

ORIGINAL RESEARCH

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The immune receptor Tim-3 acts as a trafficker in a Tim-3/galectin-9 autocrine loop in human myeloid leukemia cells

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ABSTRACT

The immune receptor Tim-3 is often highly expressed in human acute myeloid leukemia (AML) cells where it acts as a growth factor and inflammatory receptor. Recently, it has been demonstrated that Tim-3 forms an autocrine loop with its natural ligand galectin-9 in human AML cells. However, the pathophysiological functions of Tim-3 in human AML cells remain unclear. Here, we report for the first time that Tim-3 is required for galectin-9 secretion in human AML cells. However, this effect is cell-type specific and was found so far to be applicable only to myeloid (and not, for example, lymphoid) leukemia cells. We concluded that AML cells might use Tim-3 as a trafficker for the secretion of galectin-9 which can then be possibly used to impair the anticancer activities of cytotoxic T cells and natural killer (NK) cells.

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Introduction

The T cell immunoglobulin and mucin domain 3 (Tim-3) is a plasma membrane-associated protein which is highly expressed in acute myeloid leukemia (AML) cells and is therefore considered as a potential target for anti-AML therapy.^{1,2} Although the biochemical activities of Tim-3 have recently been elucidated,³⁻⁷ the functional role of this protein/receptor in AML cells remains unclear and the reasons for striking increases in Tim-3 levels in these cells are also not yet understood. Galectin-9, which was found to be a natural Tim-3 ligand,^{3,5} was recently suggested to form an autocrine loop, where newly released galectin-9 interacts with Tim-3 expressed on the cell surface.³ Furthermore, in leukocytes Tim-3 is known to be shed by proteolytic enzymes including metalloproteinases.⁸ This leads to the dissociation of Tim-3 from the plasma membrane as a soluble protein, which then can be detectable in human blood plasma.^{8,9} The physiological role of this shedding remains unclear as well as the functions of soluble Tim-3. However, it is known that galectin-9 is capable of impairing the immunological activities of cytotoxic T cells as well as natural killer (NK) cells^{1,10,11} In cytotoxic T cells galectin-9 was found to induce apoptotic death,¹¹ while in NK cells it mainly affected cytokine production thus impairing their cytotoxicity function.¹⁰ These effects might therefore help AML cells to escape immune attack by both cytotoxic T cells and NK cells.^{1,10,11}

Given the above, we hypothesized that Tim-3 might act as a trafficker for galectin-9 in AML cells. We found that galectin-9 is expressed in the AML cell line THP-1, in primary human AML cells, and in primary healthy human leukocytes (although in this case the expression was considerably lower). Galectin-9 is capable of forming a stable complex with Tim-3 in cell lysates. We also detected that in THP-1 and U-937 cell lines as well as in primary healthy leukocytes the surface presence of Tim-3 is much lower compared to primary human AML cells. The same effect was observed for galectin-9. Galectin-9 knockdown in U-937 cells reduced their total Tim-3 levels. Furthermore, Tim-3 knockdown in U-937 cells did not show reductions in intracellular galectin-9 levels but galectin-9 secretion was dramatically reduced. We therefore suggested that Tim-3 is required for galectin-9 secretion in AML cells. Primary human chronic lymphoid leukemia (CLL) cells were also capable of secreting galectin-9 but expressed very low amounts of Tim-3. These results suggest that Tim-3 might be required for galectin-9 secretion in AML cells. Using ELISA, we were able to detect galectin-9 and soluble Tim-3 in human blood plasma obtained from healthy donors. Furthermore, capturing soluble Tim-3 on ELISA plates also led to detection of galectin-9. These results suggest the presence of Tim-3-galectin-9 complexes in human blood plasma but do not indicate whether they are formed inside secreting cells, on their surface, or constitutively present in blood plasma.

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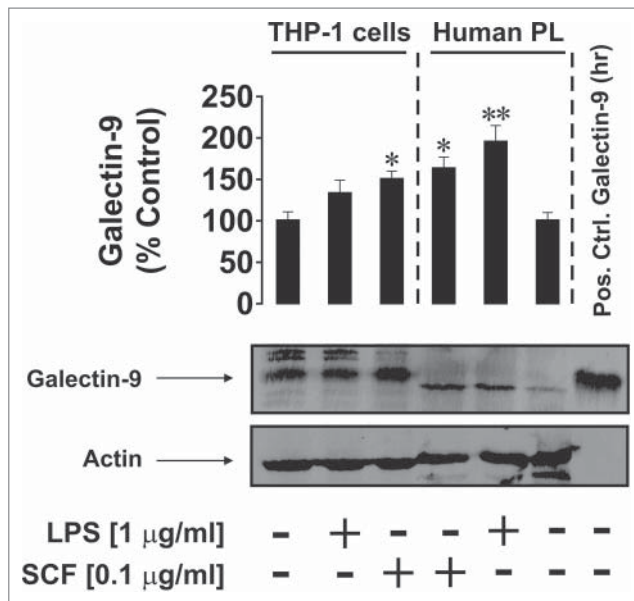


Figure 1. Effects of LPS and SCF on galectin-9 protein expression in THP-1 cells and PHL (abbreviated as PL in the figure). Cells were treated for 4 h with the indicated concentrations of LPS and SCF, then harvested and galectin-9 protein expression levels were analyzed by Western blot as outlined in Materials and Methods. Images are from one experiment representative of three which gave similar results. Human recombinant galectin-9 (R&D Systems) was used as a positive control. Data are mean values \pm SEM of three independent experiments; * p < 0.05; ** p < 0.01 vs. control.

Results

Galectin-9 is expressed in healthy primary human leukocytes and malignant human myeloid cells and forms a complex with Tim-3 in both cell types

We first assessed whether healthy primary human leukocytes (PHL) and malignant human myeloid cell lines express galectin-9 and if its expression could be affected by pro-inflammatory stimulation as well as growth factor-induced biological responses. For this purpose, we used PHL which were isolated from buffy coat blood obtained from healthy donors and THP-1 human myeloid leukemia cells which were reported to express galectin-9.⁶

We found that THP-1 cells produced more galectin-9 compared to PHL, however, lipopolysaccharide (LPS, ligand of pro-inflammatory Toll-like receptor (TLR) 4¹²) and stem cell growth factor (SCF—a major haematopoietic cytokine responsible for the promotion of leukemia progression¹³) significantly upregulated galectin-9 levels in PHL (LPS was however shown, to be a stronger activator). Interestingly, galectin-9 bands in PHL cells appeared slightly lower on the gel compared to THP-1 cells and the positive control (Fig. 1), suggesting potential differences in post-translational processing. Only SCF was able to significantly increase galectin-9 levels in THP-1 cells (Fig. 1).

We then asked whether galectin-9 is capable of forming a complex with Tim-3 in cell lysates. For this purpose, we coated MaxiSorp ELISA plates with rabbit capture antibody directed against galectin-9 followed by the addition of cell lysates (see Materials and Methods). After incubation, galectin-9 was extracted and detected by Western blot analysis using anti-galectin-9 rabbit antibody. Tim-3 was also detected using highly specific mouse antibody. We found that in both THP-1

and PHL cells Tim-3 and galectin-9 gave positive staining at above 70 kDa which corresponds to the sum of Tim-3 and galectin-9 molecular weights detected in these cells by Western blot analysis (Fig. 2). Traces of free galectin-9 were still detectable, while Tim-3 was almost undetectable. The fact that SDS-PAGE electrophoresis did not separate these proteins indicates the formation of a stable complex. Since this did not occur with recombinant proteins expressed in *E. Coli* (which lack glycosylation), it is reasonable to surmise that the lectin (galectin-9) binds to sugar moieties on Tim-3 protein. The fact that both un-complexed proteins were detected by Western blot in fresh cell lysates suggests the requirement of both time and the presence of a surface (a solid phase—ELISA plate bottom in our case) to form such a steady complex.

Next, we analyzed the cellular distribution of Tim-3 and galectin-9 comparing intracellular levels with cell-surface expressions. We used THP-1 and U-937 human myeloid cell lines, primary human AML cells (AML-PB001F) as well as PHL. We measured the total amounts of both proteins using in-cell Western assay in comparison to the amounts present on the cell surface using in-cell assay (also called on-cell assay; as described in the Materials and Methods). We observed that resting THP-1, U-937, and PHL cells expressed substantially more Tim-3 and galectin-9 intracellularly than on the surface. In contrast, primary AML cells had comparatively greater surface presence of both proteins (Fig. 3). Interestingly the ratios of $\text{Tim-3}_{\text{surface}}/\text{Tim-3}_{\text{total}}$ and $\text{galectin-9}_{\text{surface}}/\text{galectin-9}_{\text{total}}$ were quite similar to each other in all studied cell types (Fig. 3).

Tim-3 is required for galectin-9 release in human myeloid leukemia cells

We next asked whether Tim-3 is required for galectin-9 release and used U-937 cells (higher siRNA transfection efficiency could be reached with this cell line using our DOTAP transfection method) to investigate our hypothesis. We also studied if galectin-9 is required for Tim-3 production. First, we knocked down galectin-9 expression in U-937 cells as outlined in Materials and Methods. Non-treated normal and galectin-9 knockdown U-937 cells as well as those treated for 4 h with 2 µg/mL anti-Tim-3 stimulatory antibody⁵ were analyzed. Successful knockdown of galectin-9 was confirmed by Western blot analysis (Fig. 4A) as well as quantitative real-time reverse transcription PCR (qRT-PCR; Fig. S1). We found that galectin-9 knockdown reduced cellular Tim-3 levels as well as affecting galectin-9 levels (both intracellular and released (Fig. 4A)). Previously, we reported that Tim-3 activates the mTOR pathway resulting in upregulation of glycolysis and release of pro-angiogenic vascular endothelial growth factor (VEGF). We found that galectin-9 knockdown upregulated stimulatory phosphorylation of mTOR (position S2448, Fig. 4B). This result was in line with the upregulation of glycolysis and VEGF release (Fig. 4B). Interestingly, we could not observe a significant increase in intracellular levels of methylglyoxal (MGO, Fig. 4B) in either case, indicating that the efficiency of glycolysis correlates with the activity of glycolytic enzymes, since MGO is spontaneously formed out of dihydroxyacetone phosphate when glycolytic enzymes are oversaturated. MGO catalyzes

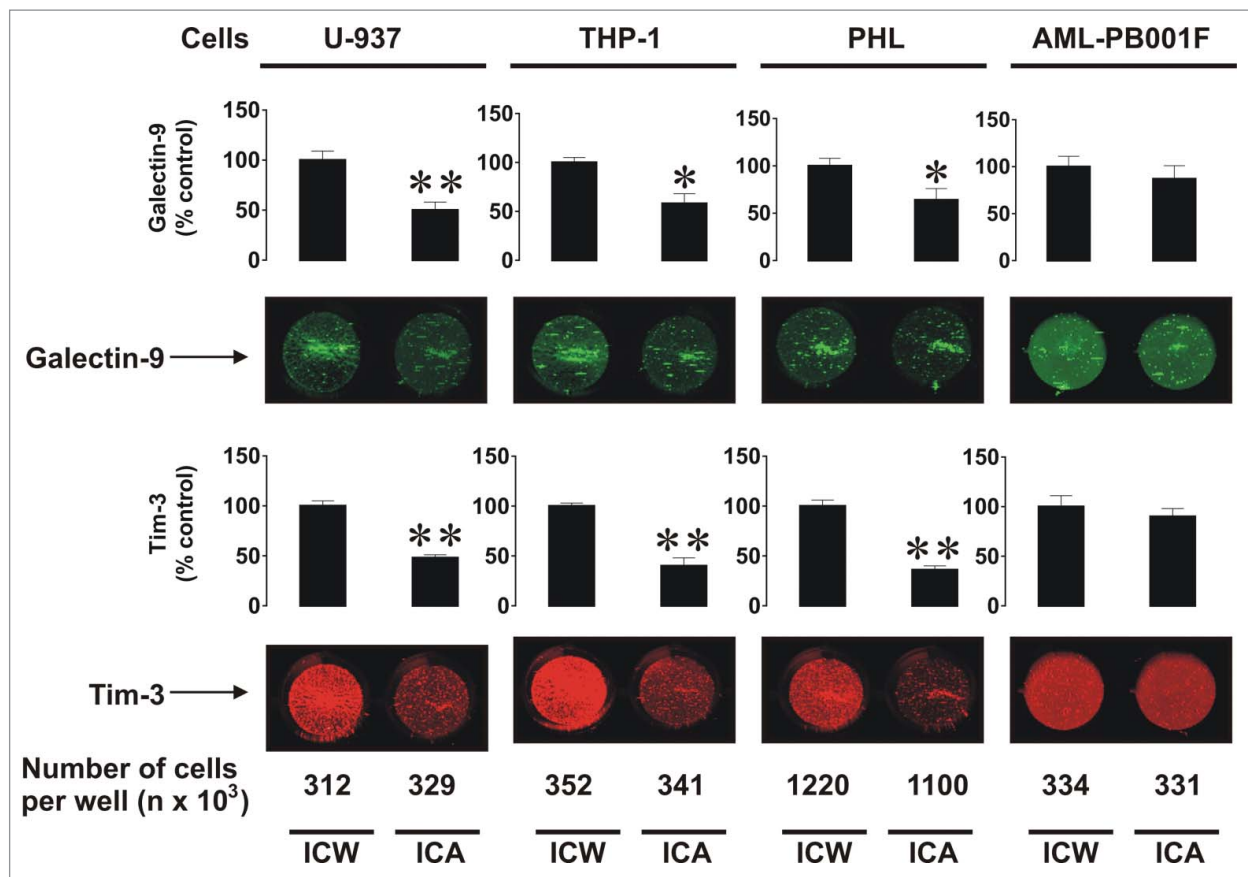


Figure 3. Comparative analysis of total and surface-based Tim-3 and galectin-9 proteins in human myeloid leukemia cell lines, PHL, and primary human AML cells. The indicated numbers of resting U-937 and THP-1 cells, PHL and primary human AML cells (AML-PB001F) were subjected to in-cell assay (ICA, detecting surface proteins) or in-cell Western (ICW, total protein detection) analysis as described in Materials and Methods. Images are from one experiment representative of five which gave similar results. Data are mean values \pm SEM of five independent experiments; * $p < 0.05$; ** $p < 0.01$ vs. control.

galectin-9. In AML cells, but not in CLL cells, the amount of secreted galectin-9 was significantly increased by 4 h of exposure to 2 μ g/mL anti-Tim-3 (Fig. 6A). Both PHL and AML cells expressed Tim-3 but this expression was much higher in AML cells compared to PHLs (Tim-3/actin ratios were analyzed, Fig. 6B). CLL cells expressed almost undetectable amounts of Tim-3 but still they were capable of releasing galectin-9, indicating that they probably use a different trafficker for this protein.

Finally, we observed that blood plasma of healthy donors contained both galectin-9 and soluble Tim-3 as measured by ELISA. Intriguingly, when capturing Tim-3 on the ELISA plate and applying detection antibody directed against galectin-9 we were able to detect a clear signal (Fig. 6C) which was not applicable when intact cell culture medium or galectin-9 standards were used. This means that a complex formed by non-cell-associated soluble Tim-3 and galectin-9 is present in the blood plasma of healthy donors. Our previous experiments demonstrated that anti-Tim-3 antibody does not compete with galectin-9.⁵

Discussion

Galectin-9 is a typical galectin family member which has a tandem structure.^{10,14} It has been reported to specifically interact with the immune receptor Tim-3 inducing mTOR

pathway activation as well as forming an autocrine loop with Tim-3 in human AML cells.^{3,5} Due to very high expression levels of Tim-3 in AML cells this protein is currently considered as a target for anti-AML therapy. Furthermore, galectin-9 was reported to specifically target cytotoxic T cells and NK cells and impair their ability to attack and kill leukemia cells.^{10,11,15}

Our present study clearly shows that human myeloid leukemia cells express and secrete galectin-9 (Figs. 1, 3, and 7 and Fig. S1). Importantly, galectin-9 production is upregulated by pro-inflammatory stimulation (LPS) and growth factor (SCF) action in both healthy human leukocytes and AML cells (THP-1 cell line). Release of galectin-9 was clearly dependent on the Tim-3. Tim-3 knockdown in U-937 cells led to significant reductions in galectin-9 secretion compared to normal (wild type) U-937 cells (Fig. 5A). Tim-3 protein levels were also reduced (but not significantly) in galectin-9 knockdown U-937 cells compared to wild type cells (Fig. 4A). However, Tim-3 mRNA levels were significantly downregulated in galectin-9 knockdown U-937 cells (Fig. S1) suggesting that transcription of the Tim-3 gene depends on intracellular levels of galectin-9. Interestingly, Tim-3 knockdown did not affect intracellular galectin-9 levels in U-937 cells, as measured by Western blot analysis, but dramatically reduced the capability of these cells to secrete galectin-9. These results suggest that galectin-9 levels influence Tim-3 expression at a genomic level, whereas Tim-3

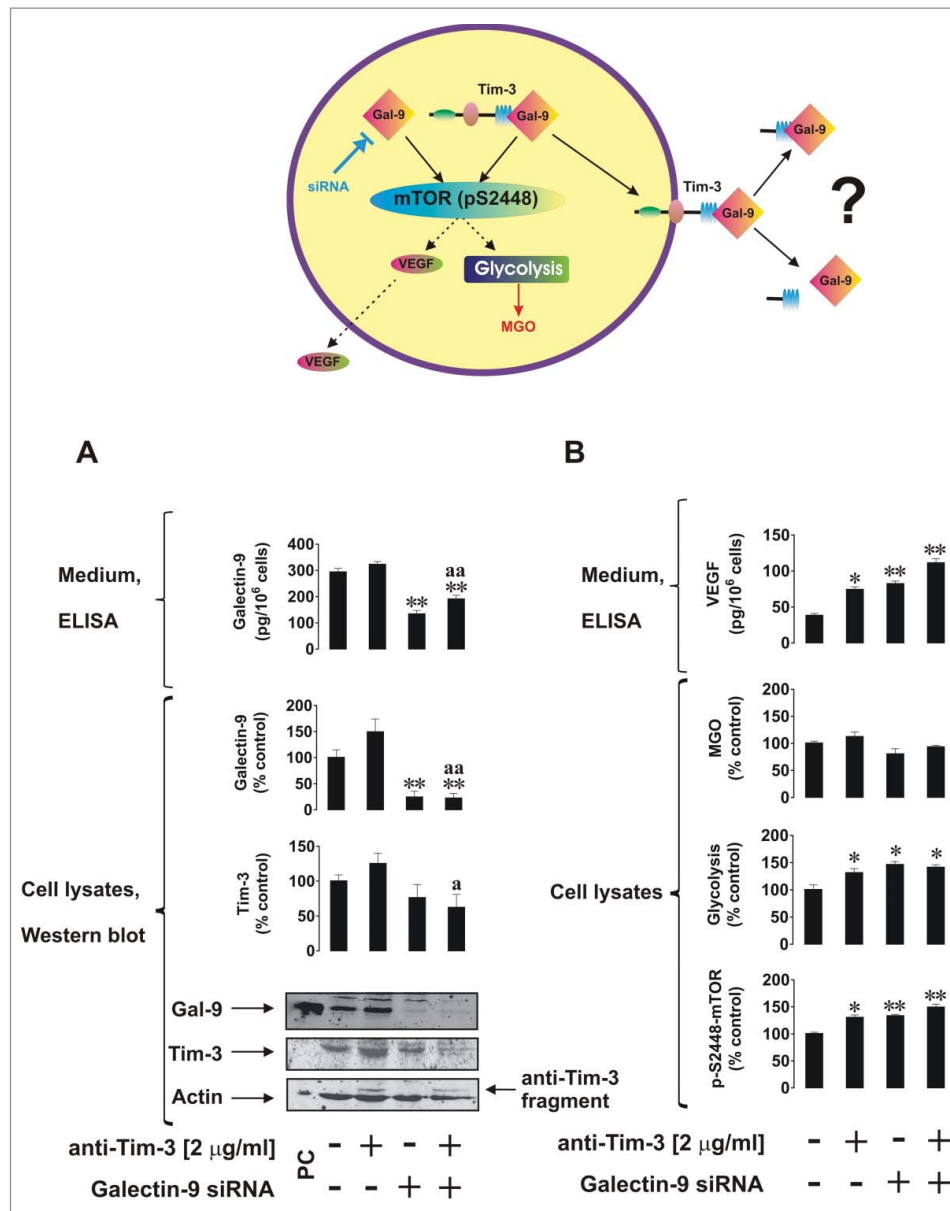


Figure 4. The effects of galectin-9 knockdown on Tim-3 expression and the mTOR pathway. Non-treated normal (wild type) and galectin-9 knockdown U-937 cells, as well as those which underwent 4 h treatment with 2 μg/ml anti-Tim-3 stimulatory antibodies, were analyzed for Tim-3 and galectin-9 levels, galectin-9 release (A), pS2448 mTOR intracellular levels, glycolysis, MGO, and VEGF secretion (B). Images are from one experiment representative of three which gave similar results. Data are mean values ± SEM of 3–5 independent experiments; * $p < 0.05$; ** $p < 0.01$ vs. control; ^a $p < 0.05$; ^{aa} $p < 0.01$ vs. anti-Tim-3.

protein controls the capability of myeloid leukemia cells to secrete galectin-9.

Recent evidence demonstrated that **A** disintegrin **and** metalloproteases (ADAM) 10 and 17 control shedding of Tim-3 from the cell surface in HEK-293 cells and human primary CD14⁺ monocytes.⁸ This suggests that Tim-3 might undergo shedding when it is in complex with galectin-9, which allows dissociation of the Tim-3 fragment together with the full-length galectin-9 protein. This hypothesis might explain why LPS reduces cellular Tim-3 surface expression^{6,8} taking into account our results demonstrating LPS-induced upregulation of galectin-9 levels. Our previous study demonstrated that Tim-3 protein production is not reduced by LPS⁵ while surface presence is affected.^{6,8} This might be the result of increased intracellular galectin-9 levels leading to enhanced galectin-9 secretion

which, as we have described above, requires Tim-3. ADAM10/17 or other proteolytic enzymes can then possibly shed the Tim-3/galectin-9 complex. Further evidence also supports the rapid shedding process such as *via* ligand-induced Tim-3-mediated activation of the mTOR pathway.

Although Tim-3 is a weaker signal transducer compared to Kit (SCF receptor) or TLR4, a moderate level of mTOR activation could be the result of swift removal of the extracellular domain which terminates the signal prematurely compared to those observed for non-cleaved receptors (as TLR4 or Kit). Furthermore, in support of a Tim-3/galectin-9 complex shedding scenario, other factors, such as HIV-1 infection, lead to increases in both soluble Tim-3 and galectin-9 levels in blood plasma.^{9,16} Also, phorbol 12-myristate 13-acetate (PMA) was found to upregulate galectin release in Jurkat T cells,¹⁷ where

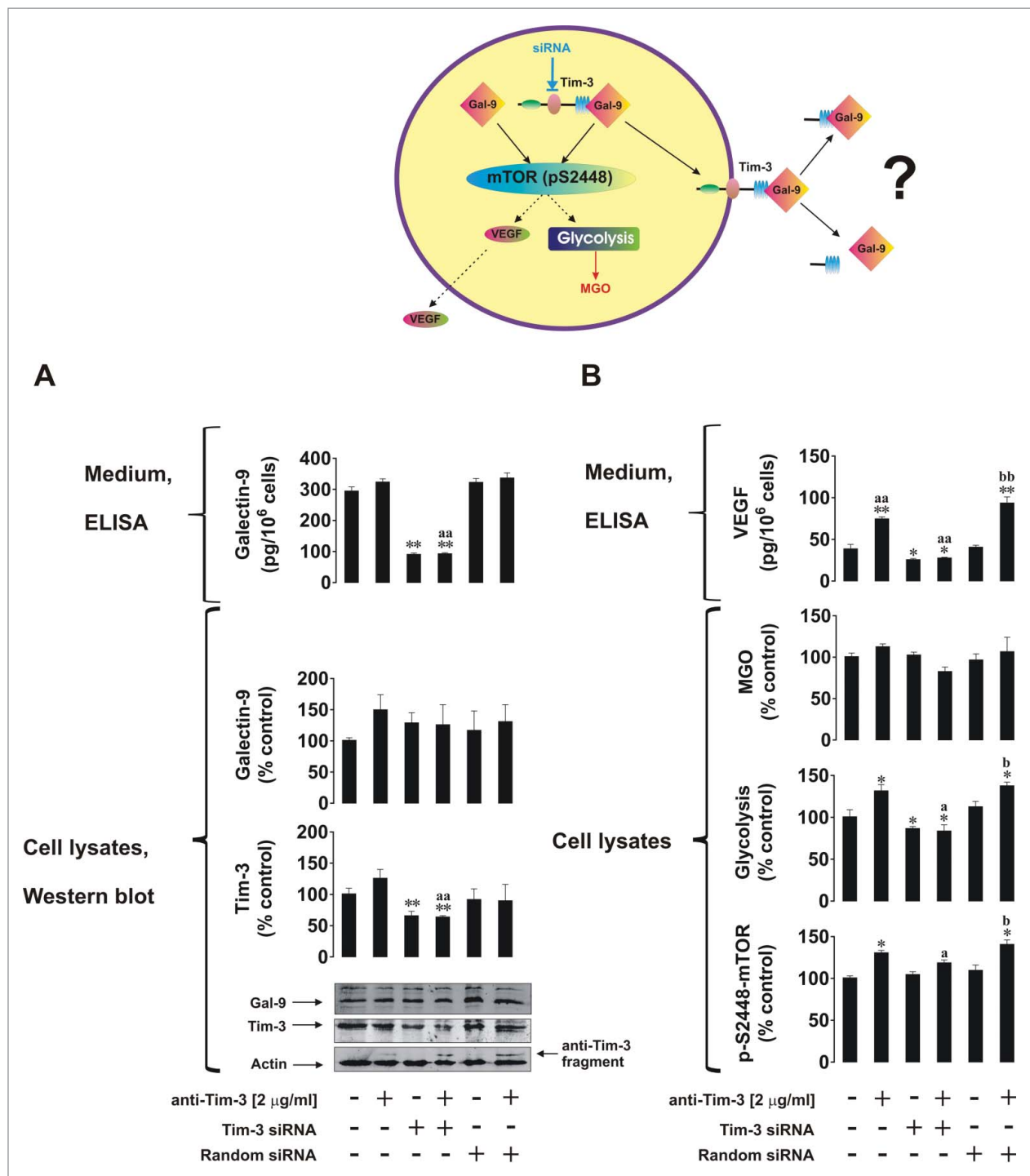


Figure 5. Tim-3 is required for galectin-9 secretion in human myeloid leukemia cells. Non-treated normal (wild type), Tim-3 knockdown and transfected with random siRNA U-937 cells, as well as the same cells which underwent 4 h treatment with 2 μg/mL anti-Tim-3, were subjected to analysis for Tim-3 and galectin-9 levels, galectin-9 release (A), pS2448 mTOR intracellular levels, glycolysis, MGO, and VEGF secretion (B). Images are from one experiment representative of three which gave similar results. Data are mean values ± SEM of 3–5 independent experiments; **p* < 0.05; ***p* < 0.01 vs. control; ^a*p* < 0.05; ^{aa}*p* < 0.01 vs. anti-Tim-3; ^b*p* < 0.05; ^{bb}*p* < 0.01 vs. random siRNA.

it also upregulates Tim-3 expression/externalisation.¹⁸ PMA is also known to promote Tim-3 proteolytic shedding.⁸ Matrix metalloproteinase inhibitor BB-94 (ADAM10 was also found to be sensitive to it¹⁹) was able to abolish PMA-induced galectin-9 release in Jurkat T cells (immortalised T lymphocytic cell line derived from T cell leukemia¹⁷), suggesting that proteolysis is probably involved in shedding Tim-3 complexed with galectin-9.

Our results also demonstrated that CLL bone marrow mononuclear cells (containing elevated levels of CD4⁺ T cells with a cytotoxic phenotype²⁰) are capable of secreting galectin-9 but they only produce very low amounts of Tim-3 (Figs. 6A and B). This suggests that Tim-3 is a cell type-specific trafficker of galectin-9 in AML cells, while other cell types (such as CLL bone marrow mononuclear cells) might use other galectin-9-specific proteins, which can be externalized. In support of this,

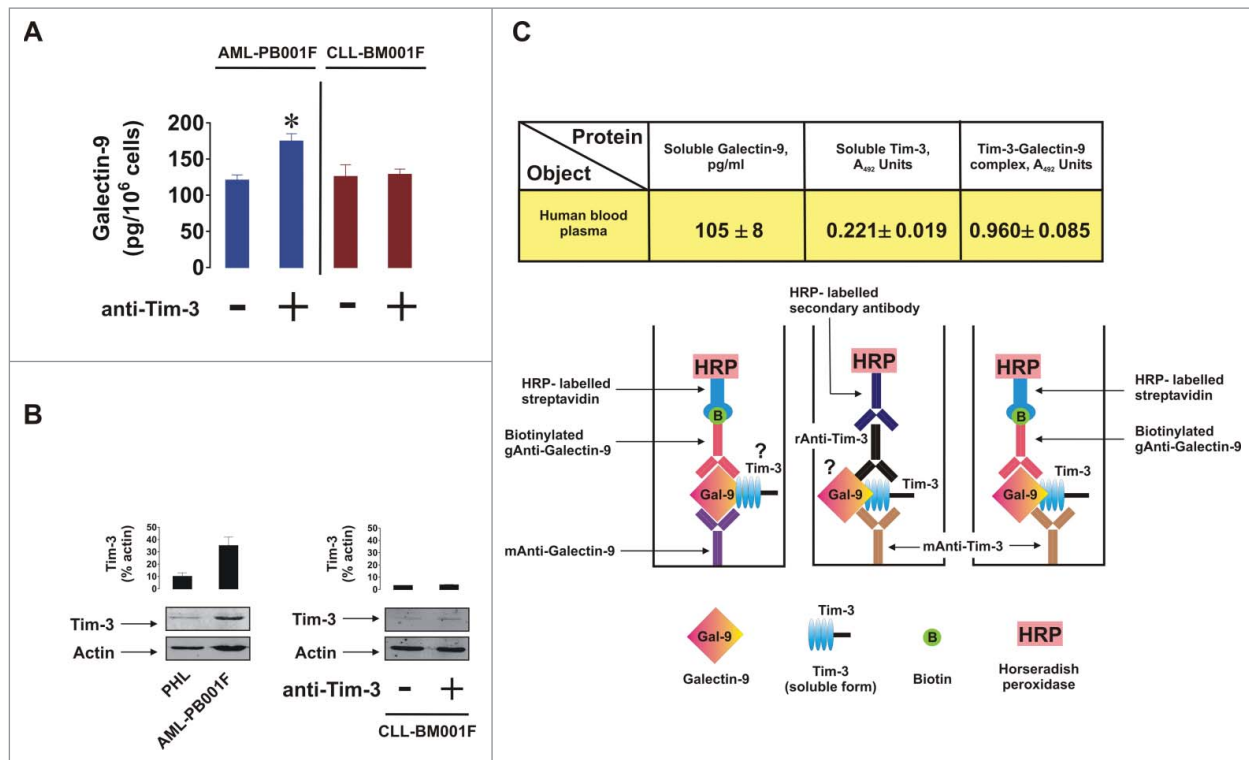


Figure 6. Galectin-9 and Tim-3 levels in primary human AML, CLL, and PHL cells as well as in blood plasma. (A) Non treated primary human AML and CLL cells, as well as those exposed for 4 h to anti-Tim-3, were precipitated and medium was subjected to measurement of galectin-9 by ELISA. Data represent mean values ± SEM of three independent experiments; **p* < 0.05 vs. control. (B) PHL, primary human AML, and CLL cells were subjected to Western blot analysis for Tim-3. Images are from one experiment representative of four which gave similar results. Data are mean values ± SEM of four independent experiments. (C) Blood plasma of six healthy donors was subjected to ELISA assays for galectin-9, soluble Tim-3, and Tim-3-galectin-9 complex (for details, please see Materials and Methods and diagrams underneath the table). Data are mean values ± SEM of 3–5 independent experiments.

there is evidence that galectin-9 may cause Tim-3-independent effects (including apoptosis) in lymphoid cells.^{10,21}

In human blood plasma obtained from healthy donors, we found that both galectin-9 and soluble Tim-3 are present (Fig. 6C). However, anti-Tim-3 detection antibodies also gave

rise to a positive signal to visualize the product captured by anti-galectin-9 antibody (Fig. 6C). This clearly indicates that a Tim-3/galectin-9 complex is present in human blood plasma. However, our results do not provide any indication at which point the complex was formed.

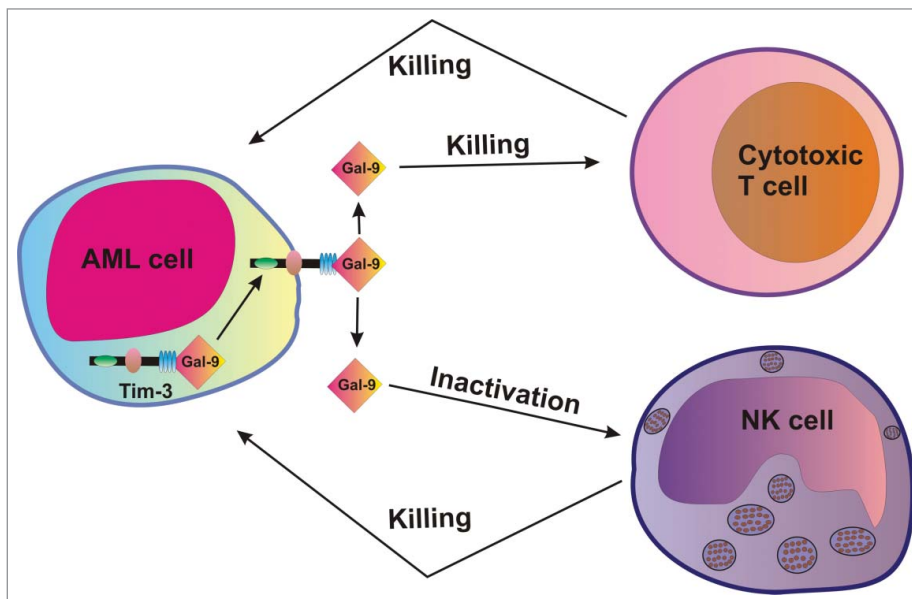


Figure 7. Tim-3-dependent secretion of galectin-9 in human AML cells could potentially impair the anti-leukemic activity of cytotoxic T cells and NK cells.

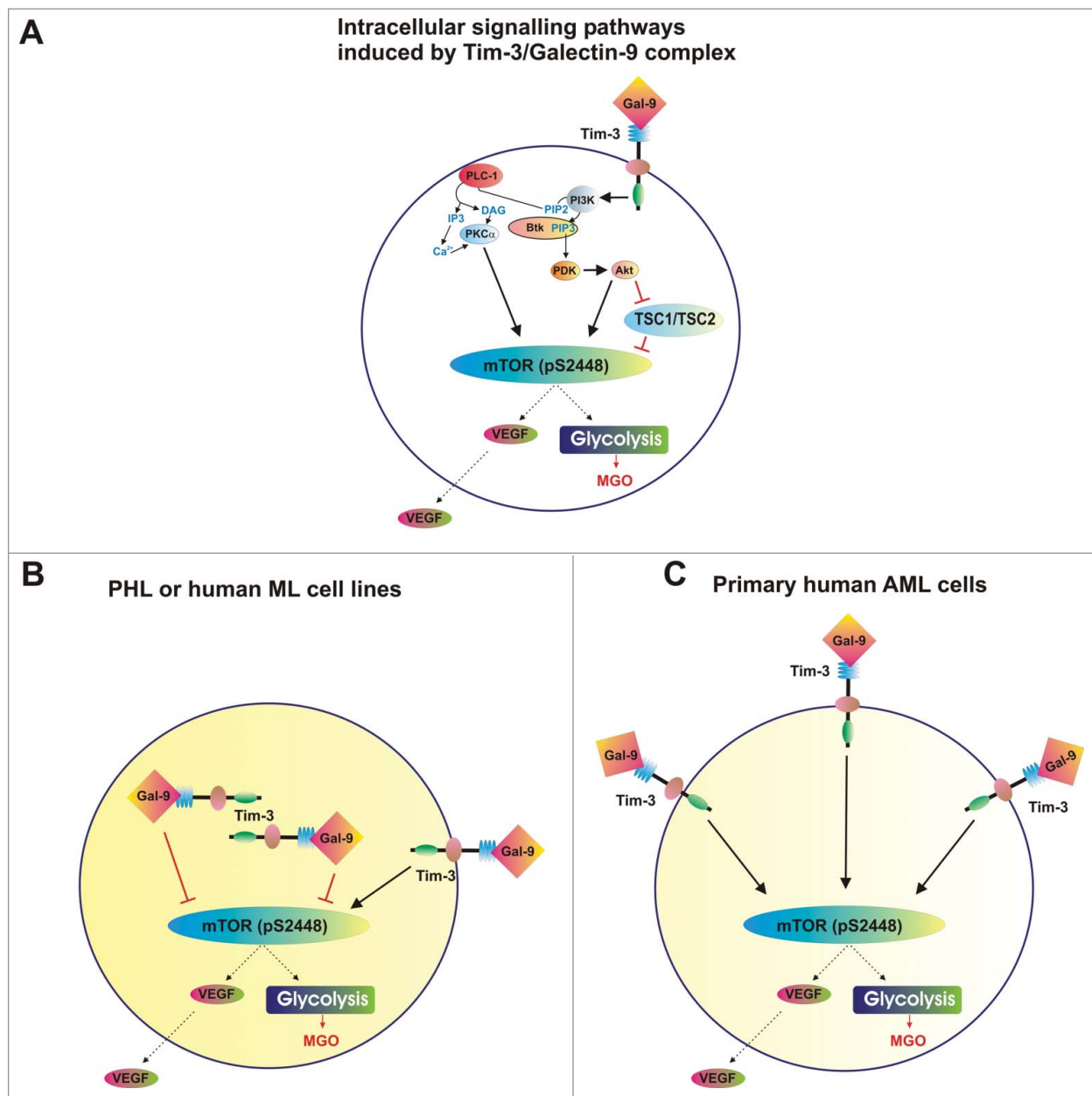


Figure 8. Distribution of complexes and corresponding effects of Tim-3 and galectin-9 in healthy and malignant human white blood cells. (A) Scheme demonstrating intracellular signalling pathways induced by Tim-3-galectin-9 complexes. (B) Tim-3/galectin-9 distribution and corresponding effects in PHL and human myeloid leukemia cell lines (THP-1 and U-937). (C) Tim-3/galectin-9 distribution and corresponding effects in primary human AML cells.

Our results suggest that Tim-3 is required for galectin-9 secretion in AML cells and this mechanism may be then used to impair the anticancer activity of cytotoxic T cells and NK cells, which might allow malignant blood cells to escape immune attack. Our hypothesis is summarized in the Fig. 7.

Our findings also demonstrated that decrease in intracellular galectin-9 level increases mTOR activity and its downstream effects (efficient glycolysis and VEGF production, Fig. 4A). In myeloid leukemia cell lines as well as in PHL, the activity of the mTOR pathway is always lower than in primary AML cells.^{4,5} One of the reasons for this might be externalisation of the Tim-3/galectin-9 complex in primary AML cells (confirmed in our studies—see Fig. 3). In this case, while there are lower amounts

of galectin-9 inside the cells Tim-3/galectin-9 complexes are externalized on the cell surface, which can initiate signaling events before further modification or proteolytic shedding. These effects are presented in Fig. 8, which summarizes Tim-3 signal transducing activities in PHL/AML cell lines compared with primary human AML cells.

Our results also highlight that both Tim-3 and galectin-9 are promising therapeutic targets for possibly curing AML. Absence of the Tim-3/galectin-9 autocrine loop or the timely capture and subsequent inactivation of biologically active galectin-9 may reduce the ability of AML cells to impair the anticancer activities of NK cells and cytotoxic T cells. This may therefore result in the natural eradication of cancer cells by functional NK cells and cytotoxic T cells.

Materials and methods

Materials

RPMI-1640 medium, foetal bovine serum and supplements, LPS (from *P. aeruginosa*), DOTAP transfection reagent, primers and galectin-9 silencing RNA (siRNA) were purchased from Sigma (Suffolk, UK). Maxisorp™ microtitre plates were provided either by Nunc (Roskilde, Denmark) or were a kind gift of Oxley Hughes Ltd (London, UK). Mouse monoclonal antibodies directed against mTOR and β -actin as well as rabbit polyclonal antibodies against phospho-S2448 mTOR, galectin-9, Tim-3, and HRP-labeled rabbit anti-mouse secondary antibody were purchased from Abcam (Cambridge, UK). Goat anti-mouse and goat anti-rabbit fluorescence dye-labeled antibodies were obtained from Li-Cor (Lincoln, Nebraska USA). ELISA-based assay kits for the detection of VEGF were purchased from Bio-Techne (R&D Systems, Abingdon, UK). All other chemicals purchased were of the highest grade of purity.

Primary human AML and CLL cells

Primary human AML mononuclear blasts (AML-PB001F, newly diagnosed/untreated) were purchased from AllCells (Alameda, CA, USA) and handled in accordance with manufacturer's instructions. Primary human bone marrow derived CLL mononuclear cells (CLL-BM001F, newly diagnosed/untreated leukemia) were also obtained from AllCells (Alameda, CA, USA) and handled in accordance with manufacturer's protocol. Cells from six different patients were analyzed following ethical approval (REC reference: 16-SS-033).

THP-1 and U937 human myeloid cell lines

THP-1 human myeloid leukemia monocytes and U937 human myeloid monocytes were obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 media supplemented with 10% foetal bovine serum, penicillin (50 IU/mL) and streptomycin sulfate (50 μ g/mL).

Primary human leukocytes obtained from healthy donors (buffy coats)

PHL were obtained from buffy coat blood (originated from healthy donors undergoing routine blood donation) which was purchased from the National Health Blood and Transfusion Service (NHSBT, UK) following ethical approval (REC reference: 16-SS-033). Mononuclear-rich leukocytes were obtained by Ficoll-density centrifugation according to the manufacturer's protocol. Cell numbers were counted using a haemocytometer and diluted accordingly with HEPES-buffered Tyrode's solution before the indicated treatments.⁴

Stem cell factor

Human SCF protein was produced in *E. Coli* and purified in accordance with previously published protocols.²⁰

Transfer of Galectin-9/Tim-3 siRNA into U-937 cells and qRT-PCR

We employed a galectin-9-specific siRNA target sequence (uga ggu gga agg cga ugu ggu ucc c) which was previously described.¹⁴ For Tim-3 knockdown, we used commercially available siRNA purchased from Santa Cruz Biotechnology, CA, USA, as described before.⁷ As a control, we used corresponding random siRNA (uac acc guu agc aga cac c dtdt²²). Transfection into U-937 cells was performed using DOTAP reagent according to the manufacturer's protocol.

To monitor galectin-9 and Tim-3 mRNA levels, we used qRT-PCR.²¹ Total RNA was isolated using a GenElute™ mammalian total RNA preparation kit, followed by a target protein mRNA reverse transcriptase-polymerase chain reaction (RT-PCR) performed in accordance with the manufacturer's protocol (Sigma). This was followed by quantitative real-time PCR. Primer selection was as follows: Galectin-9, 5'-CTTTCAT-CACCACCATTCTG-3', 5'-ATGTGGAACCTCTGAGCACTG-3' Tim-3, 5'-CATGTTTTTCACATCTTCCC-3' actin, 5'-TGACG GGGTCACCCACACTGTGCCCATCTA-3', 5'-CTAGAAGC ATTTGCGGTCG-ACGATGGAGGG-3'. Reactions were performed using a LightCycler® 480 real-time PCR system and respective SYBR Green I Master kit (obtained from Roche, Burgess Hill, UK). Analysis was performed according to the manufacturer's protocol. Values representing galectin-9 and Tim-3 mRNA levels were normalized against β -actin.

Western blot analysis

Expressions of Tim-3 and galectin-9 were determined by Western blot analysis and compared to β -actin in order to determine equal protein loading, as previously described.⁴ Li-Cor goat secondary antibodies, conjugated with fluorescent dyes, were used in accordance with manufacturer's protocol to visualize target proteins (using a Li-Cor Odyssey imaging system). Western blot data were quantitatively analyzed using Odyssey software and values were subsequently normalized against those of β -actin.

Detection of phospho-S2448 mTOR in cell lysates by ELISA

Phosphorylation of mTOR was analyzed by ELISA as described before.²³ Briefly, 96-well ELISA plates were coated with mouse anti-mTOR antibodies followed by blocking with 2% BSA. Cell lysates were then added to the wells and incubated at room temperature for 2 h (with constant agitation). Plates were then washed with Tris-Buffered Saline containing Tween 20 (TBST). Following washing with TBST buffer plates were incubated with HRP-labeled goat anti-rabbit IgG for 1 h at room temperature. Plates were then washed using TBST and bound secondary antibodies were visualized by the peroxidase reaction (ortho-phenylenediamine/H₂O₂).

In-cell Western and in-cell (on-cell) assay

We employed a standard Li-Cor in-cell Western assay to analyze total Tim-3 and galectin-9 expressions in the studied cells.⁴ The in-cell (also called on-cell) assay was employed to

characterized Tim-3 and galectin-9 surface presence in the studied cells.

Characterization of Tim-3 and galectin-9 interactions in cell lysates

An ELISA-based analysis was used where plates were first coated with anti-galectin-9 antibody followed by blocking with 2% BSA. Cell lysates were then applied and incubated for at least 4 h at room temperature after which plates were extensively washed with TBST. A glycine-HCl pH lowering buffer (pH 2.0) was then applied to extract the bound proteins. Extracts were mixed with equal volumes of lysis buffer (pH 7.5) and with 4× sample buffer for SDS-PAGE at a ratio of 1:3. Samples were then subjected to Western blot analysis, using rabbit anti-galectin-9 and mouse anti-Tim-3 primary antibodies, as described above. The plate was subjected to analysis with galectin-9 detection antibody followed by standard ELISA steps to ensure that no galectin-9 remained in the wells. Signals obtained were similar to those where PBS was used instead of the sample confirming that all the protein was extracted.

Detection of galectin-9 and VEGF secretion, soluble forms of Tim-3 and Tim-3-galectin-9 complexes

Galectin-9 and VEGF levels released into the medium were analyzed by ELISA (R&D Systems assay kits) according to the manufacturer's instructions. Soluble Tim-3 was detected using similar approach where mouse anti-Tim-3 (mAnti-Tim-3) was employed as a capture antibody and rabbit anti-Tim-3 (rAnti-Tim-3)—for detection (Fig. 6C, scheme). HRP-labeled anti-Rabbit secondary antibody (Abcam) was used to visualize the reaction. Tim-3-galectin-9 complexes in blood plasma were detected by ELISA. Mouse anti-Tim-3 (mAnti-Tim-3) was employed as a capture antibody and biotinylated goat anti-galectin-9 (gAnti-Galectin-9, R&D Systems) as detection antibody. The reaction was visualized using HRP-labeled streptavidin (R&D Systems; Fig. 6C, scheme). In all cases plates were washed using TBST and bound secondary antibodies visualized by the peroxidase reaction (ortho-phenylenediamine/H₂O₂).

Characterization of glycolysis and MGO levels

Glycolytic degradation of glucose was analyzed using a colorimetric assay as described before.²⁰ Briefly, the approach was based on the ability of cell lysates, used as a multi-enzyme preparation, to convert glucose into lactate in the absence of oxygen (this was achieved by employing an anaerobic chamber). Cell lysates were incubated for 1 h at 37°C with 1% glucose solution within the anaerobic chamber. 2% trichloroacetic acid solution was then used to precipitate proteins. This was followed by carbohydrate precipitation using saturated CuSO₄ solution in combination with Ca(OH)₂ (at a final concentration of 60 mg/mL). Lactate was then converted into acetic aldehyde using concentrated H₂SO₄ at 90°C for 1 min and cooled the samples down on ice. We then detected acetaldehyde using the veratrole (1,2-dimethoxybenzene) test. MGO was also detected colorimetrically following biochemical modifications.^{24,25} Briefly, MGO was condensed with reduced glutathione (GSH, 1 mM)

for 10 min at 37°C. The complex was then converted into lactate by Glyoxalases I and II at pH 8.0 (Glyoxalase I converted the complex in D-lactoyl-L-glutathione, which was then transformed into lactate by Glyoxalase II²⁶). The whole process is highlighted in Fig. S2). Lactate was then measured colorimetrically as described above.

Statistical analysis

Each experiment was performed at least three times and statistical analysis was conducted using a 2-tailed Student's *t* test. Statistical probabilities (*p*) were expressed as **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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