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Cortisol facilitates immune escape of human acute myeloid leukaemia cells by inducing latrophilin 1 expression

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Progression of acute myeloid leukaemia (AML) – the most severe blood/bone marrow cancer – is determined by the ability of malignant cells to escape host immune surveillance. However, the systemic regulation mechanisms underlying this phenomenon remain largely unknown. In this study we have discovered a fundamental systemic biochemical strategy which allows AML cells to employ physiological body systems to survive and escape immune attack. We found that AML cells use a crucial human adrenal cortex hormone (cortisol) to induce the expression of the neuronal receptor latrophilin 1 (LPHN1) which facilitates exocytosis. This receptor interacts with the blood plasma protein FLRT3 (fibronectin leucine rich transmembrane protein 3) to cause secretion of the immune suppressor galectin-9 which impairs the anti-cancer activities of cytotoxic lymphoid cells.

AML is a cancer of the blood and bone marrow which originates from self-renewing malignant immature myeloid cells and rapidly becomes a systemic, and very often fatal, malignancy. AML cells employ body systems to produce factors required for their proliferation/disease progression. This includes employment of stem cell factor (SCF), a major hematopoietic growth factor controlling AML progression and thus becoming highly oncogenic. Expression and release of SCF can be triggered by AML cells via cytokines (e.g. interleukin-1β). Recent evidence clearly demonstrated that AML cells are also capable of impairing the activities of cytotoxic lymphoid cells (e.g. natural killer (NK) cells and cytotoxic T cells). One of the biochemical mechanisms underlying this phenomenon lies in the ability of AML cells to secrete the protein called galectin-9. This tandem type galectin binds the immune receptor Tim-3 and induces a variety of intracellular and cell-to-cell signalling events leading to the inactivation of NK cells as well as killing of cytotoxic T cells. We recently reported that the process of galectin-9 secretion in AML cells is stimulated by the unique G protein-coupled receptor LPHN1, which normally functions in neurons facilitating exocytosis. LPHN1 is also found in haematopoietic stem cells (HSCs).
but its expression disappears at early stages of their maturation\(^4,7\). However, upon malignant
transformation, AML cells preserve their abilities to express LPHN1 and produce high levels
of galectin-9 and Tim-3, where the latter is involved in trafficking galectin-9 during the
secretion process (HSCs express neither galectin-9 nor Tim-3\(^4\)).

It is currently unknown which molecular mechanisms trigger high levels of LPHN1
expression in primary human AML cells, and in general mechanisms of upregulation of
LPHN1 expression at the genomic level remain unclear. It is also unknown whether FLRT3,
a natural LPHN1 ligand\(^4,8\), is present in human blood plasma and other tissues associated
with AML. Unravelling these mechanisms is crucial in order to understand the pathways that
control the ability of AML cells to protect themselves against cytotoxic lymphoid cells and
thus was the aim of the present study.

**Results and Discussion**

In order to investigate the effects of cortisol on LPHN1 transcription, we exposed primary
and THP-1 human AML cells, primary human HSCs and primary healthy human leukocytes
to 1 µM cortisol for 24 h followed by quantitative real-time PCR analysis of LPHN1 mRNA
levels. We found that all cell types, except primary healthy leukocytes, transcribed detectable
amounts of LPHN1 mRNA and in all these cases levels were significantly upregulated by
treatment with cortisol (Figure 1A). In both THP-1 and primary human AML cells LPHN1
protein levels were also clearly upregulated (Figure 1B and C). In contrast, primary human
healthy leukocytes did not express detectable amounts of LPHN1 protein and this was not
altered by the effects of cortisol (Figure 1D). Comparative analysis of LPHN1 protein
expression in primary human AML cells, THP-1 cells and primary human healthy leukocytes
is shown in Supplementary figure 1.
Cortisol treatments did not upregulate galectin-9 secretion in any of these cell types (Figure 1 B-D), suggesting that LPHN1 needs to be activated by a ligand to induce galectin-9 release.

Analysis of blood plasma levels of cortisol in AML patients vs healthy donors (samples were collected at the same time of the day to avoid the influence of circadian dynamics) demonstrated that its levels were significantly higher in the blood plasma of AML patients compared to healthy donors (Figure 1E). Galectin-9 levels were also substantially higher in AML patients (Figure 1F), which is in line with our previous observations\(^4\). Furthermore, there was no correlation between cortisol and galectin-9 levels in the blood plasma of healthy donors, while in AML patients there was a clear correlation (Figure 1G), suggesting that galectin-9 secretion in this case might be linked to LPHN1 expression.

If LPHN1 is expressed on the surface of blood cells, it can also be shed by proteolysis and therefore appear in the plasma. LPHN1 was immunoprecipitated, extracted and subjected to Western blot analysis from blood plasma samples from AML patients using several LPHN1 antibodies. A clear fragment was detectable at around 67-68 kDa, smaller fragments were detectable as well, but only in AML plasma, while in the blood plasma of healthy donors there was no evidence of the presence of LPHN1 fragments (Figure 1H). These fragments were also detectable by ELISA (Figure 1I, see Materials and Methods for description of the ELISA format).

As reported before\(^4\), we observed that exposure of THP-1 AML cells to 10 nM FLRT3 for 16 h resulted in a significant increase in galectin-9 secretion (Figure 2A). This effect was not detectable in primary healthy human leukocytes (Figure 2A). Importantly, 1 h pre-exposure of THP-1 cells to rabbit polyclonal antibody recognising LPHN1 (clone name RL1\(^9\)) prior to 16 h treatment with 10 nM FLRT3 attenuated FLRT3-induced galectin-9 release, confirming involvement of LPHN1 in this process (Figure 2A). The antibody employed specifically
recognised target molecules on the surface of THP-1 cells (Supplementary figure 2). We used mouse neuroblastoma cells NB2A, which do not express LPHN1\textsuperscript{10}, as a negative control and measured the interaction of the antibody with the cell surface using a Li-Cor on-cell assay as described in the Materials and Methods (please see supplementary information). Exposure of THP-1 cells to 1 µg/ml RL1 for 16 h did not affect galectin-9 secretion levels (data not shown) suggesting that this antibody lacks a LPHN1 agonistic effect.

Interestingly, we found that blood plasma of both healthy donors and AML patients contains approximately equal amounts of secreted FLRT3 (most likely by proteolytic shedding) with a molecular weight of approximately 55 kDa (which corresponds to the molecular weight of FLRT3 shed from the cell surface by proteinases\textsuperscript{11}). Another specific band was seen at around 27-28 kDa which most likely corresponds to a smaller cleavage fragment of the FLRT3 extracellular domain (Figure 2B). The amounts of this smaller fragment were also equal in blood plasma of healthy donors and AML patients (Figure 2B). To explore which blood plasma-based ligands can induce galectin-9 secretion in AML cells we cultured THP-1 cells in RPMI-1640 medium containing antibiotics (as outlined in Materials and Methods – see supplementary information) replacing 10 % foetal bovine serum (FBS) with blood plasma from either healthy donors or AML patients. Cells were incubated for 16 h with or without 30 min pre-incubation with anti-FLRT3 antibody in order to neutralise FLRT3 activity. Galectin-9 secretion levels were significantly higher in the presence of both sources of human blood plasma compared to FBS (negative control). Anti-FLRT3 antibody attenuated galectin-9 secretion (Figure 2C). Binding of LPHN1 and FLRT3 was further confirmed using SRCD spectroscopy. We found that the two proteins interact with each other with high affinity, inducing conformational change in both proteins, as seen from far UV synchrotron radiation circular dichroism (SRCD) spectra (Figure 2D). This is further confirmation of the high
affinity interaction of LPHN1 and FLRT3 observed in previous studies\(^8\) using different techniques.

Taken together, our results demonstrate, for the first time, that cortisol upregulates LPHN1 expression at the transcriptional level, thus stimulating its translation in human AML cells. AML leads to a decreased blood plasma glucose levels\(^5\), which normally leads to upregulation of secretion of corticotropin-releasing hormone (CTRH) by hypothalamus\(^12\). CTRH induces secretion of adrenocorticotropic hormone (ACTH) by pituitary gland\(^12\). ACTH upregulates cortisol production by the adrenal cortex\(^12\). Cortisol is then employed by AML cells. In healthy human leukocytes cortisol is not capable of inducing LPHN1 transcription/translation, possibly because of gene repression. Interaction of AML cell-derived LPHN1 with released FLRT3 available in blood plasma facilitates the secretion of galectin-9. The latter protects AML cells against immune attack which could otherwise be performed by NK cells as well as cytotoxic T cells (Supplementary figure 3). Importantly, LPHN1 fragments are present in the blood plasma of AML patients but not in healthy donors. These fragments were detectable by both Western blot analysis and ELISA, which indicates the possibility of detection of these fragments for rapid AML diagnosis, although differential verification tests have yet to be performed. Our results suggest a fundamentally novel mechanism used by AML cells in order to progress the disease. They use a common endogenous human hormone (cortisol) to induce LPHN1 expression by employing a widely available ligand (FLRT3, which is always present in blood plasma) in order to escape host immune surveillance. Thus, AML cells employ crucial functional systems of the human body to support their survival and attenuate the anti-cancer activities of cytotoxic lymphoid cells. Our work indicates that galectin-9 and secreted FLRT3 are the most promising targets for anti-AML immune therapy.
Materials and Methods are presented in Supplementary Information.

Acknowledgements

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Conflict of interest

The Authors have no conflict of interest to declare
References


Figure legends.

Figure 1. Cortisol induces LPHN1 expression in human AML cells and haematopoietic stem cells but not in primary healthy human leukocytes. Primary human AML, THP-1 and haematopoietic stem cells as well as primary healthy leukocytes were exposed to 1 µM cortisol for 24 h followed by analysis of LPHN1 gene transcription by quantitative real-time PCR (A) and Western blot analysis (B – primary AML cells, C – THP-1 cells and D – PHL). For PHL, lysates of LPHN1 overexpressing NB2A cells were used as a positive control. ELISA was used to measure secreted galectin-9 levels. Blood plasma of ten healthy donors and ten AML patients was collected at the same time of the day to ensure comparability of cortisol levels. Cortisol (E) and galectin-9 (F) levels were measured by ELISA and correlation between the levels of these two proteins was analysed (G). Soluble LPHN1 fragments were immunoprecipitated and detected by Western blot (H) and ELISA (I), as outlined in Materials and Methods. Images are from one experiment representative of four – six which gave similar results. Data represent mean values ± SEM of six – ten independent experiments. ; *p < 0.05; **p < 0.01; ***p < 0.01 vs. control.

Figure 2. FLRT3 induces galectin-9 secretion in AML cells in a LPHN1-dependent manner. THP-1 cells and PHL were exposed to 10 nM human recombinant FLRT3 for 16 h, followed by detection of secreted galectin-9 by ELISA. In THP-1 cells, the treatment was performed with or without 1 h pre-exposure to 1 µg/ml RL1 anti-LPHN1 polyclonal antibody
The levels of released FLRT3 fragments were analysed in the blood plasma of healthy donors and AML patients using Western blot (B). THP-1 cells were exposed for 16 h to 10% blood plasma either from healthy donors or AML patients, with or without pre-treatment with FLRT3 neutralising antibody. Levels of secreted galectin-9 were analysed using ELISA. (C). Secondary structure and conformational changes of LPHN1, FLRT3 and the complex of the two proteins were characterised using SRCD spectroscopy as outlined in Materials and Methods (D). Images are shown from one representative experiment of four which gave similar results. Data are shown as mean values ± SEM from four independent experiments; *p < 0.05; **p < 0.01 vs. control.

Figure 1
Cortisol facilitates immune escape of human acute myeloid leukaemia cells by inducing latrophilin 1 expression

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Materials and Methods

Materials

RPMI-1640 medium, foetal bovine serum, supplements as well as basic laboratory chemicals were purchased from Sigma-Aldrich (Suffolk, UK). Maxisorp™ microtitre plates were obtained from Nunc (Roskilde, Denmark) and Oxley Hughes Ltd (London, UK). Human recombinant FLRT3, mouse monoclonal antibody against LPHN1 and rabbit antibody against FLRT3 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). PAL1 and RL1 rabbit polyclonal antibodies against LPHN1 were described previously\textsuperscript{1,2}. Rabbit antibody against native LPHN1 was obtained from Abcam (Cambridge, UK). Goat anti-mouse and goat anti-rabbit fluorescence dye-labelled antibodies were obtained from LI-COR
ELISA-based assay kits for the detection of galectin-9 were purchased from Bio-Techne (R&D Systems, Abingdon, UK). A soluble extracellular fragment of LPHN1, LPH-51, was produced and purified as described before. Briefly, NB2a cells stably expressing LPH-51 were cultured in serum-free medium for 48 h. 30 mL of the medium was then incubated overnight at 4°C with 500 μL of anti-V5-antibody agarose (Sigma-Aldrich). After incubation, the column was washed with 10 column volumes of PBS. Bound protein was fractionally eluted with 5 volumes of elution buffer containing 50 mM triethylamine and 150 mM NaCl (pH 12). The eluted protein was immediately neutralized with 150 mM NaCl, 1 M HEPES (pH 7.2). The fractions were then analysed by Western blotting and combined. The protein was concentrated using Amicon centrifugal ultrafiltration units with a 30,000 molecular weight cut-off (Sigma-Aldrich). All other chemicals purchased were of the highest grade of purity commercially available.

**Cell lines and primary cells**

THP-1 human myeloid leukemia monocytes cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 medium (R8758 – Sigma-Aldrich) with L-glutamine and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture) supplemented with 10% foetal bovine serum, penicillin (50 IU/ml) and streptomycin sulphate (50 μg/ml). Untransfected mouse neuroblastoma cells (NB2A) and those overexpressing the full-size LPHN1 (LPH-42) were handled as described earlier.

Primary human AML cells were obtained from the sample bank of the University Medical Centre Hamburg-Eppendorf (Ethik-Kommission der Ärztekammer Hamburg, reference:
Cells were incubated in IMDM medium containing 15% BIT 9500 serum substitute, 100 µM mercaptoethanol, 100 ng/ml stem cell factor (SCF), 50 ng/ml FLT3, 20 ng/ml G-CSF, 20 ng/ml IL-3, 1 µM UM729 and 500 nM stemregenin 1 (SR1) as described before.3

Primary human blood plasma samples

Blood plasma from healthy donors was generated by centrifugation of peripheral blood provided by the National Health Blood and Transfusion Service (NHSBT, UK) following ethical approval (REC reference: 16-SS-033). Primary human AML plasma samples were obtained from the sample bank of University Medical Centre Hamburg-Eppendorf (Ethik-Kommission der Ärztekammer Hamburg, reference: PV3469).4

Western blot analysis

LPHN1 protein levels were analysed using Western blotting. β-actin staining was used to confirm equal protein loading as described previously (4, 6, 14). LI-COR goat secondary antibodies (dilution 1:2000), conjugated with fluorescent dyes, were used in accordance with manufacturer's protocol to visualise target proteins (using a LI-COR Odyssey imaging system). Western blot data were quantitatively analysed using Odyssey software and values were subsequently normalised against those of β-actin.1,4,5

Enzyme-linked immunosorbent assays (ELISAs) and immunoprecipitation

Secreted galectin-9 was measured by ELISA using R&D Systems kits according to manufacturer’s protocol. Plasma cortisol was measured by ELISA using the Salimetrics assay kit according to the manufacturer’s protocols (Salimetrics, Suffolk, UK.)

For immunoprecipitation of LPHN1 fragments from human blood plasma, plates were coated with mouse monoclonal LPHN1 antibody following the principle and protocol described
previously. This was followed by blocking with 2% BSA. Samples were then applied and incubated for at least 4 h at room temperature after which plates were extensively washed with Tris buffered saline with Tween-20 (TBST). 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 4x sample buffer for SDS-PAGE at a ratio of 1:3. Samples were then subjected to Western blot analysis (5), using rabbit PAL1 anti-LPHN1 antibody. Alternatively, PAL1 antibody was applied as detection antibody, followed by visualisation using HRP-labelled anti-rabbit secondary antibody (Abcam, Cambridge, UK).

**On cell assays**

We employed a standard LI-COR on cell assay to characterise interaction of RL-1 antibody with the surface of THP-1 cells.

**Synchrotron radiation circular dichroism (SRCD) spectroscopy**

Human recombinant LPHN1 and FLRT3 were analysed, either alone or in combination with each other, using SRCD spectroscopy at beamline B23, Diamond Light Source (Didcot, UK). (The B23 is equipped with a highly collimated microbeam allowing the use of small aperture long path length microcuvettes). SRCD measurements were carried out using 0.01 µM sample of soluble LPH-51 in a 1 cm path length cell of 3 mm aperture diameter using a Module B instrument with 1 nm increment, 1 s integration time and 1.2 nm bandwidth at 23 °C. The cuvette capacity was 60 µl. Titration experiments were conducted as described previously using standard far-UV measurements.

**Statistical analysis**
Each experiment was performed at least three times and statistical analysis was conducted using a two-tailed Student's t-test, where appropriate. Multiple comparisons were performed using an ANOVA test. Post-hoc Bonferroni correction was applied. Statistical probabilities (p) were expressed as * where p<0.05; **, p<0.01 and *** when p<0.001. Coefficient of determination (R^2) was calculated using GraphPad Prism software in order to assess correlation.

References


Supplementary Figures

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Supplementary figure 1. Comparative analysis of LPHN1 protein expression in primary human AML, THP-1 cells and PHL. Lysates of each cell type were subjected to Western blot analysis as outlined in the Materials and Methods. Images are from one experiment representative of three which gave similar results. Data represent mean values ± SEM of three independent experiments; **p < 0.01 vs. AML cells.
Supplementary figure 2. Cell surface detection of LPHN1. Wild type NB2A cells (negative control) and THP-1 cells were subjected to an on-cell assay using anti-rabbit Li-Cor secondary antibody. Images are from one experiment representative of three which gave similar results.
Supplementary figure 3. Physiological cross-links leading to cortisol-induced upregulation of LPHN1 expression in AML cells followed by facilitation of galectin-9 secretion in a FLRT3-dependent manner. AML is associated with a decreased blood plasma glucose levels, which normally leads to upregulation of secretion of corticotropin-releasing hormone (CTRH) by hypothalamus. CTRH induces secretion of adrenocorticotropic hormone (ACTH) by pituitary gland. Secreted ACTH upregulates cortisol production by the adrenal cortex, thus leading to cortisol-induced upregulation of LPHN1 levels in AML cells.
Galectin-9, secreted in FLRT3-LPHN1-dependent manner attenuates anti-cancer activity of cytotoxic T cells (CTC) and NK cells.