Xynapse™-T Protocol: Stimulation of Human Antigen-Specific T Cells

Products

Xynapse™-T, Cat. No. X[W/F/Z/X]xxxxxxPxxx

Purpose

Xynapse™-T reagents are non-cellular antigen presenting molecules designed to specifically stimulate human antigen-specific T cells in biological samples or TCR engineered T cell products. Xynapse™-T reagents can be used as convenient substitutes for antigen presenting cells in a variety of applications such as cytokine production assays, enrichment of TCR engineered T cells or rare T cells in biological samples and functional characterization of T cell responses to antigen challenge.

This protocol describes how Xynapse™-T can be used to stimulate antigen-specific T cells in PBMCs, or isolated T cells samples.

Material provided

Xynapse™-T consist of a polymer backbone carrying an optimized number of human MHC-peptide complexes and CD28 engager molecules.

Xynapse™-T reagents are available with a broad range of MHC class I & II, CD1d and MR1 alleles of human origin: immudex.com/resources/mhc-alleles-list/

Material required (not provided)

For T cell stimulation with Xynapse™-T

TexMACS™ Medium (Miltenyi Biotec)

Human IL-2

Human Il-7

Human IL-15

Sterile filtered PBS, 10 % FCS, pH 7.2.

For flow cytometry analysis

MHC Dextramer®
(https://immudex.com/products/basic-research/mhc-i-dextramer-flow-cytometry/)

MHC Dextramer® Negative Controls
(https://immudex.com/resources/dextramer-controls/)

4 mL Falcon disposable 12 x 75-mm test tubes or equivalent LoBind® Eppendorf tubes

Stain and Wash buffer: PBS, 1-5 % FCS, pH 7.4

100 µM d-Biotin (e.g. Avidity, cat# BIO200) diluted in PBS, pH 7.4

Antibodies against relevant cell surface markers:

For $CD8^+$ T, $CD4^+$ T and NKT cells (e.g., CD3, CD4 and CD8)

For MAIT cells (e.g. CD3, CD4, CD8 and CD161)

Optionally other desired antibodies and live-dead dye.

For cytokine production assays
BD Cytofix/Cytoperm Plus Kit with BD (Cat. No. 554715) containing:
BD GolgiStop™ (monensin)
Fixation/Permeabilization solution
BD Perm/Wash™ Buffer

Antibodies against cytokines of interest

Procedure

All steps in the protocol must be performed in a laminar flow cabinet using sterile techniques.

Sample preparation
Prepare either PBMCs or isolated T cells:
1. To the PBMC sample (cryopreserved or freshly isolated) add 10 mL of sterile PBS, 10 % FCS. Centrifuge for 10 min at 300 x g. Discard supernatant.
2. Repeat step 1.

Keep prepared cells cold at 4 °C or prepare isolated T cells by using a human T cell isolation kit according to the manufacturer's recommendation.

Antigen-specific T cell stimulation

Stimulation of antigen-specific T cells with Xynapse™-T can be performed using culture plates with different well sizes. Table 1 shows the recommended number of T cells (or PBMCs) to seed, and the culture volume to use in different cell culture plate formats. The recommended working concentration of Xynapse™-T added to the seeded cell culture is 1:100 (v:v) irrespective of the frequency of target T cells.

Culture plate	**Growth area**	**Max. cell culture volume**	**Total CD3+ T cell number (or PBMC cell number)**	Xynapse™-**T to add per well**
96 well	0.31 cm^2	0.2 mL	0.3×10^6 (0.6×10^6)	2 µL
48 well	1 cm^2	1 mL	1×10^6 (2×10^6)	10 µL
24 well	2 cm^2	2 mL	2×10^6 (4×10^6)	20 µL
12 well	4 cm^2	4 mL	4×10^6 (8×10^6)	40 µL
6 well	10 cm^2	5 mL	10×10^6 (20×10^6)	50 µL

Table 1. We recommend seeding T cells at an optimal surface density of 1×10^6 cells per cm^2 and PBMCs with up to 2×10^6 per cm^2 and stimulating with 1:100 (v:v) of Xynapse™-T reagent.

The volumes given below are for the stimulation in a 48-well plate of up to 1×10^6 T cells or of 2×10^6 PBMCs in a total volume of 1 mL TexMACS™ medium.

Activation in a 48-well plate

3. Count the $CD3^+$ T cell sample (or PBMC sample).
4. Adjust the cell density of CD3+ T cells to $1x10^6$ cells/mL or of PBMCs to $2x10^6$ cells/mL in TexMACS™ medium.
5. Seed 1 mL of cell suspension in wells of a 48 well culture plate.
6. Add 10 µL of the Xynapse™-T reagent and mix gently by pipetting.
7. Incubate at 37 °C, 5 % CO_2.

After this step the response of T cells to antigen challenge can be measured with assays, such as activation induced (surface) marker assays (AIM), cytokine production assays (see example below "Cytokine production assays by intracellular flow cytometry") and degranulation assay. When to harvest T cells for analysis depends on the expression kinetics of the marker of interest.

8. On day 2–3 gently replace 50 % (500 µl) of the cell culture supernatant with 500 µL TexMACS™ medium supplemented with human IL-2 (50 U/mL), with minimal displacement of cells from the surface of the well. Incubate at 37 °C, 5 % CO_2.
9. On day 5 and every second day forward, gently replace 50 % of the cell culture supernatant with 500 µL of TexMACS™ medium supplemented with human IL-2 (50 U/mL), human IL-7 (10 ng/mL) and human IL-15 (10 ng/mL). Incubate at 37 °C, 5 % CO_2.
10. Inspect the cell culture daily and add fresh medium if required. If the cell density becomes too high, split the cell suspension into two equal parts and add TexMACS™ medium supplemented with human IL-2 (50 U/mL), human IL-7 (10 ng/mL) and human IL-15 (10 µg/mL).
11. Grow the cells for up to 14 days.

Note:
For stimulation of TCR engineered T cells the protocol may need to be individually optimized for the specific cell product with respect to titration of Xynapse™-T reagent and culture condition of the cells.

Analysis of antigen-specific T cell expansion by flow cytometry

Proliferation of Xynapse™-T activated T cells can be detected and enumerated by flow cytometry analysis of stimulated cells stained with corresponding fluorescently labelled MHC Dextramer® reagents. Compare the frequency of antigen specific T cells in Xynapse™-T treated samples with an appropriate control sample e.g. cells treated with a Xynapse™-T negative control reagent[a].

Monitor enrichment of antigen-specific T cells on days 5-14. The preferred time of analysis depends on the initial frequency of antigen-specific T cells in the unstimulated sample. Expansion of naturally frequent antigen-specific T cells (1 % or more of all T cells) can be evaluated at day 5-7, while expansion of rare antigen-specific T cells (~ 0,1 % of all T cells or below) require longer proliferation time (10-14 days).

1. Transfer 1-3 x 10^6 cells (2-5 x 10^4 for engineered cells) from the culture well to a FACS tube.
2. Add Stain and Wash buffer to a total volume of 4 mL and centrifuge for 10 min at 300 x g. Discard the supernatant.
3. Wash the stimulated cells by adding 4 mL Stain and Wash buffer, centrifuge for 10 min at 300 x g. Discard the supernatant.
4. Resuspend cells in 50 µL of Stain and Wash buffer, store at 4 °C.

5. To prepare a pool of pMHC specific and corresponding negative control MHC Dextramer® mix the following reagents in an empty 1.5 mL LoBind® Eppendorf tube:
   a. Add 0.2 µL of 100 µM d-Biotin per Dextramer® reagent.
   b. Add 10 µL of each Dextramer® reagent.
   c. Add 0.6 µL of 10x PBS per Dextramer® reagent.
   NB: When staining with a single Dextramer® reagent, a) and c) can be omitted.
6. Vortex the Dextramer® pool briefly. The Dextramer® pool must be used directly after preparation and cannot be stored.
7. Centrifuge the pool at 10.000 x g for 1 min. to avoid transferring any potential precipitate.
8. Add the Dextramer® pool to the cell sample and vortex briefly.
9. Incubate in the dark at room temperature:
   a. MHC I, CD1d, MR1 Dextramer®: 10 min. incubation.
   b. MHC II Dextramer®: 30 min. incubation
10. Add relevant antibodies in the volume/concentration according to manufacturer's instructions for sorting of relevant T cells.
11. Incubate at 4 °C in the dark for 20 min.
12. Wash cells by adding 2 mL Stain and Wash buffer. Centrifuge at 300 x g for 5 min. and remove the supernatant. Repeat washing for a total of 2 washes.
13. Resuspend the pellet in the desired volume of Stain and Wash buffer suitable for your flow cytometer.
14. Proceed to analyze the samples on a flow cytometer or store at 2-8 °C in the dark. For optimal results, do not store the samples for longer than 2 hours before acquisition.

Notes:

a. As a negative control we recommend a Xynapse™-T reagent with MHC monomers displaying a highly diverse peptide pool (https://immudex.com/resources/dextramer-controls/). Alternatively, a Xynapse™-T reagent presenting an irrelevant peptide can be used.
b. Upon Xynapse™-T stimulation, a transient decrease in T cell receptor in the cell membrane occurs. Therefore, detection of activated T cells with MHC Dextramer® can be challenging for a period of 6-48 hours after stimulation.
c. We recommend including a live/dead marker, to exclude dead or apoptotic cells from the analysis. Live-dead staining can be performed at the beginning or end of the staining procedure according to manufacturer's instructions.
d. Staining with antibodies against CD3 and CD8 has a negative impact on simultaneous or subsequent staining with MHC I Dextramer®. In most cases it is therefore highly recommended to stain with MHC I-, MR1- and CD1d-Dextramer® before staining with CD3 and CD8 antibodies. Simultaneous staining will reduce the Dextramer® staining intensity significantly.
e. Staining can be performed using 96-well microtiter plates. In that case after antibody incubation make 4 sequential washes using 200 µL Stain and Wash buffer per well. Centrifuge at 300 x g for 5 min. between each wash and remove supernatant.
f. Detailed protocol and tips and tricks for MHC Dextramer® staining can be found here: https://www.immudex.com/resources/protocols/

Cytokine production assays by intracellular flow cytometry

1. Follow the stimulation protocol to step 7.
2. Add BD GolgiStop™ to the cell culture according to the BD protocol.
3. Incubate at 37 °C for 3-6 h.
4. Stain cells with specific MHC Dextramer® reagents and antibodies to relevant cell surface markers as described under "Analysis of antigen-specific T cell expansion by flow cytometry" steps 1-12 above.
5. Add 200 µL of BD Fixation/Permeabilization solution to each tube and resuspend cells, incubate for 15-30 min. at 4 °C in the dark.
6. Add 1,8 mL Stain and Wash buffer. Centrifuge at 500 x g for 5 min. Remove supernatant.
7. Add 2 mL Stain and Wash buffer. Centrifuge at 500 x g for 5 min. Remove supernatant.
8. Dilute BD Perm/Wash™ Buffer 10x in Milli-Q water for use in the following steps below.
9. Add 2 mL BD Perm/Wash™ Buffer. Centrifuge at 500 x g for 5 min. Remove supernatant.
10. Add 2 mL BD Perm/Wash™ Buffer and incubate for 15 min at 4 °C in the dark.
11. Centrifuge at 500 x g for 5 min. Remove supernatant.
12. Add 50 µL of BD Perm/Wash™ Buffer.
13. Add antibodies against cytokines (e.g. IFN-g, TNF-a, IL-2) according to manufacturer's instructions, incubate 20 min at 4 °C in the dark.
14. Add 2 mL BD Perm/Wash™ Buffer to each tube, Centrifuge at 500 x g for 5 min. Remove supernatant.
15. Repeat the above washing step.
16. Resuspend cells in 400 µL Stain and Wash buffer and analyze cells by flow cytometry.

Example 1. Enrichment of MART-1 specific T cells with Xynapse™-T

In this example isolated CD3+ T cells were stimulated with Xynapse™-T HLA-A*0201/MART-1 reagent and expansion of specific cells was evaluated after 13 days by flow cytometry.

Human CD3+ T cells were isolated from healthy donor PBMCs using the "Pan T cell isolation kit, Human" (Miltenyi Biotec, Cat. 130-096-535) according to the manufacturer's instruction and resuspended in TexMACS™ medium ($1.5x10^6$ cells/mL) without added cytokines.

200 µL $CD3^+$ T cells ($0.3x10^6$ cells) were transferred to a 96 well culture plate and stimulated with 2 µL Xynapse™-T HLA-A*0201/MART-1 reagent for a duration of 13 days. 50 % of the medium was replaced with fresh TexMACS™ medium supplemented with human IL-2 (50 U/mL) at day 3. From day 5, 50 % of the medium was replaced with TexMACS™ supplemented with human IL-2 (50 U/mL), human IL-7 (10 ng/mL) and human IL-15 (10 µg/mL) every second day.

On day 13 the expanded cell culture was harvested and stained with eBioscience™ Fixable Viability Dye eFluor™ 780 (ThermoFisher Cat. 65-0865-14). Subsequently cells were stained with MHC Dextramer® HLA-A*0201/MART-1, PE reagent (Cat No. WB02162), and MHC Dextramer® HLA-A*0201/ALIAPVHAV, APC Negative Control reagent (Cat No. WB02666), plus antibodies for the cell surface markers (CD3, CD4, CD8) according to above mentioned "Analysis of antigen-specific T cell expansion by flow cytometry" procedure. Finally stained cells were analyzed by flow cytometry.

Gating strategy: Viable lymphocytes were gated on scatter signals and the viability stain allowed exclusion of dead cells. After gating of $CD8^+$ T cells expanded HLA-A*0201/MART-1 Dextramer® positive cells were detected as shown below.

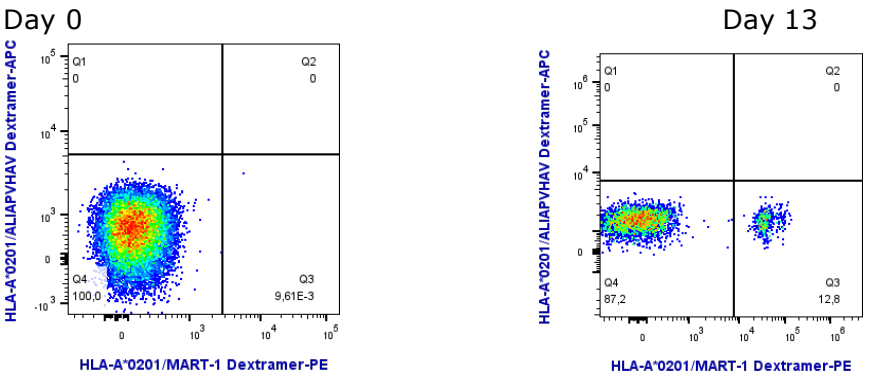

Fig 1. Enrichment of rare MART-1 specific T cells with Xynapse™-T
Flow plots showing frequency of MART-1 specific $CD8^+$ T cells at days 0 and 13 after Xynapse™-T stimulation. Undetectable at day 0, MART-1 T cells make up almost 13 % of $CD8^+$ T cells at day 13.

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