This protocol was developed by partners of the GAPVAC consortium and has been published in Hilf et al. Nature 565, 240–245, 2019

Supplementary Methods 2: Harmonized GAPVAC TIL isolation and expansion protocol from glioblastoma tumour specimens

Preparation of tumour fragments (all laboratories).

- Transfer fresh tumour material that is obtained from the operation room immediately (packed in a sterile gauze that is soaked) in sterile medium[1] or PBS in a sterile container and transport it at room temperature to the laboratory.
- Within the biosafety cabinet, put the tumour into a 9 cm diameter sterile petri□dish. Wash it once with medium or with PBS and discard all bloody or necrotic areas.
- Note the consistency, size and/or weight of the tumour material.
- Use sterile scalpel and forceps to cut the tumour into small fragments (1 to 2 mm^3). Note that the tumour should be processed within 3 hours, but preferably within 2 hours after it was removed from the patient.

Preservation of tumour fragments (only for laboratories that cannot culture TIL).

- Dissociate the tumour fragments as explained above and freeze them down according to the protocol used for freezing PBMC[2]. The expansion of TIL from fresh tumour samples is preferred.
- Ship the samples to the designated GAPVAC partner TIL laboratory for your facility.

Expansion of TIL from tumour fragments (for laboratories that can culture and analyse TIL).

- Quickly transfer 1-4 fragments per well in 24 well plates. Try to transfer at least 48 fragments to the 24 well plates. Make sure that the tumour fragments do not dry during processing.

- Add a total volume of 2 mL TIL medium[1] plus IL-2[3] (6000 or 1000 IU/mL, either one is fine but do not change it in between) per well.
- Add the soluble agonistic anti-CD3 antibody to each well (OKT3 at 30 ng/mL)[4]. Alternatively, one may use washed CD3/CD28 beads (4 µL/well)[5]; however, it is strongly preferred to use the anti-CD3 antibody[6]. Make sure that these stimuli do not contain preservatives as these may inhibit the growth of T cells.
- Refresh medium every 2-3 days by removing 1 mL of medium and adding 1 mL fresh medium plus IL-2 (either 12000 or 2000 IU/mL → final concentration of either 6000 or 1000 IU/mL). Refresh 3 times per week. Do not provide new anti-CD3 stimulation.
- Split wells into two wells of a 24 well plate when the cells at the bottom of the well form a confluent layer. Add fresh medium up to 2 mL with IL□2 (final concentration of 6000 or 1000 IU/mL). Do not mix the cultures of different expanding initial wells, maintain them as separate cultures. However, if more than 4 different initial wells grow out, pool certain wells in order to have a maximum of 4 different cultures per patient.
- If required, cells can be transferred to 6 well plates; re-suspend TIL cultures carefully and transfer 4 mL TIL suspension per well and add equal amount of fresh TIL medium with IL-2 (final concentration of either 6000 or 1000 IU/mL). Do not provide new anti-CD3 antibody.
- Culture up to $5x10^7$ cells for as many as possible different individual cultures from one patient as possible. Stop the expansion protocol after approximately 28 days of culture to obtain at least one culture with a size of $10x10^6$ cells or earlier when more than 2 cultures have reached this size. No more than 4 different cultures are required.
- Perform a phenotypic cell analysis for CD3, CD4, CD8, CD16, CD56, TCRαβ and TCRγδ by flow cytometry, use a dead cell marker.
- Cryopreserve on liquid nitrogen using a PBMC freezing protocol[2].

Notes

[1] TIL media that can be used are:

1) RPMI + Glutamax with 10% heat-inactivated human AB serum and 1,25 µg/µL Fungizone as well as 1% penicillin/streptomycin.

2) IMDM + Glutamine + 7.5% heat- inactivated human AB serum and 0.5% penicillin/streptomycin (penicillin 10.000 Units/mL, streptomycin 10.000 µg/mL mixture).

[2] Freezing protocol:

Use the freezing protocol that is also used for the freezing of PBMC samples in your laboratory. Consider the following guidelines: 1) Store between 5-10 million cells per vial, if possible freeze at least 2 vials per culture; 2) Pre-cool the cryovials; 3) make sure that the cells and the freezing medium (e.g. 90% serum + 10% DMSO) are incubated on ice or at 4°C for at least 15 minutes; 3) Use a final concentration of DMSO of 10%; 4) Add the freezing medium drop-wise to the cells and shake well before aliquoting; 5) Transfer cryovials to -80°C, if possible in a cryocontainer (Mr Frosty), then to the nitrogen tank for long term storage.

[3] IL-2 source:

Proleukine = Aldesleukine (Novartis 17152.00.00). 1 vial of $18x10^6$ IE can be dissolved in 5.970 mL of sterile water or culture medium, add 30 µl of 20% human albumin for stability (Albumin 200 g/L, Sanquin) to obtain a 0.1% solution. Then aliquot and store at -80^oC. A thawed vial can be kept at 4^oC for 3-5 days.

[4] OKT3 antibody:

Miltenyi CD3 pure – functional grade, human (clone: OKT3, cat n° 130-093-387) for research use only, content: 100 µg in 1 mL.

[5] CD3/CD28 beads:

T-cell activator, Dynal No 111.31D (or 111.32D, 111.61D). Take 4 µL for each culture well of beads (as per manufacturer's instructions, 2 µL are used to activate 8×10^4 human T cells) and wash them as instructed by the manufacturer) before adding them to the cultures.

[6] This recommendation was based on the fact that only few experiments were performed with CD3/CD28 beads.