H2LPLP-SA-LAETG	Alexa 488
4H2LPLP-SA-LAETG	Alexa 647
4 H2LPLP-SA-LAETG	Alexa 405
2 H2LPLP-SA-LAETGx2 H8-SA-LPESG	Alexa 488 x Alexa 647
2 H2LPLP-SA-LAETGx2 H8-SA-LPESG	Alexa 405 x Alexa 647
2 H2LPLP-SA-LAETGx2 H8-SA-LPESG	Alexa 488 x Alexa 405

Table 2 library table of the 1 octamer with the corresponding fluorophores

Octamer	Flurophores
(3 H2LPLP-SA-LAETGx1 H8-SA-LPESG)x(1 H2LPLP-SA-LAETGx3 H8-SA-LPESG	Alexa 647 x FITC x Alexa 405

Figure 1 All 6 tetramers and 1 octamer illustrated with their corresponding fluorophores

As illustrated in Figure 1, all platforms with the fluorophores were made and purified. Just the staining part with MHC I (SIINFEKL-H2Kb-biotinylated) for the flow cytometer was failing miserably. The MHC I did not show any signs of binding to any of the tetramer's sides. Therefore, following the incubation of the tetramers of the library with another nanobody, the samples were put through a flow cytometer for examination and comparison. Because it did work, we can deduce that there was an error with the MHC I (SIINFEKL-H2Kb-biotinylated). There are numerous reasons why it did not work, including the fact that it is extremely sensitive, freezing and thawing were effective, the biotinylation was not successful, and also the refolding

could be miscalculated. All in all, the Color-coded platform is working with different antibodies and different fluorophores on microscopy and on flow cytometer. It requires further experimentation as well as optimization, but the base is already given. There is a tremendous amount of potential in terms of the applications that can be made with the platform and the ways in which it can be further developed.

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