UNDERSTANDING THE WORLD OF MHC I MULTIMERS

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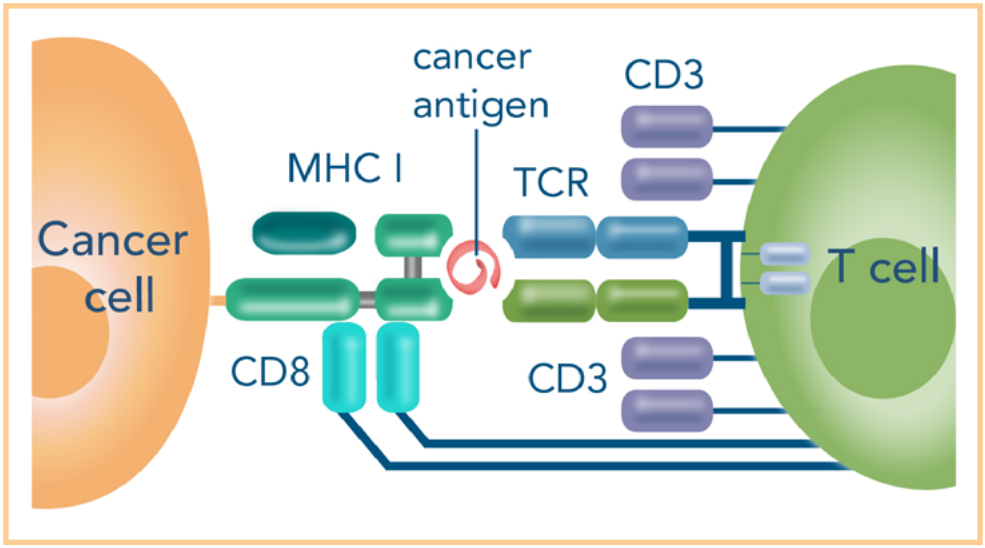
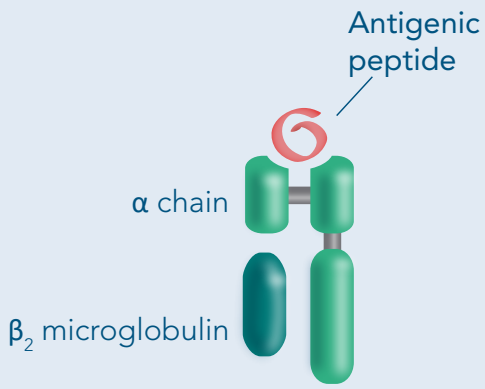
1. MHC MULTIMERS – A REVOLUTION IN IMMUNOLOGY RESEARCH

Immune cells play a crucial role in maintaining body homeostasis, which includes eliminating cancerous, damaged and infected cells.

Cytotoxic T lymphocytes (CTLs) are one of the main effector cells in this process. They express T cell receptors (TCRs) that specifically recognize altered cells. CTLs are identified by CD8 co-receptors. These co-receptors bind to the ligands of TCRs, which are major histocompatibility complex (MHC) class I molecules. They are expressed on the surface of virtually all nucleated cells and bind peptides from proteins that are processed inside the cell. Therefore, the body's own, but also viral peptides or peptides derived from mutated proteins are displayed on the outside of a cell. If these peptides presented by MHC class I molecules are recognized as foreign, a CTL with a receptor that matches the peptide will try to destroy the cell and thereby eliminate an infection or prevent cancer development.



Every TCR recognizes a unique peptide-MHC (pMHC) combination. To cover the recognition of as many different peptides as possible, the amount of different TCRs needs to be huge. This broad range of TCR variants is achieved on gene expression level. Rearrangement of three TCR gene segments, random deletion or addition of nucleotides as well as pairing of the heterodimeric TCR can theoretically yield as many as 10^{20} unique TCRs[1].



MHC I STRUCTURE

Class I MHC molecules are made up of a polymorphic heavy α-chain associated with an invariant light chain beta-2 microglobulin (β2m).

To decipher and really understand immune responses it is necessary to figure out which kind of T cells are involved and which TCRs are dominant. This knowledge can answer basic research questions but is also crucial for vaccine development. Tracking T cell populations over the duration of a virus infection or after the application of a vaccine can give important insights into an ongoing immune response.

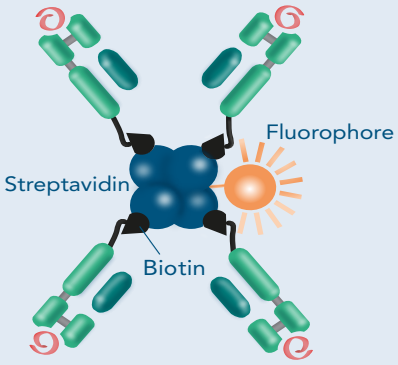
A possible analysis method to investigate antigen-specific T cells is Enzyme-linked-immuno-Spot (ELISpot). It is a functional assay that detects the number of cytokine secreting cells in a sample. Although it is a sensitive cellular assay, it does not have a high resolution, as only cytokine-producing immune responses can be measured. Another way is to use the natural ligand of TCRs, the peptide-loaded MHC molecules, coupled to a detector molecule to understand immune responses on a high-resolution level. Depending on the detector molecule, this approach could also be used to isolate single antigen-specific T cells. However, the affinity of wild type TCRs to pMHC I molecules is naturally very low with a dissociation constant (K_D) ranging from 1 µM to 100 µM. This is up to 10.000x lower than some high affinity antibodies to their target and makes their direct usage for antigen-specific T cell analysis difficult and ineffective.

The solution for using pMHC I molecules as a tool for antigen-specific T cell staining and analysis was found by Altman and colleagues in 1996: They complexed several pMHC I molecules together on a fluorescently conjugated backbone to increase the total binding strength that enabled direct staining of HIV-specific T cells. These experiments represent the proof-of-principle that multimerization of low affinity pMHC I monomers increases the avidity of the complex sufficiently to stably bind to cells. This MHC multimer approach was a breakthrough in antigen-specific T cell research and is still further developed up until today[2].

2. WHAT TYPE OF MHC I MULTIMERS EXIST?

TETRAMERS

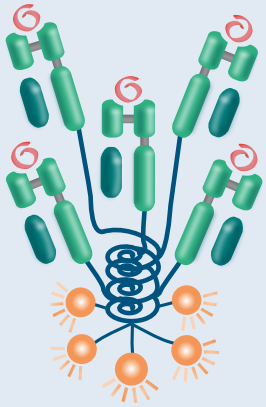
The first MHC I multimers, developed in 1996, were called MHC I tetramers. This name resulted from the four pMHC complexes that could be bound to the chosen backbone: avidin. Avidin is a tetrameric glycoprotein that is found in the egg white of e.g. chicken and binds to biotin with a very high affinity. It has four biotin binding pockets and consequently, four biotinylated pMHC I molecules can be bound. Alternatively, streptavidin can be used as a backbone for MHC tetramers. It is a non-glycosylated analogue of avidin that is derived from

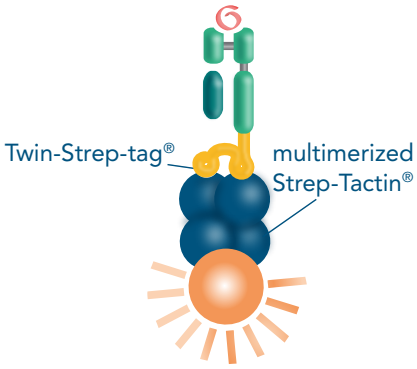


Streptomyces avidinii and shows less unspecific binding. The strong binding of avidin or streptavidin to biotinylated MHC molecules leads to the formation of stable multimeric complexes. The addition of a fluorochrome to the backbone allows these complexes to be used for antigen-specific T cell staining. Since the development of tetramers, different MHC I multimer technologies aiming at optimizing e.g. detection and isolation of antigen-specific T cells emerged. The most frequently used commercially available MHC I multimers next to tetramers include MHC pentamers, MHC streptamers, and MHC dextramers. In addition, there are also other types of multimers for example NTAmers.

PENTAMERS

In 2000, so called MHC I pentamers were developed. They contain five MHC class I complexes that are multimerized by a self-assembling coiled-coil domain. All five pMHC complexes face in the same direction resulting in a very high avidity interaction with the TCR. It also comprises up to five fluorescent or biotin tags for bright and efficient labelling compared to tetramer staining.



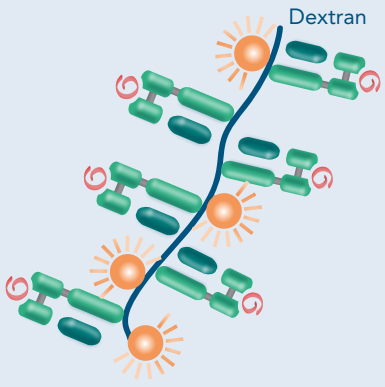


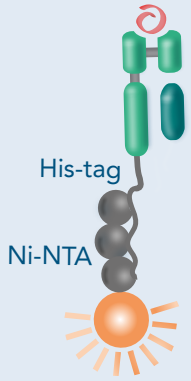
STREPTAMERS

MHC I streptamers, first published in 2002, are based on the Strep-tag® technology. In this approach, pMHC I molecules are fused to a Twin-Strep-tag®, which is a short amino acid sequence that binds to a streptavidin variant called Strep-Tactin®. Biotin causes the release of pMHC I molecules from the Strep-Tactin® backbone, because it has a much higher affinity to Strep-Tactin® than the Strep-tagged pMHC I molecules[3]. MHC I Streptamer® complexes are therefore the first commercially available reversible multimers.

DEXTRAMERS

A few years after MHC streptamers were first used in experiments, MHC dextramers were developed. They got their name from the backbone used: dextran. Several pMHC molecules and several fluorescent conjugates are bound to a dextran fiber, which allows the detection of very low affinity antigen-specific T cells that cannot be identified by other technologies.





NTAmers

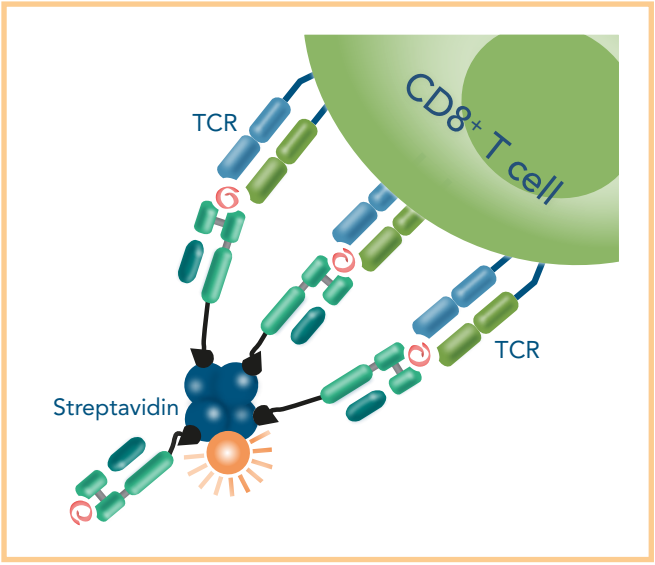
Although not commercially available, NTAmers are a second option for reversible staining of antigen-specific T cells. In this approach, pMHC I complexes are multimerized via a oligohistidine-tag (His-tag) on Ni^{2+}- nitrilotriacetic acid (NTA) moieties. Addition of imidazole disrupts the binding and dissolves the multimerization, similar to biotin in the MHC I Streptamer® technology.

3. WHAT ARE THE MAIN DIFFERENCES OF THE MHC I MULTIMERS?

Except for the underlying principles of forming the multimeric complex, the MHC multimers differ in other aspects.

VALENCY & AMOUNT OF pMHC MOLECULES THAT CAN BIND TO A CELL

The valency, i.e. the amount of pMHCs that can be bound to a backbone, is one of the factors that distinguishes one MHC multimer from the other. However, here should be considered that not only the backbone of choice plays a role in determining how many pMHC molecules potentially can bind to a cell, but also the chosen fluorophores and the orientation of pMHC molecules on the backbone. For example, to enable fluorescent staining of cells with MHC tetramers, fluorochromes phycoerythrin (PE) or allophycocyanin (APC) are frequently conjugated to the streptavidin backbone. PE and APC are relatively large (around 240 kDa and 104 kDa, respectively) and the process of chemically crosslinking them to streptavidin (around 53 kDa) leads to the coupling of more than one backbone to one fluorochrome. Consequently, the resulting staining complexes are not just tetramers anymore,

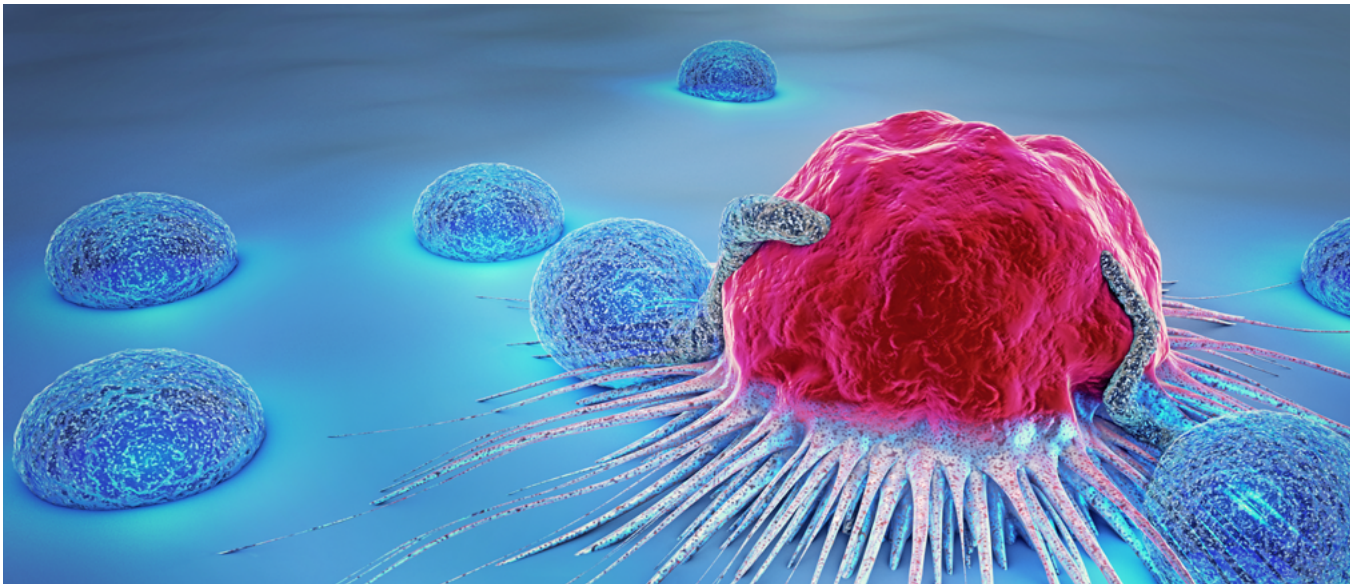


but rather oligomers. "True"-tetrameric MHC multimers can only be produced by e.g. using "tetra-grade" streptavidin-PE, or streptavidin coupled to smaller fluorochromes, such as Cy5. Similar to streptavidin-based multimer backbones, the multimer formation caused by crosslinking the fluorophores PE and APC with Strep-Tactin® also affects the actual valency of MHC I streptamers, leading to larger multi-MHC complexes.

MHC I MULTIMER	TECHNOLOGY / PRINCIPLE	REVERSIBLE
Tetramer	Biotinylated pMHC I molecules bound to streptavidin	No
Pentamer	pMHC I headgroups connected via linkers to a coiled-coil multimerization domain	No
Dextramer®	pMHC I molecules attached to a dextran backbone	No
NTAmer	pMHC I molecules with tandem His-tag bound to Ni^{2+} Nitrilotriacetic acid (NTA) moieties	Yes
Streptamer®	pMHC I molecules with Twin-Strep-tag® bound to Strep-Tactin®	Yes

The orientation of binding sites on streptavidin and Strep-Tactin® molecules also plays a role in the binding to a cell. Due to the rigid tetrahedral configuration, it is likely that only a fraction out of the available MHC molecules binds simultaneously to surface TCRs.

In contrast to MHC tetramers and streptamers, pentamers have a well-defined size. The five pMHC molecules also face in the same direction, increasing the chance that all five molecules can bind to TCRs on the cell surface. Dextramers have several pMHC molecules bound to dextran fibers. Due to the flexibility and length of the fibers, a high amount of pMHCs can bind to TCRs on the cell surface at the same time. Although the exact number of pMHC molecules is not exactly defined, it is likely that more TCRs are bound compared to MHC tetramers, pentamers or streptamers. The size of NTAmers is also variable, depending on how many NTA moieties are linked together and how many of these NTA moieties are conjugated to a fluorophore.

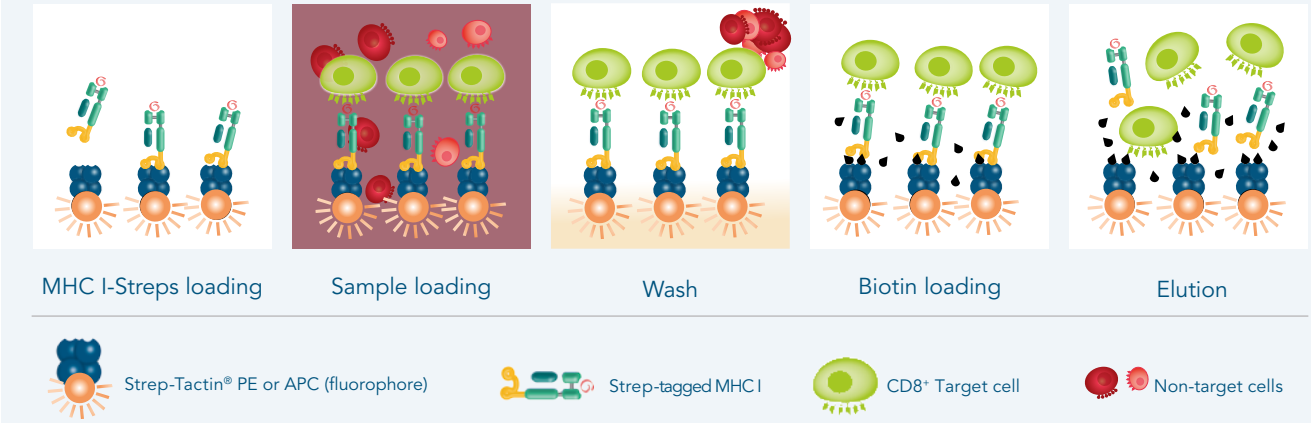


REVERSIBILITY

One feature that clearly distinguishes the currently available MHC I multimers is reversibility. Once the pMHC molecules are multimerized and added to cells, they stably bind to the TCRs on the cell surface due to their enhanced avidity. If the multimerization is not disrupted, this binding is virtually irreversible. The binding of streptavidin to biotin is one of the strongest non-covalent biological bindings known and the underlying principle of MHC tetramers. This makes them very stable, but at the same time hard to remove from the cells. Pentamers and dextramers also efficiently, but irreversibly, multimerize MHCs to enhance avidity and thereby attach stably to the cell surface. MHC I streptamers are multimerized by adding twin-strep-tagged MHC I molecules to Strep-Tactin®-fluorochrome conjugates. In contrast to other multimers, the multimerization of MHC I Streptamer® complexes is reversible. The binding of the Twin-Strep-tag® to Strep-Tactin® can be disrupted by adding biotin. This results in a loss of avidity and the dissociation of MHC I monomers from the TCRs due to their low affinity[3]. NTAmers work in a similar way as the multimerization can be reversed by imidazole, which disrupts the binding of the His-tag to N^{2+}-NTA4.

Disadvantages of irreversible binding:

- Internalization of staining complex
- Cell apoptosis
- Impaired proliferative capacity
- Altered cell functionality
- TCR-ligand dissociation assay not possible



MHC I Streptamer® Cell Staining Workflow

THE MHC I STREPTAMER® CELL STAINING WORKFLOW

includes the following steps: MHC I-Streps have to be loaded on fluorescently conjugated Strep-Tactin®. The resulting complexes are used to label cells for subsequent flow cytometric analysis and/or cell sorting. After washing away all unwanted cells, biotin addition releases MHC I-Streps from fluorescent Strep-Tactin® and causes the spontaneous dissociation of MHC I-Streps from the cell surface (elution).

4. WHY IS REVERSIBILITY AN IMPORTANT FEATURE OF MHC I MULTIMERS?

Studying antigen-specific T cells beyond simply determining their frequency requires sorting or isolation, and possibly even *in vitro* or *in vivo* experiments. For further downstream approaches it is crucial that the cells keep their original phenotype as closely as possible. However, using MHC I multimers that remain attached to the cells after isolation can lead to internalization of the staining complexes and to apoptosis[5,6] when culturing cells at 37 °C. This in turn affects the proliferative capacity as well as *in vitro* and *in vivo* functionality[3], which complicates investigating antigen-specific immune responses of T cells. Therefore, all labeling reagents that are required for sorting or isolation of these cells should be removed prior to downstream experiments, which makes reversibility an important feature of MHC I multimers.

Furthermore, reversible pMHC multimer constructs can also be used to characterize T cells and their T cell receptors. The functional profile of a T cell is in large parts imprinted in the TCR, which determines the binding strength to their cognate pMHC. The properties of the TCR:pMHC interaction guides the T cell, as it is the driving force in thymic selection, T cell differentiation and consequently its response upon encountering pathogenic peptides. A TCR-ligand dissociation assay allows to calculate the half-life of TCR:pMHC complexes *in situ*, thus under consideration of any additional influence from co-receptors and surface molecules. This calculation has been shown to be predictive of *in vivo* functionality. In the experimental setup, reversible pMHC monomers themselves are labeled with a fluorophore allowing to track their dissociation from TCRs on living T cells over time. Depending on the binding strength this dissociation is different and can be measured microscopically or using a flow cytometer[7,8].

WHAT ARE POSSIBLE APPLICATIONS FOR REVERSIBLE MHC I STREPTAMER® MULTIMERS PROVIDED BY IBA LIFESCIENCES?

Like other MHC I multimer approaches, MHC I Streptamer® reagents are well suited for staining antigen-specific T cells for flow cytometric analysis. Cell populations that have a low frequency are reliably detected and can be sorted for further experiments if needed.

Since cells are completely label-free after the isolation using the MHC I Streptamer® approach, they are suitable for various applications. Cells are still functionally active and can be expanded *in vitro* followed by for example adoptive transfer experiments in mouse models.

This way, testing efficient protection of selected antigen-specific T cells from infections or cancer can be tested in an *in vivo* model. In addition, MHC I streptamers can be used to isolate and analyze TCRs recognizing e.g. cancer antigens from human samples.

Identifying well-functioning TCRs is an important step in some therapeutic approaches as their transfer into activated T cells and subsequent injection into patients is a promising clinical treatment option. For more information on this approach, watch this webinar on YouTube:

Click here

5. DRAWBACKS & PROSPECTS OF MHC MULTIMERS

MHC I multimers are a valuable tool in researching antigen-specific T cells and understanding adaptive immune responses for example in autoimmune diseases, infections, or cancer. Nevertheless, they have some common drawbacks: their production is very time-consuming and labor-intensive, as many steps are involved such as recombinant expression of proteins, purification and re-folding of the heavy and light chains with the peptide of choice. In the end, this leads to the production of only one MHC I allele with one type of peptide, which can be a limitation considering the number of existing antigens and MHC I alleles (> 22.000). In addition to potential batch-to-batch variation, this also generates high costs and reduces flexibility.

To address these drawbacks of MHC multimers production, some adaptations of the procedure were developed to make them more cost efficient and broaden their applicability. As the re-folding in the presence of a peptide is required to generate a stable MHC I complex, the production of "empty" MHC I molecules that can be loaded with a pepti-

de of choice afterwards can be a way to quickly generate multiple pMHC complexes. Removing the peptide after the re-folding and loading the MHC molecule with a peptide of choice can work, if the initial production was done for example with a labile peptide that can be degraded by UV light [9], chemical factors or elevated temperatures. Another option that was shown to stabilize MHC class I production is the use of dipeptides during the re-folding process instead of a full-length peptide. Dipeptides also support the binding of high affinity peptides of choice afterwards compared to "empty"-folded molecules. In this approach, the dipeptide has to be chosen carefully to fit the chosen MHC I allele[10,11]. The different peptide exchange technologies can broaden the applicability of MHC multimers but also still have limitations.

As another way of improving the versatile applicability of MHC multimers, the existing technologies can also be modified. For example, fusing a second tag to MHC I streptamers was explored as an option. In addition to the Strep-tag® that permits reversible multimerization, a Tub-tag was expressed. A Tub-tag is a short hydrophilic, unstructured sequence that is recognized by an enzyme called tubulin tyrosine ligase (TTL). This enzyme can be utilized to flexibly functionalize the pMHC complexes. For example, using this chemoenzymatic system to conjugate MHC I monomers to a fluorescent dye of choice allows for monitoring the dissociation from the cell surface after disrupting the multimeric complex with biotin. By having the possibility to functionalize one MHC I multimer in multiple ways, the production cost, and the batch-to-batch variability can be reduced[12].

SUMMARY

In contrast to high affinity antibodies, the binding of a peptide-loaded MHC I molecule to its cognate TCR is not stable enough to enable the direct use of this interaction for cell staining and analysis. The development of MHC multimers solved this limitation. Several pMHC molecules are bound to a fluorescently conjugated backbone to increase the total binding strength, thereby permitting direct staining and analysis of antigen-specific T cells. Currently used multimers include MHC tetramers, pentamers, dextramers, streptamers and NTAmers. They differ in the underlying mechanisms of multimerization and consequently in the amount of MHC molecules that can bind to a cell, but share the ability to precisely illuminate even tiny antigen-specific T cell populations.

The most important feature that distinguishes the MHC multimers is the reversibility of the staining i.e., the possibility to disrupt the multimerization to cause the dissociation of pMHC molecules from the cell surface. The optimization of different MHC multimer features is still ongoing, focusing on making the production procedures more cost effective and the application of MHC multimers more flexible.

If you are already planning your experiments using **MHC Streptamers**, but still have some questions, just drop an email to **strep-tag@iba-lifesciences.com**
We are here to help.

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