Cell Selection & Expansion

Application Note

ANTIGEN-SPECIFIC T CELL STAINING

The effect of different backbone affinities on staining sensitivity

Introduction

Antigen-specific T cells are key players in immune responses against infection and cancer. Their T cell receptor binds to major histocompatibility complex class I (MHC I) molecules that present peptides on the cell surface. This interaction can be used to identify and select T cells that recognize specific viruses or mutated cells by engineering MHC I molecules and using them as staining reagents. However, the binding affinity of a single TCR to a peptide-loaded MHC I (pMHC I) is too low to enable cell selection as a single molecule. Multimerizing several pMHC I monomers on a common backbone leads to a sufficient increase in binding strength. These so-called MHC I multimers are a frequently used tool in immunology research for antigen-specific T cell staining and isolation.

In our MHC I Streptamer® approach, MHC I molecules are fused to a short peptide sequence, the Twin-Strep-tag® **(Fig. 1A)**. Via this tag, these MHC I-Streps bind to a streptavidin variant, Strep-Tactin®, which serves as backbone for the generation of multimers called Streptamers **(Fig. 1B)**. Depending on the Strep-Tactin® conjugate, fluorescent staining or different cell isolation methods are possible. A central feature of the MHC I Streptamer® approach is the possibility to remove the staining complex from the receptor and thereby prevent internalization. Due to its higher affinity, biotin triggers the release of MHC I-Streps from Strep-Tactin®, resulting in their monomerization and subsequent spontaneous dissociation from the cell surface **(Fig. 1C)**.

Flow cytometry is a widely used method to analyze the frequency of antigen-specific T cells in a sample. The naturally low affinity interaction of ligand and receptor as well as the scarcity of these cells among all T cells highlight the importance of sensitive detection reagents. Here it can help to choose bright fluorophores such as phycoerythrin (PE) and allophycocyanin (APC) to achieve the best separation of positive and negative populations. It is also advantageous to choose a backbone that generates a stable multimer, especially for very low affinity interactions.

In this application note we compared our common MHC I Streptamer® cell staining approach using Strep-Tactin® PE or APC to a modified Strep-Tactin® version called Strep-Tactin®XT. Strep-Tactin®XT

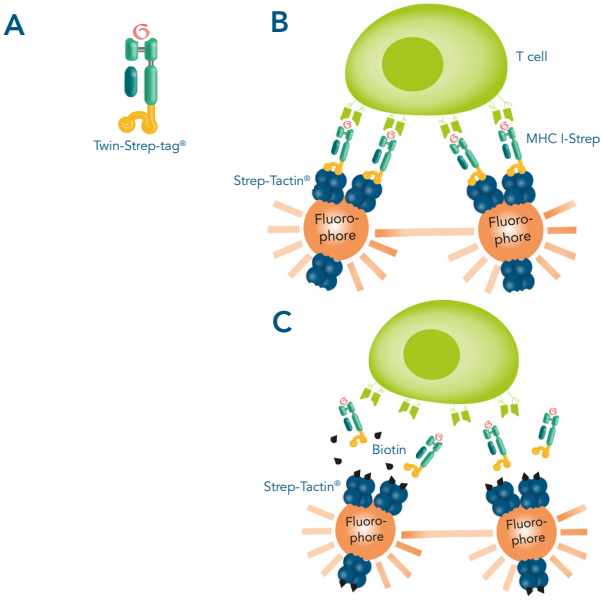

Fig 1. The MHC I Streptamer® staining principle. (A) The MHC class I molecules used for multimer generation are fused to a Twin-Strep-tag® (= MHC I-Streps) (B) Several MHC I-Streps are multimerized on a fluorescent Strep-Tactin® conjugate to form Streptamers for stable binding to cells. (B) Biotin addition releases the MHC I-Streps from Strep-Tactin® causing their monomerization and subsequent spontaneous dissociation from the cell surface.

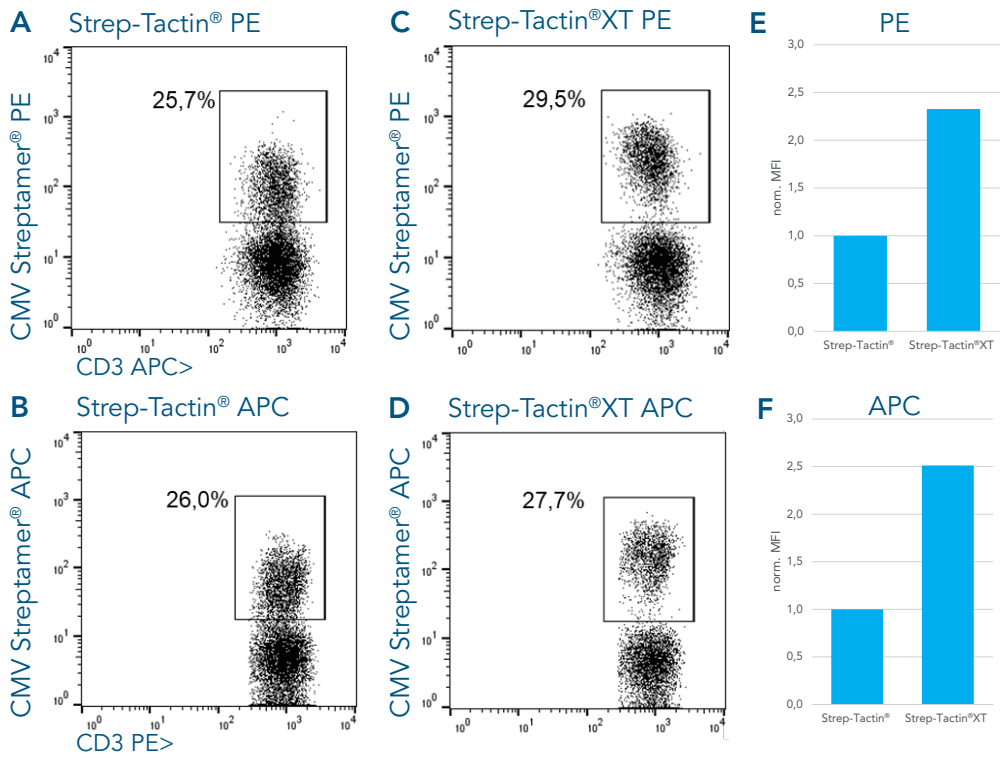

Fig 2. Figure 2: CMV-specific T cell staining with Strep-Tactin® vs. Strep-Tactin®XT. PBMCs of a HLA-A*0201 positive donor were stained with MHC I Streptamer® complexes by incubating MHC I-Strep CMV pp65 (NLVPMVATV) with Strep-Tactin® PE (A,E), Strep-Tactin® APC (B, F), Strep-Tactin®XT PE (C,E), or Strep-Tactin®XT APC (D,F). Cells in FACS plots were pre-gated on living, $CD3^+CD8^+$ cytotoxic T cells. Doublets were excluded using FSC/SSC signals. Bar graphs show normalized mean fluorescent intensities (MFI) of living, CMV^+ T cells.

has a higher affinity to the Twin-Strep-tag® than Strep-Tactin® and can therefore form a potentially more stable multimer enabling more sensitive cell staining. We found that using Strep-Tactin®XT PE or APC indeed leads to a brighter staining compared to using Strep-Tactin® PE or APC, but that the extent of this effect is dependent on the donor.

Methods

Cell staining

For antigen-specific T cell staining, 200 ng of an MHC I-Strep refolded with a cytomegalovirus (CMV)-derived peptide was incubated with 75 ng of Strep-Tactin® PE or APC, Strep-Tactin®XT PE or APC or with Strep-Tactin®XT DY-649 (data not shown) for 15 min at 4 °C according to our MHC I Streptamer® cell staining protocol. Depending on the donor, MHC-allele HLA-A*0201 with CMV pp65 peptide NLVPMVATV or MHC-allele HLA-B*0702 with CMV pp65 peptide TPRVTGGGAM was used. Negative controls for evaluation of background staining of fluorescent conjugates were included (data not shown). 1 x 10^7 PBMCs in 100 µl buffer were added to the staining complexes. After 20 min incubation at 4 °C, cells were washed and antibodies against CD4, CD14, CD3 and CD8 (all BioLegend) added. Propidium iodide (PI) staining was used to discriminate live from dead cells.

Removal of staining reagents

After staining, cells were incubated with 1 mM or 5 mM biotin for 10 min at 4 °C twice to remove staining reagents. Incubation of Strep-Tactin® PE (data not shown) and APC-stained cells with 1 mM biotin served as positive control.

Results

A high affinity backbone leads to a more sensitive staining of antigen-specific T cells

Antigen-specific T cell staining requires a method that reliably detects low affinity interactions. Since monomeric MHC molecules do not stably bind to a TCR, an increase in avidity is required. We compared multimerization of MHC I molecules on two backbones that exhibit different affinities towards the Twin-Strep-tag® (TST) of the MHC molecules. Strep-Tactin® and Strep-Tactin®XT bind to the TST with a nanomolar and picomolar affinity respectively, suggesting that the stability of formed Streptamers differs, which possibly affects the staining sensitivity of these multimers. To test this, we compared CMV-specific T cell staining with Strep-Tactin® PE/APC to Strep-Tactin®XT PE/APC **(Fig. 2)**. We found that using Strep-Tactin®XT-contain-

ing MHC I Streptamers resulted in a brighter mean fluorescence intensity (MFI) and a better separation of positive from negative cell populations **(Fig. 2C+D)** than Strep-Tactin®-containing MHC I Streptamers **(Fig. 2 A+B)**. For both PE and APC conjugates, this increase in fluorescence was around 2.5-fold **(Fig. 2E+F)**, demonstrating that the higher affinity Strep-Tactin®XT backbone improves the staining sensitivity of MHC I Streptamers. However, the use of large and bright fluorophores such as PE and APC is still essential, since Strep-Tactin®XT conjugated to small and less bright DYOMICS fluorophores could not reliably stain antigen-specific T cells (data not shown).

The extent of increased brightness of the high affinity backbone is donor-dependent

To check if the initially observed increase in staining sensitivity is also observed across different individuals, we compared a CMV-positive donor with MHC I allele HLA-A*0201 to one with MHC I allele HLA-B*0702 **(Fig. 3)**. We chose donors with a comparable frequency of CMV-positive T cells to exclude any effects caused by different cell numbers. Similar as shown in **Fig. 2**, we observed a clear increase in staining brightness for antigen-specific T cells from an HLA-A*0201-positive donor if MHC I Streptamers were generated with Strep-Tactin®XT PE **(Fig. 3B+C)** compared to using Strep-Tactin®PE **(Fig. 3A+C)**. For this donor, the increase in MFI of the CMV-positive T cell population was more than 6-fold **(Fig. 3C)**. For a donor with MHC I allele HLA-B*0702, using Strep-Tactin®XT PE led to a formation of a more homogeneous cell population compared to Strep-Tactin® PE **(Fig. 3D+E)**. However, the overall increase in staining brightness of the CMV-positive population was only 1.5-fold **(Fig. 3F)**. All in all, five independent donors demonstrated a mean staining difference of around 3-fold (data not shown). These results highlight that although we observed the general trend for brighter staining with Strep-Tactin®XT, the extent of this effect can vary depending on the donor.

5 mM biotin releases Strep-Tactin®XT conjugates from the cell surface

The multimerization of MHC molecules is an effective method to utilize them as staining reagents, but their strong binding to the TCR can lead to internalization and cell death if subsequent culture of cells at 37°C is necessary. Therefore, the removal of the selection reagents prior to further experiments helps to improve

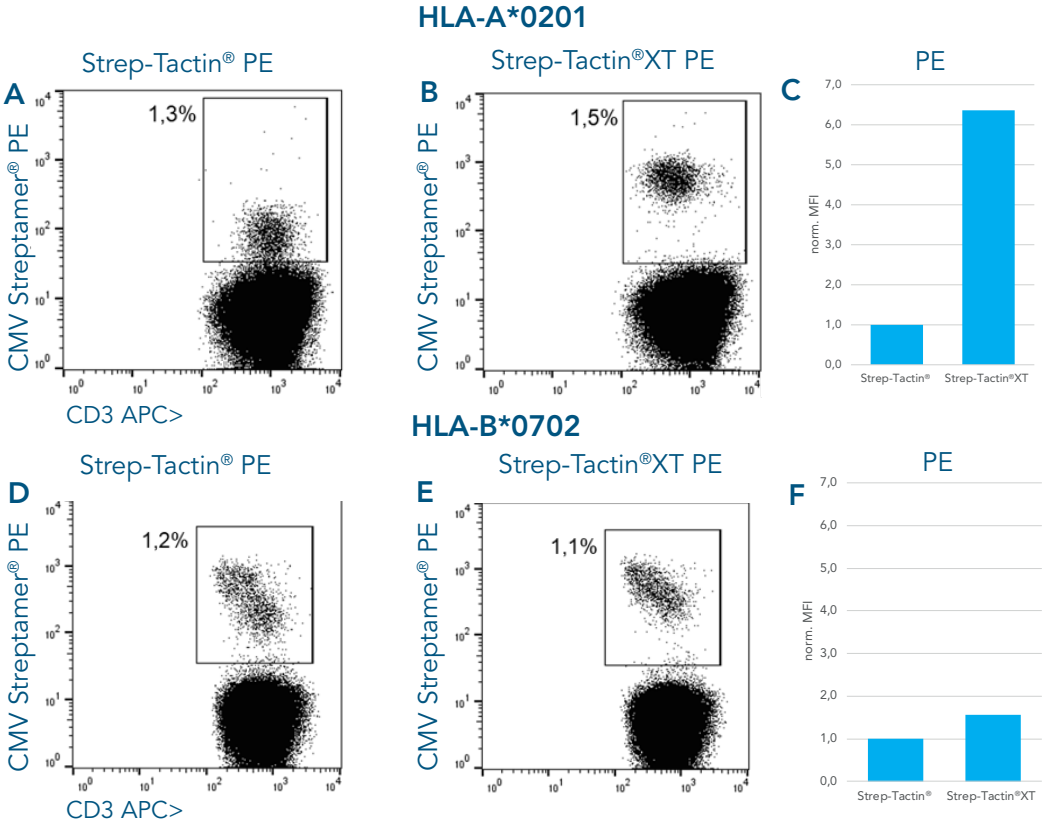

Fig 3. HLA-A*0201- vs. HLA-B*0702-MHC I Streptamer® staining. PBMCs of a HLA-A*0201 (A-C) and a HLA-B*0702 positive donor (D-F) were stained with MHC I Streptamer® complexes by incubating MHC I-Strep CMV pp65 (NLVPMVATV) or MHC I-Strep CMV pp65 (TPRVTGGGAM), respectively, with Strep-Tactin® PE (A,D) or Strep-Tactin®XT PE (B,E). Cells in FACS plots were pre-gated on living, $CD3^+CD8^+$ cytotoxic T cells. Doublets were excluded using FSC/SSC signals. Bar graphs show normalized mean fluorescent intensities (MFI) of living, CMV^+ T cells.

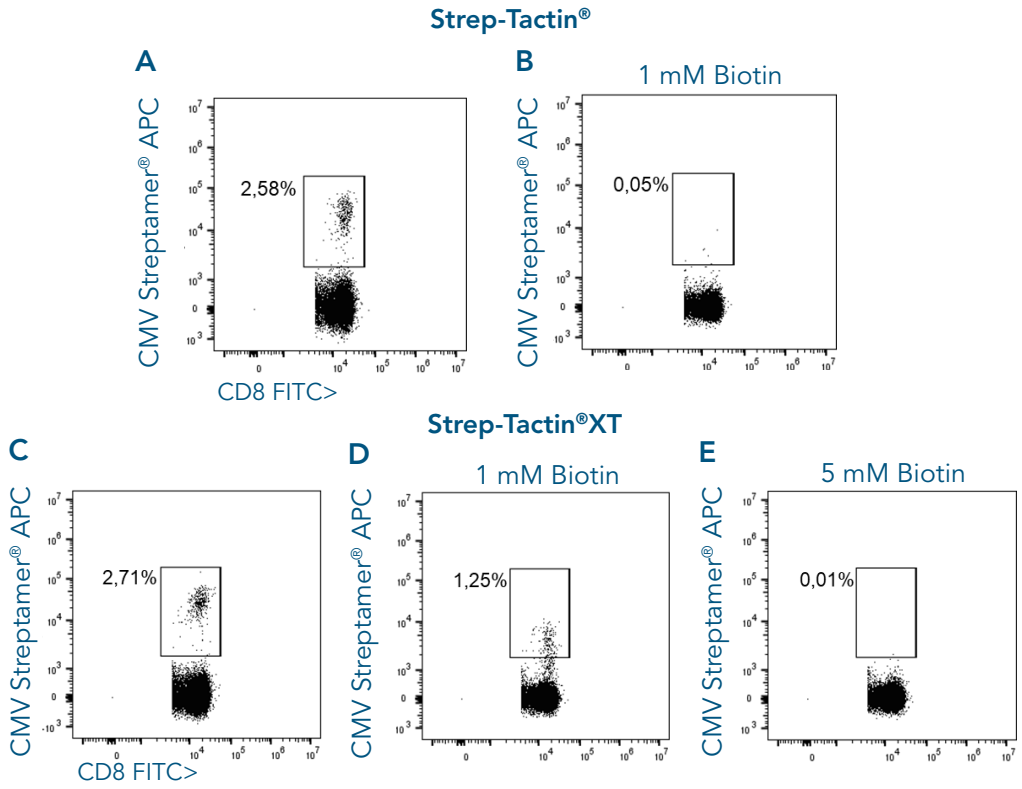

Fig. 4 Removal of Strep-Tactin® and Strep-Tactin®XT conjugates from the cell surface. PBMCs of a HLA-B*0702 positive donor were stained with MHC I Streptamer® complexes by incubating MHC I-Strep CMV pp65 (TPRVTGGGAM) with Strep-Tactin® APC (A) or Strep-Tactin®XT APC (C). Reagents were removed from Strep-Tactin®-stained cells using 1 mM biotin (B, positive control). (C-E) Strep-Tactin®XT-stained cells (C) were incubated with 1 mM (D) or 5 mM (E) biotin. Cells in FACS plots were pre-gated on living, $CD8^+$ cytotoxic T cells. Doublets were excluded using FSC/SSC signals.

the outcome. For our common MHC I Streptamer® approach using Strep-Tactin® conjugates, this can simply be done by the addition of 1 mM biotin. Biotin disrupts the multimeric complexes and thereby causes the release of all reagents from the cell surface **(Fig. 4A+B)**. Since Strep-Tactin®XT binds to the Twin-Strep-tag® of our MHC I molecules with a higher affinity than Strep-Tactin®, we tested if the generated multimeric complex is still reversible as well **(Fig. 4C-E)**. We found that incubation of Strep-Tactin®XT APC-stained cells with 1 mM biotin is not sufficient to cause a complete release of staining reagents **(Fig. 4D)**. However, increasing the biotin concentration to 5 mM was enough to remove the MHC I Streptamer® complexes entirely **(Fig. 4E)**. The results demonstrate that although Strep-Tactin®XT leads to the formation of more stable multimers, the complexes are easily removed from the cell surface, making them a suitable and preferred alternative for staining with Strep-Tactin® conjugates.

Conclusion

Taken together, Strep-Tactin®XT PE and APC enable a more sensitive staining than Strep-Tactin® PE and APC in our MHC I Streptamer® approach. In addition, Strep-Tactin®XT conjugates are still reversible, although they lead to the formation of a more stable multimer. These features make them the conjugate of choice when it comes to selecting low frequent and low affinity antigen-specific T cells.

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