# **Restoration of T cell Effector Functions, Preferential Depletion of Regulatory T cells, and Direct Killing of Tumor Cells: The** **Multiple Mechanisms of Action of anti-TIGIT Antagonists**

**Supplementary Methods**

**CD155 and CD226 staining by IHC**

Staining of CD155, pancytokeratins and CD226 were done on adjacent sections of the disease-specific TMAs. The IHC staining was performed on a Discovery Ultra staining Module (Ventana Medical Systems Inc., Tucson, AZ, USA). First, paraffin was removed by incubation with the EZ prep buffer at 69°C for 3 cycles of 8 minutes. Then, the slides were incubated with Discovery cell Conditioner 1 (CC1, pH 9) buffer at 95°C for 32 minutes for antigen retrieval. The inhibition of endogenous peroxidases was performed by adding one drop of Inhibitor CM and incubating for 8 minutes. Blocking of aspecific sites was achieved by adding a blocking solution composed of 5% BSA, 2% non-fat milk, 0.1% Tween, 1% human IgG in TBS (for CD155 and PanCK) and with 5% normal goat serum in TBST (0.15% Tween20) (for CD226) for 4 minutes. Monoclonal mouse antibody anti-pancytokeratins (M351501-2 Dako; 1:50 dilution in Dako Real antibody diluent), monoclonal rabbit anti-CD155 (13544S/1 Cell Signaling Technology; 1:50 dilution in Dako Real antibody diluent) and monoclonal rabbit anti-CD226 were incubated for 1 hour at 37°C, followed by 12 min-incubation with anti-mouse (5269652001, Roche) or anti-rabbit (5269679001, Roche) IgG HRP linked antibody respectively for 16 minutes. Finally, 3,3’-Diaminobenzidine (DAB) was added to the sections and slides were counterstained with Hematoxylin II and bluing reagents. Slides were cleaned, dehydrated by passing them in several baths with an increasing gradient of ethanol followed by Histoclear (for 15 seconds each). Slides were mounted by adding one drop of mounting medium and a coverslip. Slides were scanned at a 20x magnification with a Nanozoomer scanner (Hamamatsu). Quantification was performed with a computer-assisted image analysis with the Visiopharm® software (version 2017.1, Denmark) equipped with the Tissuearray® module. Following the overlay of the PanCK scan on the CD155 scan, the areas of PanCK+ were delimited and the number of CD155+ cells with different intensities of expression were counted (low/medium or high). The final output calculated for CD155 is the % of CD155 high in the PanCK+ area considered as the tumor area. For CD226, the % of tissue area positive for CD226 on the total area was counted. For individual cases having multiple tissue cores included in the TMAs, the mean percentages of CD155 positive cells or mean CD226 positive area were considered.

**TIGIT receptor quantification**

To quantitate TIGIT receptor cell surface expression, QuantibriteTM beads (BD Biosciences), containing 4 different populations of beads labeled with a specific number of PE molecules, were resuspended following manufacturer’s instructions and acquired by FACS with the same settings as the samples under analysis. The number of molecules per cell for each TIGIT+ cell population, analysed with anti-TIGIT clone MBSA-43 PE-labeled, was calculated by extrapolation of the corresponding geometric MFI for PE on the standard curve obtained with the ~~g~~MFI for PE and the provided PE molecules per bead population provided in the Quantibrite beads kit.

**FACS staining of murine material**

Cells were washed in PBS and stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Invitrogen) for 20 minutes at 4°C. After washing, cells were treated for 15 minutes at RT with Fc block (anti-CD16/CD32, eBioscience) before incubation with antibody cocktail as specifies in Supplementary table 1. Cells were washed and fixed overnight at 4°C, treated with Fc block for 15 min at RT, followed by intracellular staining with FoxP3-APC in permeabilization buffer for 30 min at 4°C. Cells were washed and resuspended in FACS buffer prior to acquisition. For *ex vivo* stimulation, cells were treated as previously described (22). Data were acquired on a MacsQuant flow cytometer (Miltenyi Biotech) and analyzed with FlowJoTM V10.1.

**Murine cell lines**

The CT26 (ATCC® CRL-2638TM), Hepa1-6 (CrownBio), and EL4 (ATCC® TIB-39TM) tumor cells were maintained in vitro as a monolayer culture in RPMI (CT26) or DMEM (Hepa1-6 and EL4) medium supplemented with 10% FBS and 1% HEPES buffer at 37ºC, 5% CO2. Cells in exponential growth phase were harvested and quantitated by cell counter before tumor inoculation. Transductions were performed at GIGA Institute (U. Liège). EL4 (ATCC® TIB-39TM) lymphoma cells were transduced at GIGA Institute (U.Liège) with pLV EF1A mTigit-IRES-EmGFP vector to stably express mouse TIGIT (EL4-mTIGIT) or control vector pLV EF1A-EmGFP encoding GFP alone (EL4-GFP). Transduced EL4 were cloned and grown in medium with 13.33 mg/mL of Blasticidin (ThermoFisher Scientific). PanO2 (NCI 0507406 p3) were transduced with pLV EF1A mTigit-IRES-EmLuc vector to stably express Luciferase (PanO2-Luc).

**NK and macrophages depletion**

CT26 tumor model was performed as described in material and method. NK depletion was performed using anti-Asialo-GM1 antibody (146002, Biolegend) administered IP every 5 days at the dose of 20 µl, PBS was used as control and NK depletion was verified by flow cytometry. For macrophage depletion, clodronate or PBS liposomes were generated and given IP on day 7 and 9 post tumour inoculation and once weekly thereafter. Macrophage depletion was confirmed using flow cytometry. Anti-PD-1 ( 200 mg/mouse, BioXCell BE0146) and anti-TIGIT (200 mg/mouse) were administered IP when tumors were palpable at days 10, 13 and 16 after tumor cell inoculation.

**CT26 and EMT6 rechallenge**

Rechallenge experiments were performed on mice that experienced complete regression. CT26 tumor cells were inoculated as described above on day 70 after the first tumor inoculation. EMT6 cells (ATCC® CRL-2755TM) were inoculated in the intramammary gland at day 90 after the first CT26 inoculation at the dose of 0.1 x 106 cells/mouse. A cohort of naïve mice was inoculated in parallel to confirm tumor cell viability and ability to grow.

### In vitro cytotoxicity of anti-TIGIT on EL4-mTIGIT

RAW 264.7 (ATCC® TIB-71TM) were plated in presence of 12 ng/mL of recombinant mouse IFNg for overnight stimulation. EL4-mTIGIT target cells were plated in a 96-well plate, 20000 cells/well, and RAW 264.7 effector cells were added in dedicated wells to a 5:1 effector:target ratio. Anti-TIGIT Ab was added in a 3-fold serial dilution starting at 1 mg/mL; mIgG2a isotype was used as negative control at 1 mg/mL. The plate was incubated overnight at 37°C, 5% CO2. Cells were then washed and resuspended in FACS buffer and acquired on a MacsQuant flow cytometer for GFP expressing cells. Data were analyzed with FlowJo software.

**Analysis of public datasets**

The following public microarray datasets were used for analysis of TIGIT expression: Breast, Cervix, Colon, Endomentrium, Kidney, Lung, Ovary, Prostate and Thyroid (GSE2109); Glioblastoma (GSE7696); Glioma (GSE16011); Neuroblastoma (EGAS00001001953); Myeloma (GSE2658); B-ALL (GSE11877); T-ALL (GSE26713); AML (GSE17855); Hodgin Lymphoma (GSE17920); NK Lymphoma (GSE19067); DLBCL (GSE31312); CLL (GSE39671); PTCL (GSE58445); Follicular Lymphoma (GSE93261) and Mantle Cell Lymphoma (GSE93291). All datasets were acquired from the R2 Genomics Analysis and Visualization Platform (R2: [http://r2.amc.nl](http://r2.amc.nl/)).

**Table 1 : Antibodies and flow cytometry panels**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Antibody | Clone | | | Reference | | Supplier | |
| TIGIT expression in human PBMCs and TILs (Figure 1 and 2) | | | | | | | |
| anti-CD127-APC | A019D5 | | | 351316 | | Biolegend | |
| anti-CD25-PE-Cy7 | BC96 | | | 302612 | | Biolegend | |
| anti-CD3-BV650 | SK7 | | | 563999 | | BD Horizon | |
| anti-CD39-PE-Dazzle594 | A1 | | | 328224 | | Biolegend | |
| anti-CD4-APC-R700 | RPA-T4 | | | 564975 | | BD Horizon | |
| anti-CD45-BB515 | HI30 | | | 564585 | | BD Horizon | |
| anti-CD45RO-BB515 | UCHL1 | | | 564529 | | BD Horizon | |
| anti-CD56-BV711 | 5.1H11 | | | 362542 | | Biolegend | |
| anti-CD73-BV421 | AD2 | | | 562430 | | BD Horizon | |
| anti-CD8-BV510 | SK1 | | | 563919 | | BD Horizon | |
| anti-TIGIT-PE | MBSA43 | | | 12-9500-42 | | eBioscience | |
| anti-IL-2-APC | MQ1-17H12 | | | 17-7029-82 | | eBioscience | |
| anti-IFNɣ-BV711 | 4S.B3 | | | 564793 | | BD Horizon | |
| anti-TNFa-PE-Cy7 | MAb11 | | | 25-7349-82 | | eBioscience | |
| In vitro ADCC assay on human PBMCs from cancer patients (Figure 4) | | | | | | | |
| anti-CD19-AF700 | HIB19 | | | 56-0199 | eBioscience | | |
| anti-TCRab-PerCP-Cy5,5 | IP26 | | | 306723 | eBioscience | | |
| anti-CD4-BV510 | SK3 | | | 562970 | Biolegend | | |
| anti-CD8-APC-Cy7 | SK1 | | | 344714 | Biolegend | | |
| anti-CD25-BV605 | 2A3 | | | 562660 | Biolegend | | |
| anti-CD127-APC | A019D5 | | | 351316 | Biolegend | | |
| anti-CCR7-BV421 | G043H7 | | | 353208 | Biolegend | | |
| anti-CD45RO-PE-Cy7 | UCHL1 | | | 304229 or 304230 | Biolegend | | |
| TIGIT phenotyping on gamma delta T cells (Figure 3) | | | | | | |
| anti-CD27-APC-R700 | M-T271 | | 565116 | | BD Biosciences | |
| anti-granulysin-AF488 | RB1 | | 558254 | | BD Biosciences | |
| anti-CD28-BV605 | 28.2 | | 562976 | | BD Biosciences | |
| anti-CD279-BV605 | EH12.1 | | 563245 | | BD Biosciences | |
| anti-perforin-BV421 | dG9 | | 563393 | | BD Biosciences | |
| anti-CD45-BB515 | HI100 | | 564552 | | BD Biosciences | |
| anti-granzymeB-AF700 | GB11 | | 560213 | | BD Biosciences | |
| anti-TCRɣδ-APC | REA591 | | 130-113-508 | | Miltenyi Biotec | |
| anti-CD3-BV421 | UCHT-1 | | 562426 | | BD Biosciences | |
| anti-Vδ2-PE-Vio770 | REA771 | | 130-111-012 | | Miltenyi Biotec | |
| anti-TIGIT-PE | MBSA43 | | 16-9500-82 | | eBioscience | |
| anti-TCR Vd1-FITC | REA173 | | 130-100-534 | | Miltenyi Biotec | |
| Phenotyping of dissociated mouse CT26 tumors (Figure 5) | | | | | | |
| anti-CD45-APC-eFluor780 | 30-F11 | 103138 | | | Bio Legend | |
| anti-CD4-FITC | RM4-5 | 11-0042-82 | | | eBioscience | |
| anti-CD8-PE-Cy7 | 53-6.7 | 25-0081-82 | | | eBioscience | |
| anti-FoxP3-APC | FJK-16s | 17-5773-82 | | | eBioscience | |
| anti-IFNɣ-APC | XMG1.2 | 17-7311-82 | | | eBioscience | |
| Measure of TIGIT expression on Sezary syndrome samples by flow cytometry (Figure 6) | | | | | | |
| anti-CD19-AF750 | J3-119 | A94681 | | | Beckman Coulter | |
| anti- CD3-Krome Orange | UCHT1 | B00068 | | | Beckman Coulter | |
| anti- CD3-PC5 | UCHT1 | A07749 | | | Beckman Coulter | |
| anti- CD4-PC7 | SFCI12T4D11 | 737660 | | | Beckman Coulter | |
| anti- CD4-PE | 13B8.2 | A07751 | | | Beckman Coulter | |
| anti- CD45-Pacific Blue | J33 | A74763 | | | Beckman Coulter | |
| anti- CD56-PC5 | N901 | A07789 | | | Beckman Coulter | |
| anti- CD8-PC7 | SFCI21Thy2D3 | 737661 | | | Beckman Coulter | |
| anti- TIGIT-APC | MBSA43 | 17-9500-42 | | | eBiosciences | |
| anti- Vb1-FITC | BL37.2 | IM2406 | | | Beckman Coulter | |
| anti- Vb13.1-FITC | IMMU 222 | IM1554 | | | Beckman Coulter | |
| anti- Vb13.6-FITC | JU74.3 | IM1330 | | | Beckman Coulter | |
| anti- Vb16-FITC | TAMAYA1.2 | IM1560 | | | Beckman Coulter | |
| anti- Vb17-FITC | E17.5F3.15.13 | IM1234 | | | Beckman Coulter | |
| anti- Vb18-PE | BA62.6 | IM2049 | | | Beckman Coulter | |
| anti- Vb2-FITC | MPB2D5 | IM2407 | | | Beckman Coulter | |
| anti- Vb20-FITC | ELL1.4 | IM1562 | | | Beckman Coulter | |
| anti- Vb7.2-FITC | ZIZOU4 | B06666 | | | Beckman Coulter | |