SUPPLEMENTARY INFORMATION

T lymphocytes induce human cancer cells derived from solid malignant tumours to secrete galectin-9 which facilitates immunosuppression in cooperation with other immune checkpoint proteins

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Materials

RPMI-1640 and DMEM cell culture media, foetal bovine serum and supplements as well as basic laboratory chemicals were obtained from Sigma (Suffolk, UK). Microtiter plates for Enzyme-Linked Immunosorbent Assay (ELISA) were provided by Oxley Hughes Ltd (London, UK). Rabbit antibodies against galectin-9, VISTA, granzyme B and CD3 were purchased from Abcam (Cambridge, UK). Anti-actin antibodies were purchased from Proteintech (Manchester, UK). Goat anti-mouse and anti-rabbit fluorescence dye-labelled antibodies were obtained from Li-COR (Lincoln, Nebraska USA). Antibodies for flow cytometry recognising human CD4, CD8, Tim-3 and galectin-9 as well as neutralising/blocking antibodies (9S2-1 for galectin-9, RPA-T4 for CD4 and DK-25 for CD8) were purchased from BD Biosciences (NJ, USA) and Sigma (Suffolk, UK). ELISA-based assay kits/antibodies for the detection of galectin-9, VISTA, IL-2 and TGF-β were purchased from Bio-Techne (R&D Systems, Abingdon, UK). The anti-Tim-3 mouse monoclonal antibody used in this work was generated by Dr. Luca Varani [14]. All other chemicals used were of the highest grade of purity commercially available.

Cell lines and primary human cells/samples

Cell lines used in this work were purchased from either the European Collection of Cell Cultures, American Tissue Culture Collection or CLS Cell Lines Service GmbH. Cell lines were accompanied by identification test certificates. Wilms Tumour cell line WT3ab was kindly provided by Dr. C. Stock (Children's Cancer Research Institute, Vienna, Austria) and cultured as described previously [15].

Jurkat T cells, MCF-7, G401, WT-3ab, HaCaT keratinocytes and K562 were cultured in RPMI 1640 media supplemented with 10% foetal bovine serum, penicillin (50 IU/ml), and

streptomycin sulfate (50 μg/ml). LN18 cells were cultured in DMEM media supplemented with 10% foetal bovine serum, penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml).

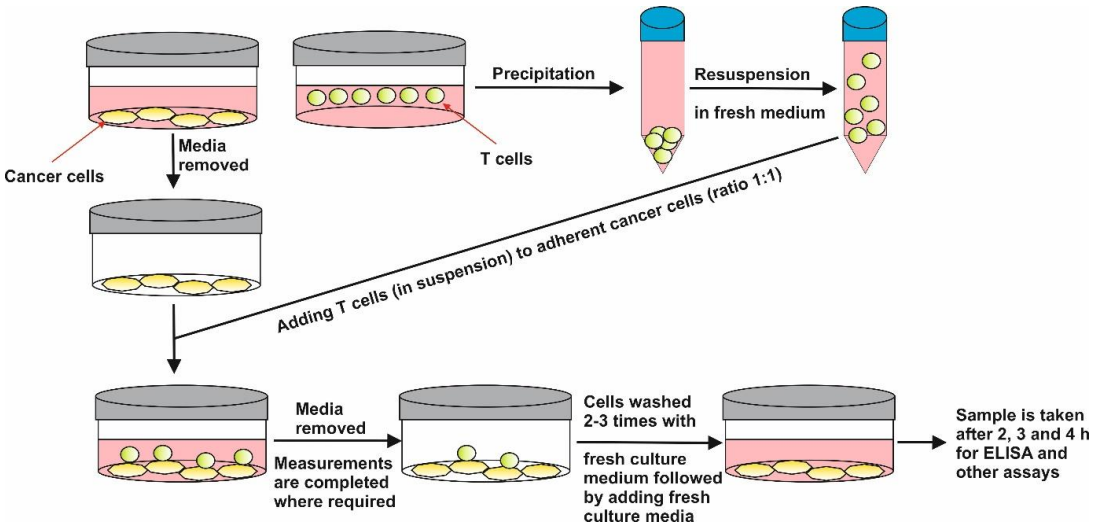
TALL-104 CD8-positive cytotoxic T lymphocytes, derived from human acute lymphoblastic leukemia (TALL), were cultured according to the ATCC instructions as described before [15]. Briefly, ATCC-formulated Iscove's Modified Dulbecco's Medium was used. To make the complete growth medium we added 100 units/ml recombinant human IL-2, 2.5 μg/ml human albumin, 0.5 μg/ml D-mannitol and 20% foetal bovine serum.

Placental tissues (CVS, chorionic villus sampling) and amniotic fluids were collected after obtaining informed written consent from pregnant women at the University Hospital Bern. Cells were prepared and cultured as described before [9].

Primary human or mouse T cells were isolated from PBMCs with a CD3 T cell negative isolation kit (Biolegend) as described before [7, 16] and resuspended in RPMI 1640 media supplemented with 10% foetal bovine serum, penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml) before co-culturing.

Adherent human cancer cells derived from solid malignant tumours and other adherent cells (HaCaT keratinocytes and primary human embryonic cells) were co-cultured for 16 h with T cells (Jurkat T, TALL-104, primary human or mouse T lymphocytes) at a ratio of 1:1. Adherent cells were cultured for 24 h prior to adding T cells. In order to ensure a 1:1 ratio, control adherent cells were cultured for 24 h and their numbers were counted using a Fuchs-Rosenthal counting chamber before plating. This was repeated 10 times for each cell type/line to ensure the correct calculation of increasing adherent cell numbers during 24 h. Primary T cells were centrifuged, resuspended in fresh medium and counted just before mixing with adherent cells. Upon completion of 16 h co-culture, conditioned cell culture medium was removed together with T cells (suspended cells). Remaining T cells and cell debris (if any)

were washed away 2-3 times using fresh cell culture medium. In some experiments, fresh medium was added to adherent cells and galectin-9 levels were analysed in this conditioned medium after 2, 3 and 4 h of culturing. The scheme of this process is presented in Supplementary figure 1.



Supplementary figure 1. Scheme demonstrating the procedure of co-culturing adherent cells with T lymphocytes used in this study.

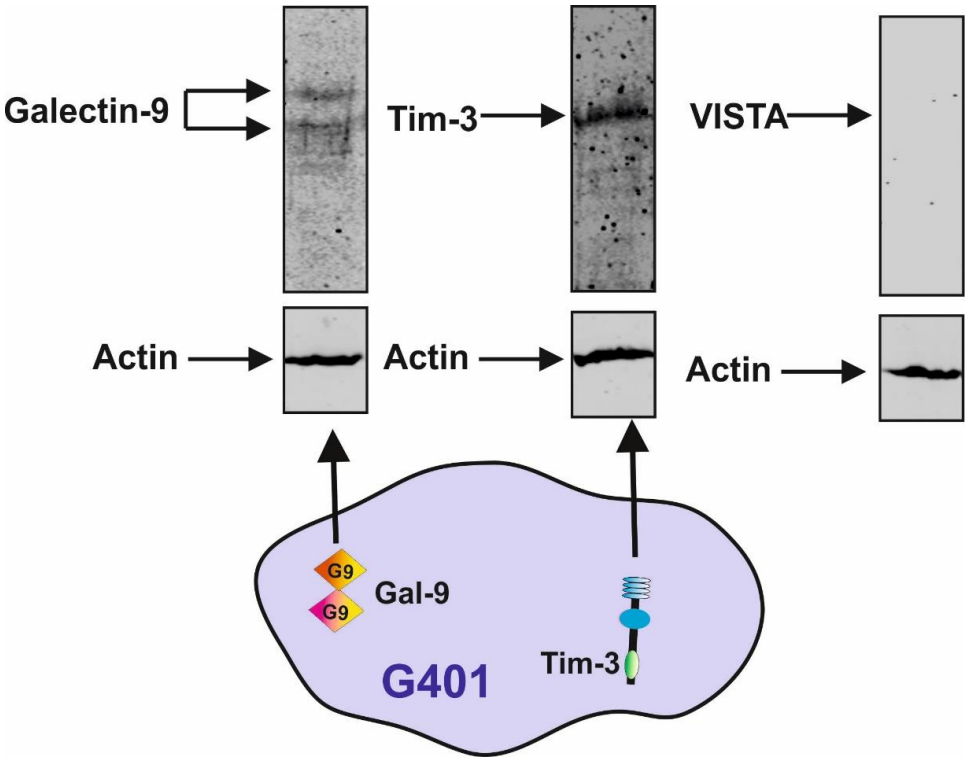
FACS analysis

FACS analysis of cell surface levels of CD4 and CD8 as well as Tim-3 and galectin-9 was performed as previously described [7]. Unstained cells or cells stained with isotype-matched IgG antibodies served as a negative control. Mean fluorescence intensities were quantitatively analysed (50 000 events for each variant) on Becton Dickinson FACS Calibur using Cell Quest Pro Software.

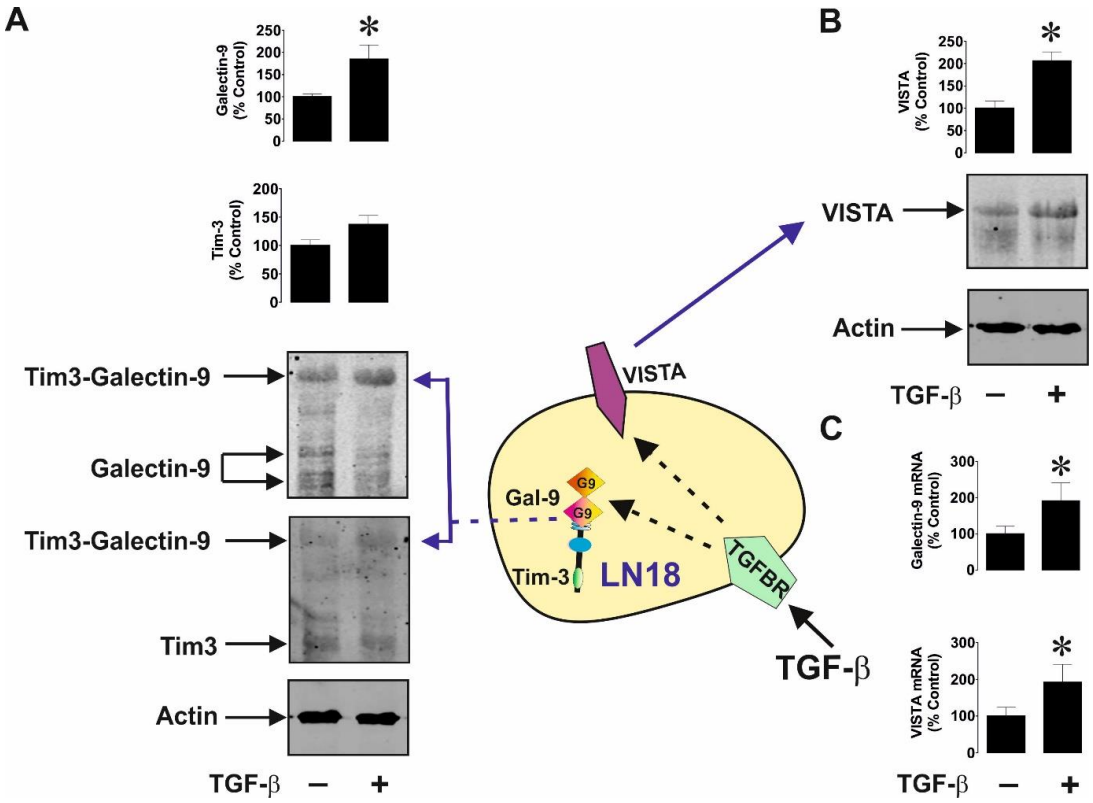
Mice

Six-week-old C57 BL16 mice (25 ± 2.5 g) were used for the indicated experiments. All procedures complied with the UK Animals (Scientific Procedures) Act (1986) and were performed under a UK Home Office Project Licence in accordance with University of Kent Policy on the Use of Animals in Scientific Research (approved by the University of Kent animal committee).

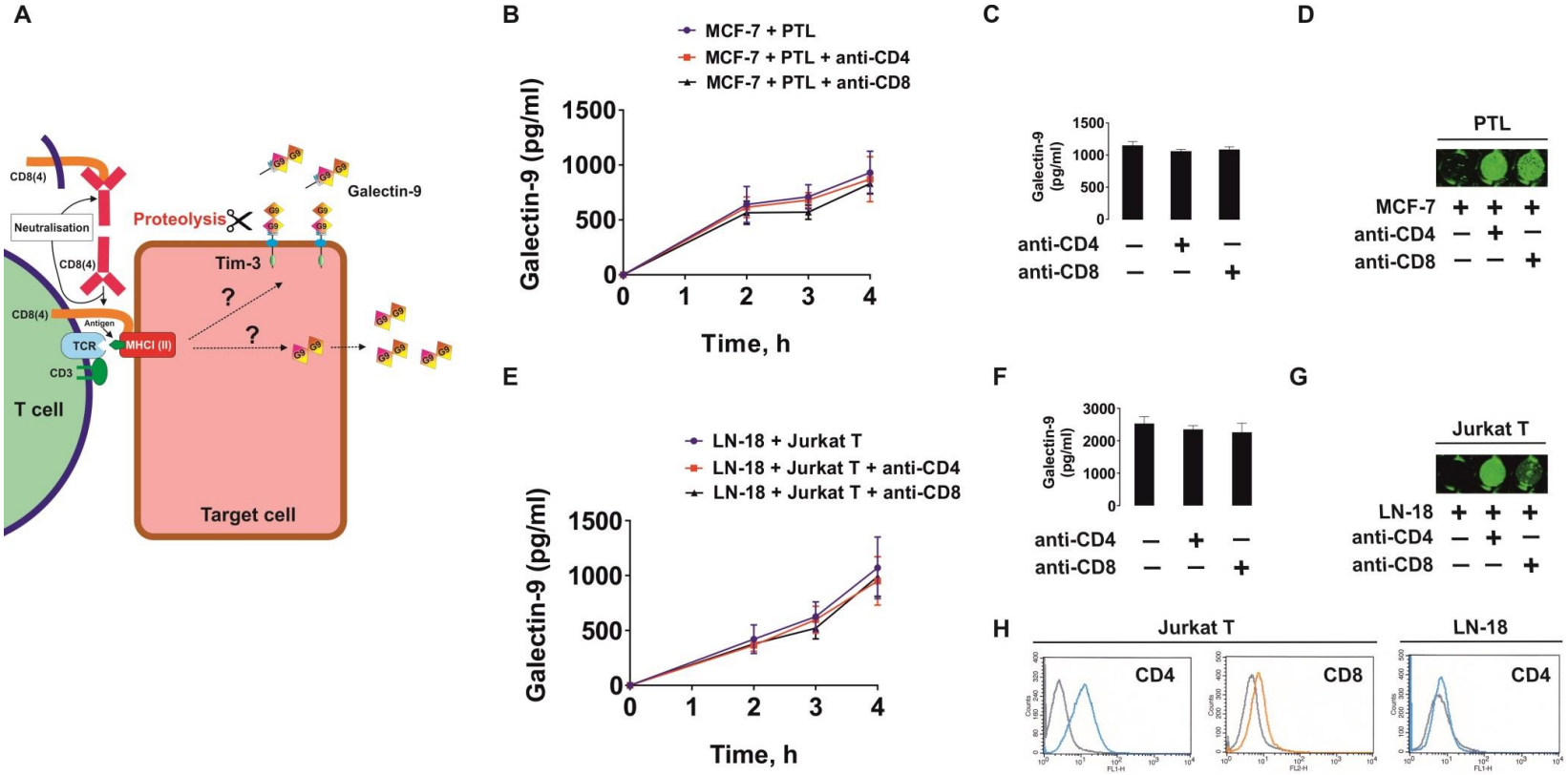
Animals were handled by authorised personnel in accordance with the Declaration of Helsinki protocols.



Supplementary figure 2. Expression of Galectin-9, Tim-3 and VISTA in G401 human kidney rhabdoid tumour cells. Expressions of Galectin-9, Tim-3 and VISTA were analysed in G401 cells using Western blot analysis. Images are from 1 experiment representative of 4 which gave similar results.



Supplementary figure 3. TGF-β induces expressions of galectin-9 and VISTA in LN-18 human glioblastoma cells. LN-18 cells were exposed to 2 ng/ml TGF-β for 24 h followed by analysis of expressions of galectin-9, VISTA and Tim-3 proteins as well as galectin-9 and VISTA mRNA levels. Images are from one experiment representative of four which gave similar results. Quantitative data represent mean values ± SEM of 4 independent experiments. *p < 0.05 vs control


A
CD8(4)
Neutralisation
CD8(4)
CD8(4)
Antigen
TCR
MHCI (II)
CD3
T cell
Proteolysis
Galectin-9
Tim-3
?
?
Target cell
B
MCF-7 + PTL
MCF-7 + PTL + anti-CD4
MCF-7 + PTL + anti-CD8
Galectin-9 (pg/ml)
1500
1000
500
0
0 1 2 3 4
Time, h
C
Galectin-9 (pg/ml)
1500
1000
500
0
anti-CD4 – + –
anti-CD8 – – +
D
PTL
MCF-7 + + +
anti-CD4 – + –
anti-CD8 – – +
E
LN-18 + Jurkat T
LN-18 + Jurkat T + anti-CD4
LN-18 + Jurkat T + anti-CD8
Galectin-9 (pg/ml)
1500
1000
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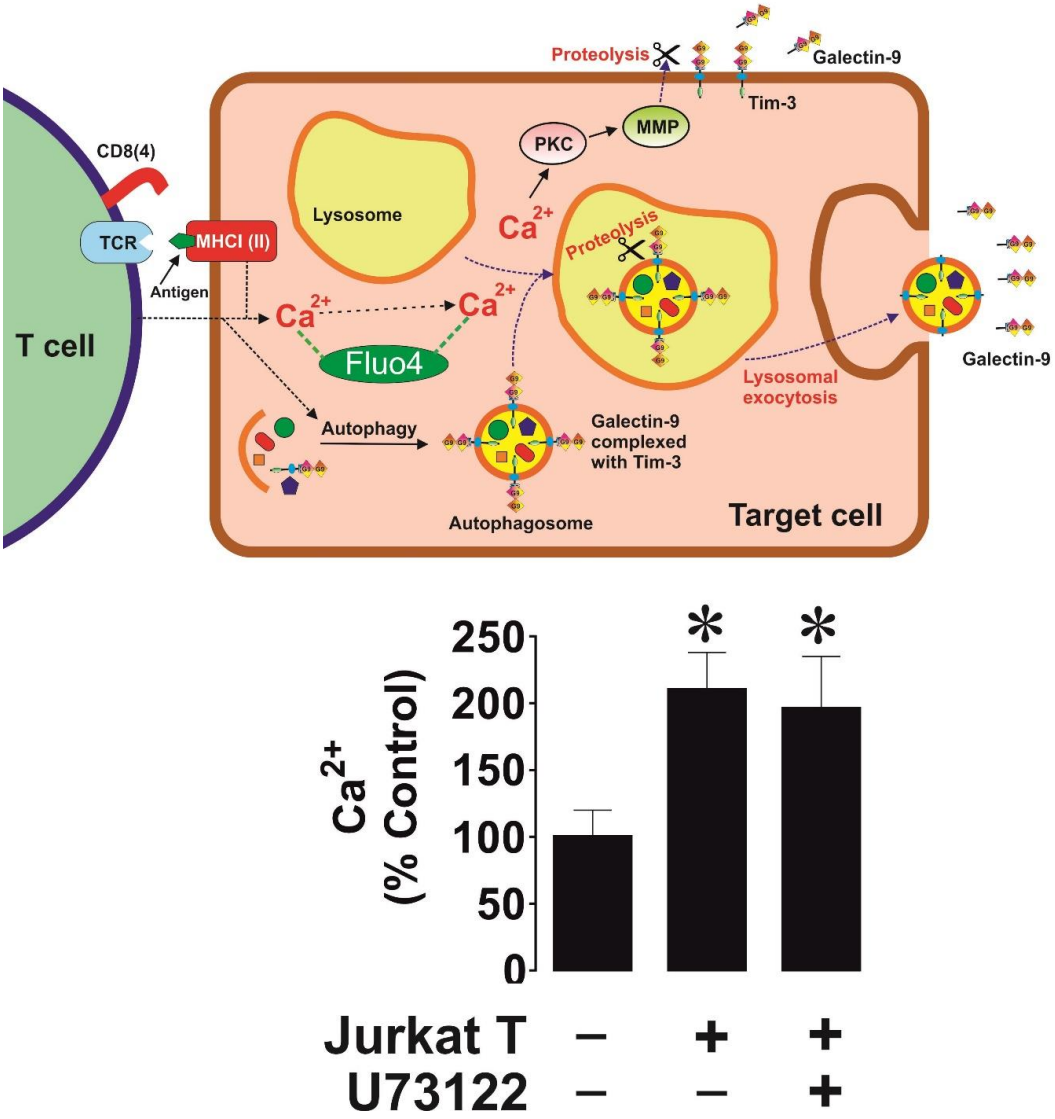
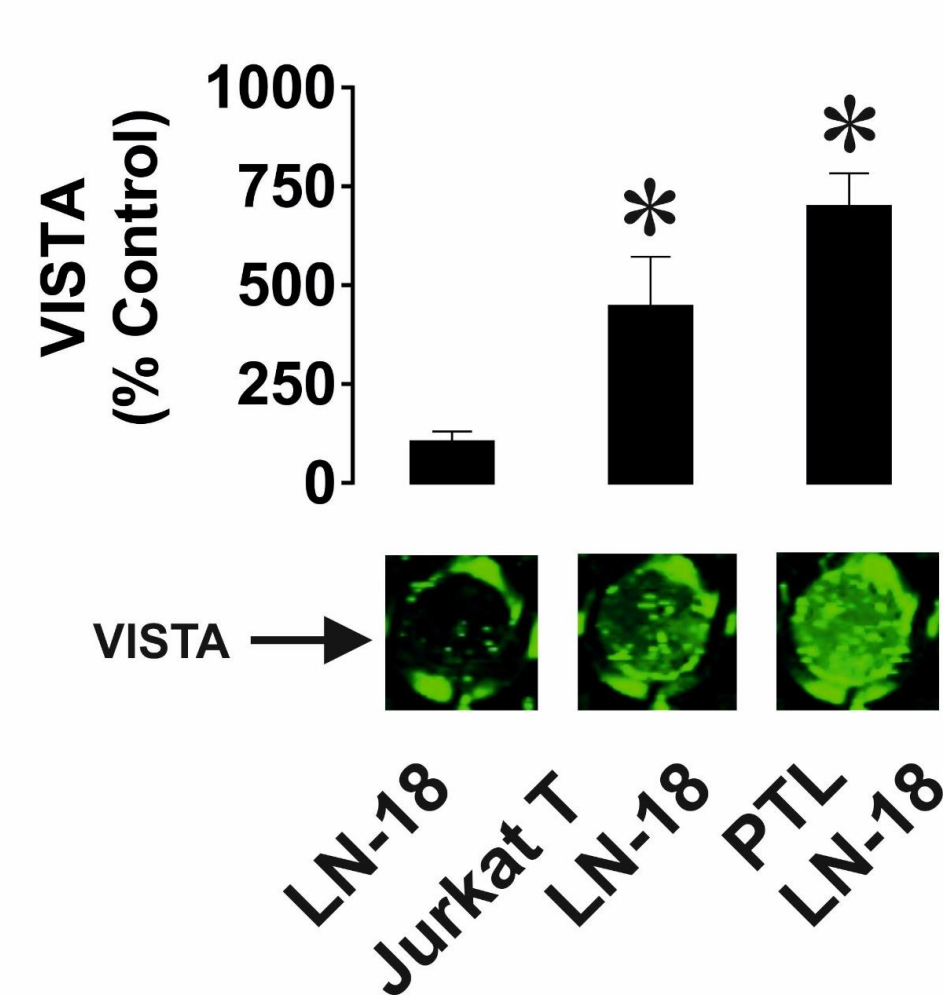
0 1 2 3 4
Time, h
F
Galectin-9 (pg/ml)
3000
2000
1000
0
anti-CD4 – + –
anti-CD8 – – +
G
Jurkat T
LN-18 + + +
anti-CD4 – + –
anti-CD8 – – +
H
Jurkat T
LN-18
CD4
CD8
CD4

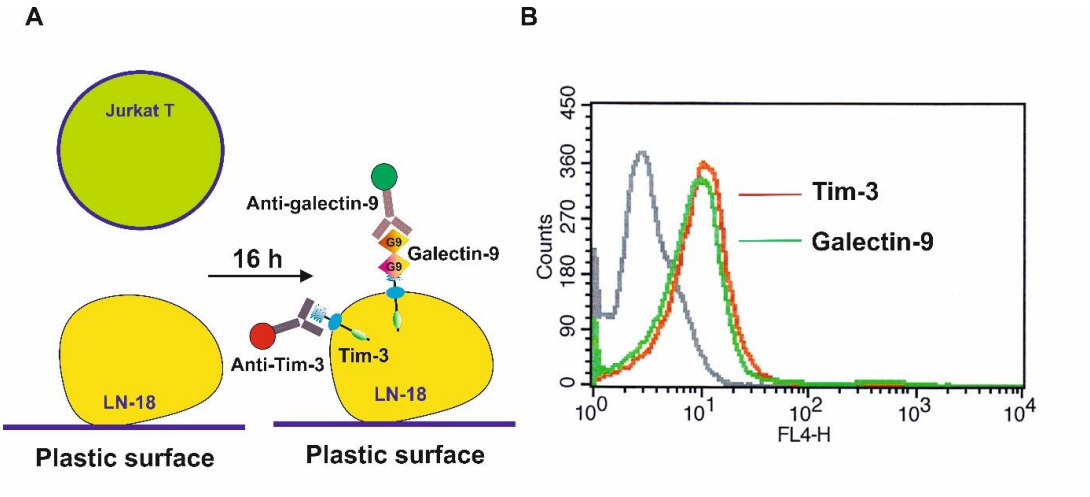
Supplementary figure 4. CD4 and CD8 proteins are not involved in T cell-induced galectin-9 secretion by human cancer cells derived from solid malignant tumours. **(A)** Human cancer cells derived from solid malignant tumours were co-cultured for 16 h with primary human T lymphocytes (PTL) or Jurkat T cells at a ratio of 1:1 in the absence or presence of CD4 or CD8 neutralising antibodies. Cells were then separated and washed with fresh culture medium followed by culture for 4 h. Galectin-9 released by MCF-7 cells co-cultured with PTL was measured at 2, 3 and 4 h **(B)** as well as in the medium collected after 16 h of co-culturing MCF-7 cells and PTL **(C)**. Binding of anti-CD4 and anti-CD8 to T cells was assessed using on-cell Western analysis (D). Galectin-9 released by LN-18 cells co-cultured with Jurkat T cells was measured at 2, 3 and 4 h and upon completion of co-culture (see above and point B) **(E)** as well as in the medium collected after 16 h of co-culturing LN-18 cells and Jurkat T cells **(F)**. Binding of anti-CD4 and anti-CD8 to Jurkat T cells was assessed using on-cell Western analysis with respective secondary antibodies (secondary antibodies alone were added to the control) **(G)**. The levels of CD4 and CD8 in Jurkat T cells and CD4 in LN-18 cells after co-culture were measured using FACS analysis (H). Images are from 1 experiment representative of five which gave similar results. Flow cytometry plots are from 1 experiment representative of 3 which gave similar results. Data are the mean values ± SEM from 4 independent experiments.



Supplementary figure 5. T cells trigger upregulation of intracellular calcium levels in MCF-7 human breast cancer cells. MCF-7 cells were co-cultured with Jurkat T cells for 16 h in the ratio 1:1. Intracellular calcium levels were measured in MCF-7 cells as outlined in Materials and Methods. Quantitative data represent mean values ± SEM of 6 independent experiments. *p < 0.05 vs control.



Supplementary figure 6. T cells induce translocation of VISTA onto the surface of LN-18 cells. LN-18 cells were co-cultured with Jurkat T or primary human T cells for 16 h in the ratio 1:1. VISTA levels on the cell surface were measured using on-cell Western analysis. Images are from one experiment representative of five which gave similar results. Quantitative data represent mean values ± SEM of 5 independent experiments. *$p < 0.05$ vs control.



Supplementary figure 7. Tim-3 and galectin-9 levels on the surface of LN-18 cells are similar upon completion of co-incubation with Jurkat T cells. (A) LN-18 cells were co-cultured with Jurkat T cells as described above. Cells were then separated and Tim-3 and galectin-9 levels on their surface were measured by FACS analysis **(B)**. Flow cytometry plots are from 1 experiment representative of 3 which gave similar results.