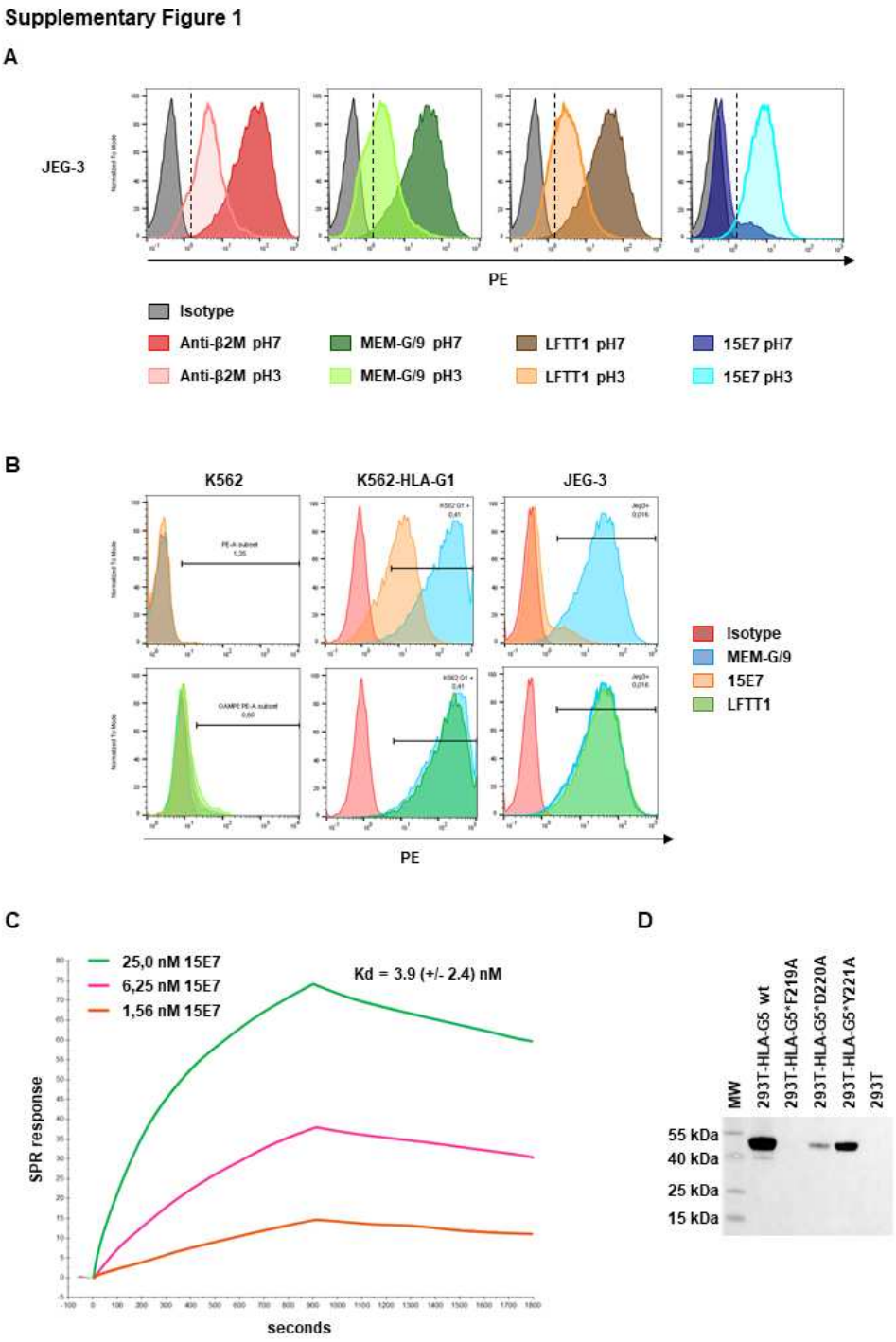
Supplementary materials



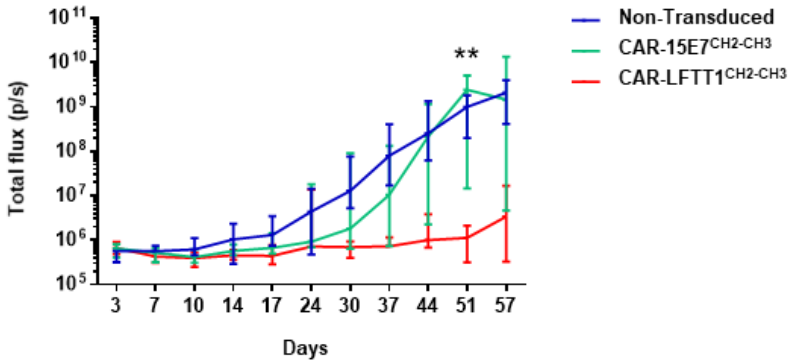
Supplementary figure 1:

Characterization of anti-HLA-G monoclonal antibodies. (**A**) LFTT1 monoclonal antibody is specific for HLA-G1/β2m-associated isoform whereas 15E7 monoclonal antibody is specific for HLA-G1/β2m-free isoforms. JEG-3 cells were incubated 2 minutes at pH=7 or pH=3 to remove the β2m association. Staining of JEG-3 cells was then performed using LFTT1 and 15E7 monoclonal antibodies in comparison to MEM-G/9 and isotype control. MEM-G/9 and LFTT1 mAbs are specific for HLA-G1/β2m-associated isoform (pH=7) whereas 15E7 mAb is specific for HLA-G1/β2m-free isoforms (pH=3). **(B)** A K562-HLA-G1 cell line expressing HLA-G1/β2m-associated and β2m-free isoform was generated. Representative figure of K562, K562-HLA-G1 and JEG-3 cell lines labelling with 15E7, LFTT1 and MEM-G/9 monoclonal antibodies in comparison to isotype control. (**C**) SPR analysis of 15E7 mAb Kd. 15E7 Kd was evaluated at 3.9nM. (**D**) Epitope mapping of 15E7 mAb. HLA-G amino acids specificity was determined on HLA-G5wt or on HLA-G5 proteins mutated for the HLA-G-α3 amino acids F, D and Y amino acids located at positions 195, 196 and 197 respectively. Each amino acids was replaced by an Alanine.

15E7 mAb was determined to be specific for amino acids F195 and D196 within the HLA-G-α3 domain.

Supplementary Figure 2

A



Supplementary figure 2:

Anti-HLA-G CAR-T cells mediated tumor control of K562-HLA-G1-Luciferase mouse model. Median of the Total Flux of each mouse in the 3 groups at different points. Significance was determined using a Mann-Whitney test (**:p<0,004) (n=6).

Supplementary Table I: Antibodies

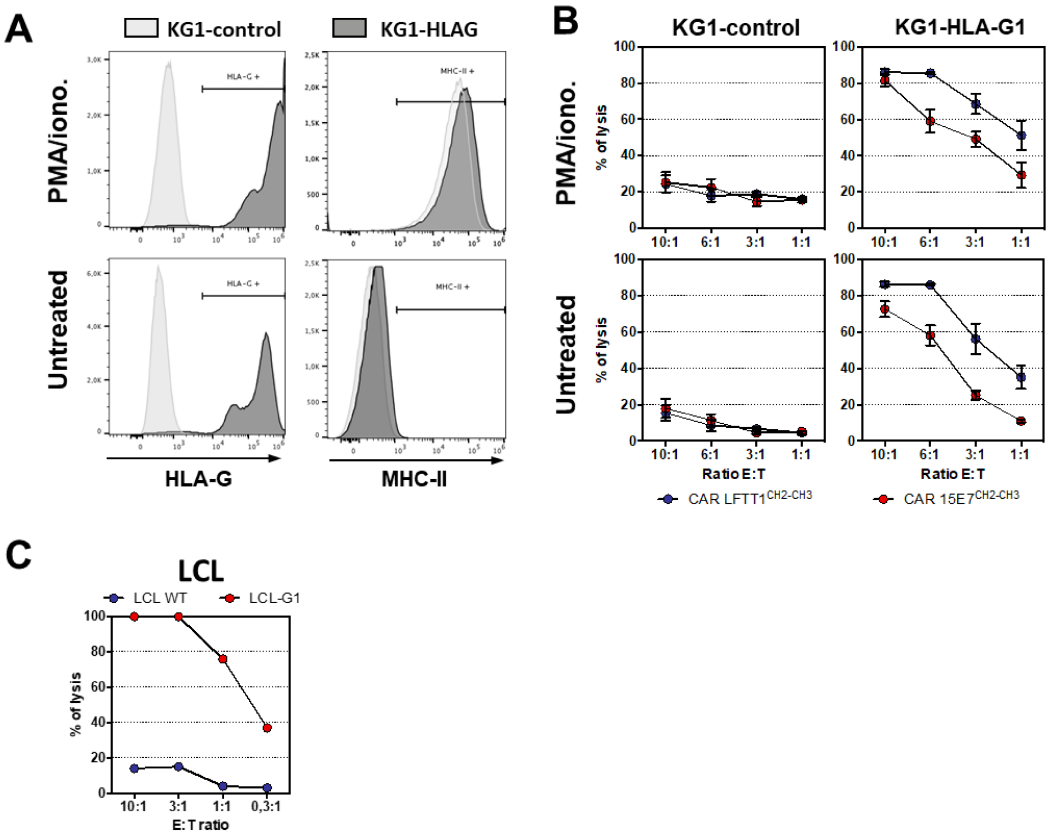
Cytometry

Antibody	Fluorochrome	Manufacturer
CD4	PerCP	BD Pharmingen
CD25	BV421	BD Horizon
CD69	BV711	
CD62L	BV421	
CD8	PE-Cy7	Biolegend
PD-1	APC	
CD19	FITC	Miltenyi Biotec
CD19	PE	
CD45RA	BV711	Invitrogen
Live/Dead	eFluor-780	Thermofisher Scientific
15E7	purified	Invectys
	PE	
LFTT1	purified	
MEM-G/9	purified	Exbio
	PE	
Anti-β2M	purified	Biolegend
GAM	PE	Biolegend

Western-Blot

Antibody		Manufacturer
GAM	HRP	Thermofisher Scientific
15E7	purified	Invectys

Supplementary figure 3:



(A) Differentiation of KG-1 cells into monocyte-derived Dendritic Cells. KG-1 cells or KG-1 expressing HLA-G1 (KG1-HLA-G1) were cultivated in presence of PMA/ionomycine or not (untreated) during 5 days. Conservation of HLA-G expression (clone MEM-G9, ThermoFisher) and MHC-II (clone L243, Biolegend) upregulation were monitored by FACS (Attune, ThermoFisher). **(B,C) Cytotoxicity of anti-HLA-G CAR-T cells against different cells lines.** CAR-T cells were cultivated in presence of target labelled-cells with different Effector : Target ratio during 24h. Then, cells were also labelled with 7-AAD and analyzed by FACS. Percentage of cell lysis was determined on conservation of Dye^{+}/$7\text{-}AAD^{-}$ population compared to a control well with target cell only. **(B)** Cytotoxicity of CAR $15E7^{CH2\text{-}CH3}$ and $LFTT1^{CH2\text{-}CH3}$ against differentiated or untreated KG-1 cells expressing or not HLA-G1 with different Effector : Target ratio. Mean of 3 different donors is represented. **(C)** Cytotoxicity of CAR $LFTT1^{CH2\text{-}CH3}$ against LCL, a lymphoblastoid cell line stably transduced with a lentiviral vector expressing HLA-G1 (-G1) or not (WT). (n=1)

Materials and methods

Antibodies

Two monoclonal antibodies were generated in-house for the purpose of this study: LFTT1 and 15E7, and their main characteristics are described in Supplementary Figure 1. LFTT1 monoclonal antibody was isolated following immunization of C57Bl/6J mice with K562-HLA-G1 cells and 15E7 monoclonal antibody was isolated following immunization of C57Bl/6J mice with the VTHHPVFDYEATLRC peptide, derived from HLA-G-α3 domain, coupled to KLH. Specificities of these monoclonal antibodies are detailed in Supplementary Table I.

15E7 specificity for HLA-G1/β2m-free isoform

Few days prior to the assay, JEG-3 cells were amplified. The cells were harvested, washed, and counted. Then, cells were incubated with 0.5mL of pH3 solution (0.2M citric acid-Na_2HPO_4) for 2 minutes, on ice, and the reaction was stopped by adding 50 mL of PBS. Before the distribution of cells into a 96-wells-plate, 1µL of Fc-Block (BD Bioscience) was added for 5.10^5 cells, incubated for 5 minutes, room temperature. Cells were labelled by MEM-G/9 (Exbio), LFTT1 (Invectys) and 15E7-PE (Invectys), the acquisition was performed with a MACSQUANT10 cytometer (Miltenyi Biotec) and results were analyzed with FlowJo software.

Epitope mapping for the 15E7monoclonal antibody

293T cells were transfected with recombinant proteins HLA-G5wt protein or HLA-G5 mutated for the HLA-G-α3 specific amino acids F195, D196 and Y197, and respectively referred as HLA-G5*F195A, HLA-G5*D196A and HLA-G5*Y197A. Transfected 293T cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (complete Mini EDTA-free; Roche, France). Proteins were separated by 12% SDS-PAGE (Invitrogen, CA, USA) and electro-transferred onto PVDF membranes (Thermo Fisher Scientific, MA, USA). The membranes were blocked by a 2-hour incubation with PBS (Sigma, MO, USA) containing 0.1% Tween20 and 5% non-fat dry milk. The membranes were then probed 2 hours with primary antibody 15E7 mAb (produced in-house) diluted at 1:2000 and washed in PBS 1x containing 0,1% Tween20. For immunodetection the membrane was subsequently probed with a secondary antibody: HRP-conjugated goat-anti-mouse IgG mAb (Thermo Fisher Scientific, MA, USA dilution 1:5000), incubated 2 hours at room temperature. Detection was carried out using

enhanced chemiluminescence reagent (Thermo Fisher Scientific, MA, USA), and detection was carried out with Chemidoc (BioRad, CA, USA).

Real-time characterization of 15E7 mAb/HLA-G interaction by surface plasmon resonance (SPR)

Experiments were performed using a Biacore T200 instrument (GE Healthcare) equilibrated at 25°C in PBS containing 1mg/ml BSA. Penta-His antibody (QiaGen) was covalently immobilized by amine coupling on two flowcells of a CM5 sensorchip (GE Healthcare), reaching a density of 6500 RU (1RU≈1 pg.mm^{-2}). Approximately 100RU of His6-tagged HLA-A (negative control) and HLA-G were then captured non-covalently from cell culture supernatants on each anti-His antibody derivatized flowcell. Different concentrations of 15E7 mAbs (4, 20 and 100nM) were then injected at 20µl/min for 900s over the two surfaces to monitor the association of the antibody/antigen complex, after which buffer was injected for another 900s to monitor the complex dissociation. Finally, the anti-His surfaces were regenerated by injecting 10mM Gly-HCL pH1.5 for 90s. Association and dissociation profiles were analyzed using the BiacoreT200 evaluation software, allowing to determine the association (k_{on}) and dissociation (k_{off}) rates of the interaction, as well as its equilibrium constant (K_d).

Activation profile assay

For KG-1 differentiation, 2x10^6 KG1-control and KG1-HLA-G1 cells were seeded in a 6-well plate in complete RPMI medium with PMA (10ng/ml, Sigma) and Ionomycine (100ng/ml, Sigma) or not during 5 days. At day 3, medium was renewed and in the case of PMA/ionomycine treatment, wells were washed 1x with PBS to remove non-adherent cells. At day 5, cells were harvested. In the case of PMA/ionomycine treatment, cells were detached with a treatment of PBS/0,1% EDTA during 10min at 37°C.

Prior cytotoxicity assay, 10^7 KG-1 cells were labelled with a CellTracker™ Deep Red Dye (Invitrogen) at 1µM in PBS during 30min then washed in complete medium. For cytotoxicity assay, 2.10^5 labelled KG-1 cells were seeded in U-bottom 96-well plates with different ratio of CAR-T cells. Cells were mixed and centrifuged during 1min at 100g before a 18h incubation at 37°C. Then, cells are washed in PBS and labelled with 7-AAD (250ng/ml, Biolegend) during 20min at room temperature and analyzed by FACS (Attune, ThermoFisher).

Similarly, prior to the assay, 3.10^4 LCL (721.221 B, ATCC) control and HLA-G^+ cells were labelled with 1µM of CFSE (CellTrace, Thermofisher) and seeded in round-bottom 96 wells. On the day of the assay, CAR-T Cells were added at various E:T ratios to either the plated CFSE-labelled LCL cells or the plated CFSE-labelled HLA-G^+ LCL cells. Cells were mixed and centrifuged during 1min at 100g before a 24h incubation at 37°C. Then, cells were washed in PBS and labelled with a Vioblue-conjugated anti-human CD45 antibody (clone REA747, Miltenyi) and a viability dye (LDCY, Invitrogen) during 30 min at 4°C. The acquisition was performed with a MACSQUANT16 cytometer (Miltenyi Biotec) and results were analyzed with FlowJo software.