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## High mobility group box 1 (HMGB1) acts as an “alarmin” to promote acute myeloid leukaemia progression

Inna M. Yasinska<sup>a,\*</sup>, Isabel Gonçalves Silva<sup>a,\*</sup>, Svetlana S. Sakhnevych<sup>a</sup>, Laura Ruegg<sup>a</sup>, Rohanah Hussain<sup>b</sup>, Giuliano Siligardi<sup>b</sup>, Walter Fiedler<sup>c</sup>, Jasmin Wellbrock<sup>c</sup>, Marco Bardelli<sup>d</sup>, Luca Varani<sup>d</sup>, Ulrike Raap<sup>e</sup>, Steffen Berger<sup>f</sup>, Bernhard F. Gibbs<sup>e,a</sup>, Elizaveta Fasler-Kan<sup>f,g</sup>, and Vadim V. Sumbayev<sup>a</sup>

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### ABSTRACT

High mobility group box 1 (HMGB1) is a non-histone protein localised in the cell nucleus, where it interacts with DNA and promotes nuclear transcription events. HMGB1 levels are elevated during acute myeloid leukaemia (AML) progression followed by participation of this protein in triggering signalling events in target cells as a pro-inflammatory stimulus. This mechanism was hypothesised to be employed as a survival pathway by malignant blood cells and our aims were therefore to test this hypothesis experimentally. Here we report that HMGB1 triggers the release of tumour necrosis factor alpha (TNF- $\alpha$ ) by primary human AML cells. TNF- $\alpha$  induces interleukin 1 beta (IL-1 $\beta$ ) production by healthy leukocytes, leading to IL-1 $\beta$ -induced secretion of stem cell factor (SCF) by competent cells (for example endothelial cells). These results were verified in mouse bone marrow and primary human AML blood plasma samples. In addition, HMGB1 was found to induce secretion of angiogenic vascular endothelial growth factor (VEGF) and this process was dependent on the immune receptor Tim-3. We therefore conclude that HMGB1 is critical for AML progression as a ligand of Tim-3 and other immune receptors thus supporting survival/proliferation of AML cells and possibly the process of angiogenesis.

### ARTICLE HISTORY

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### KEYWORDS

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High mobility group box 1 (HMGB1) is a non-histone protein localised in the nucleus, where it binds DNA in order to promote nuclear transcription processes.<sup>1</sup> In addition, HMGB1 was recently found to function as a damage-associated molecular pattern (DAMP) when released passively from either dead, dying/injured cells or secreted by immune/cancer cells in response to endogenous and/or exogenous stimuli, such as hypoxia, endotoxin etc..<sup>2-4</sup> This process is followed by participation of HMGB1 in triggering signalling events in target cells.<sup>1-4</sup> Therefore, it is often called “alarmin” in order to reflect its function as a factor secreted by cells affected by a stressor.<sup>1</sup> It has recently been found that HMGB1 levels are significantly elevated during acute myeloid leukaemia (AML, blood/bone marrow cancer).<sup>5,6</sup> Moreover, AML cells were shown to express high levels of HMGB1.<sup>5,6</sup> Elevated levels of secreted HMGB1 associated with AML progression are likely to be caused by a combination of increased expression of this protein in AML cells and conditions supporting its secretion such as hypoxia and death of the cells in the tumour microenvironment.<sup>5-7</sup>

Upon release, HMGB1 can interact with several immune receptors, including Toll-like receptors 2 and 4 (TLRs 2 and 4) as well as receptor of advanced glycation end products (RAGE).<sup>8</sup> Recent evidence has suggested a possible interaction of HMGB1 with the immune receptor Tim-3 (T cell immunoglobulin and mucin domain 3) which is highly expressed in human acute myeloid leukaemia (AML) cells.<sup>1,2</sup> However, the role of HMGB1 in leukaemia progression remains unstudied. Interestingly, signalling pathways triggered by TLRs 2/4, RAGE and Tim-3 include activation of the phosphatidylinositol-3 kinase (PI-3 K)/mammalian target of rapamycin (mTOR) pathway, which directly controls initiation of translation of proteins crucial for cell survival as well as cytokines including tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), a pleiotropic inflammatory cytokine participating in a variety of physiological processes associated with control of host immune defence and respectively haematopoiesis.<sup>9-14</sup> This pathway also triggers accumulation of hypoxia-inducible factor-1  $\alpha$  (HIF-1 $\alpha$ ), an inducible subunit of HIF-1 transcription complex, which induces glycolysis and angiogenesis on

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\*IMY and IGS have contributed equally to this work.

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genomic level.<sup>9,12</sup> However, the effects of HMGB1 on signalling events described above remain hypothetical and are not comprehensively or conceptually studied yet.

Here we report for the first time that HMGB1 triggers the release of TNF- $\alpha$  by primary human AML cells independently of Tim-3. TNF- $\alpha$  induces interleukin 1 beta (IL-1 $\beta$ ) production by healthy leukocytes and subsequent IL-1 $\beta$ -induced secretion of stem cell factor (SCF) by competent cells (for example endothelial cells). Treatment of mouse bone marrow cells with HMGB1 led to significant increase in TNF- $\alpha$ , IL-1 $\beta$  and SCF production. The levels of TNF- $\alpha$ , IL-1 $\beta$  and SCF in blood plasma of AML patients were also significantly upregulated in comparison to healthy individuals. SCF, a ligand of the Kit growth factor receptor is required for survival and proliferation of AML cells. We also show that HMGB1 induces the secretion of angiogenic protein vascular endothelial growth factor (VEGF) and this process is controlled by Tim-3. Synchrotron radiation circular dichroism (SRCD) spectroscopy confirmed that HMGB1 can specifically bind Tim-3. We conclude that HMGB1 as an alarmin participates in AML progression by interacting with Tim-3 and other immune receptors thus supporting the survival/proliferation of AML cells and possibly the process of angiogenesis.

## Materials and methods

### Materials

RPMI-1640 medium, foetal bovine serum, supplements as well as basic laboratory chemicals were purchased from Sigma (Suffolk, UK). Maxisorp<sup>TM</sup> microtitre plates were obtained from Nunc (Roskilde, Denmark) and Oxley Hughes Ltd (London, UK). Mouse monoclonal antibodies directed against HIF-1 $\alpha$ , mTOR and  $\beta$ -actin, as well as rabbit polyclonal antibodies against phospho-S2448 mTOR, RAGE and HRP-labelled rabbit anti-mouse secondary antibody were purchased from Abcam (Cambridge, UK). Antibodies against phospho-S65 and non-phosphorylated (total) eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1) were obtained from Cell Signaling Technology (Danvers, MA USA). Goat anti-mouse and goat anti-rabbit fluorescence dye-labelled antibodies were obtained from LI-COR (Lincoln, Nebraska USA). ELISA-based assay kits for the detection of TNF $\alpha$ , IL-1 $\beta$ , SCF and VEGF were purchased from Bio-Techne (R&D Systems, Abingdon, UK). Anti-Tim-3 mouse monoclonal antibody, its single chain variant as well as human Ig-like V-type domain of Tim-3 (amino acid residues 22–124) and human HMGB1 expressed and purified from *E. coli* (see below for more details) were used in our experiments.<sup>11,15</sup> All other chemicals purchased were of the highest grade of purity commercially available.

### Cell lines and primary cells

THP-1 human myeloid leukemia monocytes and MCF-7 epithelial breast cancer cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 medium (R8758 – Sigma (Suffolk, UK) 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  $\mu$ g/ml).

Primary human AML mononuclear blasts (AML-PB001 F, newly diagnosed/untreated) were purchased from AllCells (Alameda, CA, USA) and handled in accordance with the manufacturer's instructions following ethical approval (REC reference: 16-SS-033).

Bone marrow was isolated from femur bones of six-week-old C57 BL16 mice (25  $\pm$  2.5 g, kindly provided by Dr. Gurprit Lall, School of Pharmacy, University of Kent) which were used for the experiments following approval by the Institutional Animal Welfare and Ethics Review Body. Animals were handled by authorised personnel in accordance with the Declaration of Helsinki protocols. Bone marrow was isolated from femur bone heads as described before.<sup>15,16</sup> Cells were kept in RPMI 1640 medium supplemented with 10% foetal bovine serum, penicillin (50 IU/ml) and streptomycin sulphate (50  $\mu$ g/ml).

### Primary human blood plasma samples

Blood plasma from healthy donors was obtained from buffy coat blood (which originated from donors undergoing routine blood donation) 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).

### HMGB1 purification

HMGB1 was produced and purified from *E. Coli* Rosetta DE2 competent. Cells were grown at 37°C in LB medium and harvested 3 hours after induction with isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at OD<sub>600</sub> 0.7. The pellet was collected by centrifugation, re-suspended and sonicated on ice with 5  $\times$  30 seconds of pulsing and 30 seconds rest (buffer contained 20 mM TrisHCl pH 8; 150 mM NaCl; 10 mM imidazole; 2 Beta-SH and 0.2% Triton X100). Following high-speed centrifugation, HMGB1 containing supernatant was subjected to Ni-affinity and size exclusion chromatography. The protein was then eluted at a volume consistent with monomeric HMGB1.

After size exclusion HMGB1 was passed through an anion exchange (Q) column to ensure LPS removal, eluted in sodium phosphate buffer with 1.5M NaCl and finally dialysed extensively (at least 36 hours) against PBS buffer. Absence of LPS contamination was confirmed using mammalian cell lines.

HMGB1 protein was prepared for structural studies and hence highly pure. It was routinely tested for size and degradation by size exclusion (during purification) and dynamic light scattering (final product). Sample concentration was determined by UV spectroscopy. Proper folding was controlled by NMR and CD spectroscopy (all samples required a CD spectrum equivalent to that of the samples characterised with bidimensional 15 N NMR spectroscopy). This protein displayed activity similar to the human recombinant protein expressed in HEK293 cells obtained from Sigma (Suffolk, UK, SRP6265, as verified using THP-1 and primary human AML cells). The

ability of HMGB1 to interact with TLRs seen in our experiments confirmed similarities in the redox state of preparations used in our work in comparison to the pro-inflammatory form of HMGB1 released by dead/dying or stressed cancer/immune and other cells.

### Western blot analysis

Tim-3, HIF-1 $\alpha$ , phospho-S65 and total eIF4E-BP1 as well as RAGE were analysed using Western blot.<sup>13,14</sup>  $\beta$ -actin staining was used to confirm equal protein loading as described previously. 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  $\beta$ -actin. In order to quantitate levels of mTOR-dependent phosphorylation of eukaryotic initiation factor 4 E binding protein 1 (eIF4E-BP), we measured phospho-S65-eIF4E-BP and total quantity of eIF4E-BP on a different membrane to avoid the influence of possible incomplete membrane stripping following quantitative analysis. Values were normalised against those of  $\beta$ -actin for corresponding membranes. The ratio between normalised phospho-S65-eIF4E-BP and total eIF4E-BP was calculated in order to characterise eIF4E-BP phosphorylation levels. The following equation was implemented:

$$\text{pS65 - eIF4E - BP level} = \frac{[\text{pS65 - eIF4E - BP}]}{[\text{Actin}]} \div \frac{[\text{eIF4E - BP total}]}{[\text{Actin}]}$$

This ratio in control samples was considered as 100%.

### Enzyme-linked immunosorbent assays (ELISAs)

Human or mouse TNF- $\alpha$ , IL-1 $\beta$ , SCF as well as human VEGF, either in cell culture media or human blood plasma were measured by ELISA using R&D Systems kits according to manufacturer's protocols. Phosphorylation of mTOR was analysed by ELISA as previously described.<sup>13</sup>

### In cell assays and in cell Westerns

We employed a standard LI-COR in-cell Western (ICW) assay (methanol was used as permeabilization agent) to characterise Tim-3 total levels in studied cells. The in-cell (ICA, also called on-cell) assay was employed to detect Tim-3 surface presence in the cells. Following washing with PBS, cells were scanned using a LI-COR Odyssey imaging system.<sup>17</sup>

### Detection of PI-3K activity

PI-3K activity was measured in cell lysates as described previously.<sup>18</sup> Briefly, cell lysates were incubated with 30  $\mu$ l

0.1 mg/ml substrate (PI-4,5-diphosphate emulsion) in kinase assay buffer. The latter was prepared from 20 mM Tris (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 8 mM MgCl<sub>2</sub> and 40  $\mu$ M ATP in a total volume of 100  $\mu$ l at 37°C with constant agitation. Reactions were terminated by adding 1 ml of hexane/isopropanol (13:7, v:v) mixture and 0.2 ml of a mixture of 2 M KCl/HCl<sub>conc</sub> (8:0.25, v:v). After vortexing the organic phases were washed with HCl (0.5 ml; 0.1 M). This was followed by detection of phosphate groups using a colorimetric assay. The values obtained in the control samples of each experiment per 1 mg protein were counted as 100% of the PI-3 K activity. Other values were normalised and expressed as % control.

### Synchrotron radiation circular dichroism (SRCD) spectroscopy

Human recombinant Tim-3 and HMGB-1, either alone or in combination, were analysed using SRCD spectroscopy at beamline B23, Diamond Light Source (Didcot, UK). B23, equipped with a highly collimated microbeam, allows for the use of a small aperture long path length microcuvette which is unattainable with benchtop instruments due to divergent beams since samples available are of very low volume and concentrations. SRCD measurements were performed using 0.2  $\mu$ M of samples in a 1 cm path length cell of 3 mm aperture diameter and 60  $\mu$ l capacity using a Module B instrument with a 1 nm increment, 1 s integration time, 1.2 nm bandwidth at 23 °C.<sup>19-24</sup> Temperature denaturation measurements were collected over the temperatures 20°C – 95°C (in 5°C increments) for HMGB1, Tim-3 and the 1:1 mixture. The results obtained were processed using CDApps<sup>25</sup> and OriginPro<sup>®</sup>. For thermal denaturation measurements, change in CD (mdeg) at a specific wavelength was plotted against the corresponding temperature for fitting using the Gibbs-Helmholtz equation derived from Boltzmann distribution,<sup>26,27</sup> sigmoidal two-state denaturation curve to a Boltzmann distribution and the expression modified to include parameters for fitting of thermal denaturation data for the calculation of the melting temperature ( $T_m$ ). Titration experiments were conducted as described for standard far-UV measurements, with the modification of measurements collected after the addition of incremental volumes of Tim-3 stock as described previously.<sup>28</sup> The change of CD (mdeg) at single wavelength was plotted against respective ligand concentration ( $\mu$ M) using OriginPro<sup>®</sup> and fitted with the Hill binding<sup>29</sup> function to determine the  $K_d$  for binding.

### Statistical analysis

Each ELISA and cell experiment was performed at least three times and statistical analysis when comparing two events at a time was conducted using a two-tailed Student's *t*-test. 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.

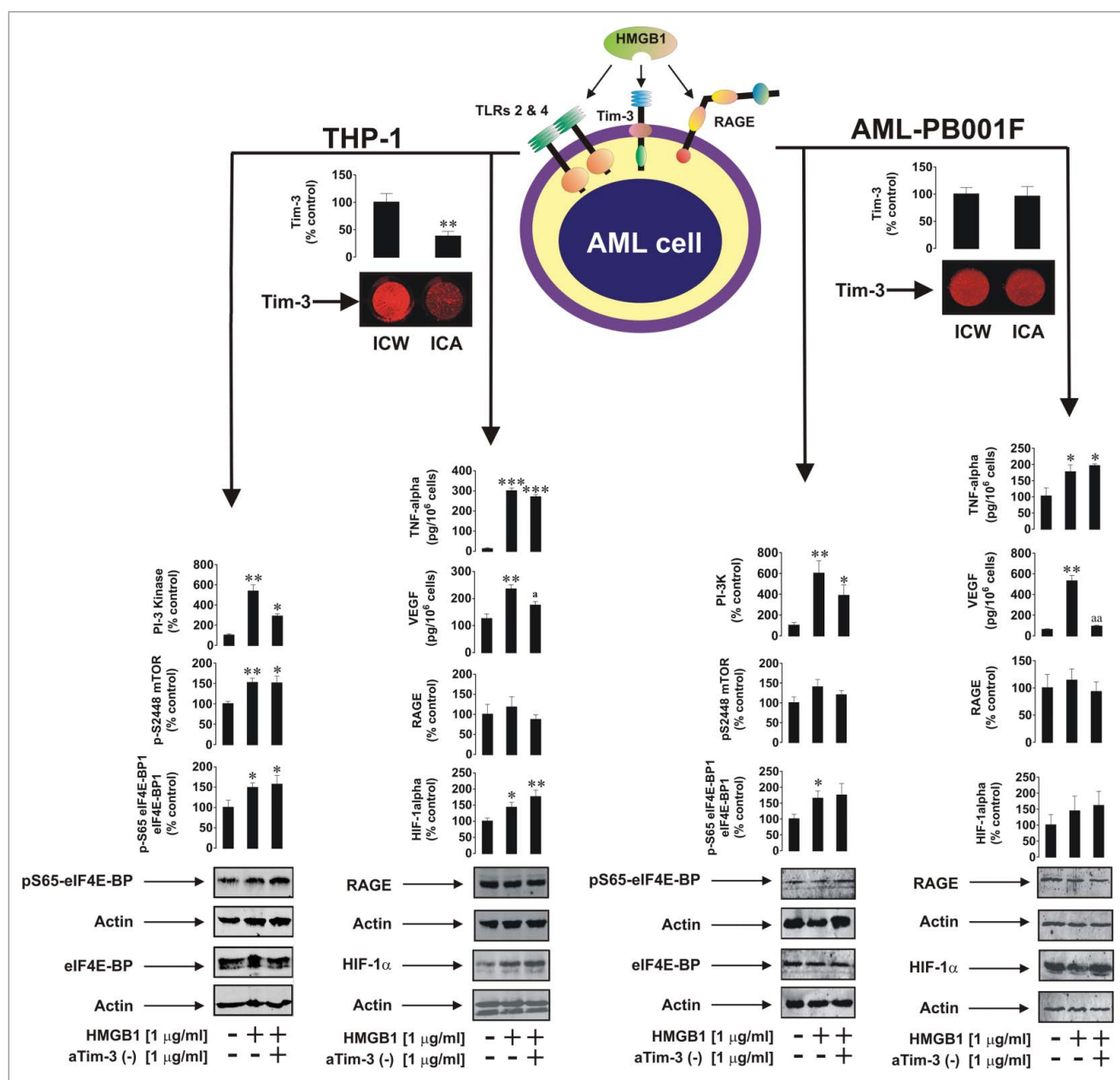
## Results

### HMGB1 induces moderate activation of PI3-K/mTOR pathway, TNF- $\alpha$ and VEGF secretion in human AML cells

We used monocytic THP-1 human acute myeloid leukaemia cells which express Tim-3, but keep most of it inside the cell (Fig. 1 left panel), and primary human AML cells (AML-PB001F) where most Tim-3 is expressed on the cell surface (Fig. 1 right panel). Cells were exposed for 4 h to 1  $\mu$ g/ml HMGB1 with or without 1 h pre-treatment with single-chain antibody against Tim-3, which does not display Tim-3 agonistic properties and also prevents the interaction of other ligands

with it. We found that HMGB1 induced activation of PI-3K in both THP-1 and primary human AML cells. In both cell types this effect was non-significantly downregulated by the presence of anti-Tim-3 antibody (Fig. 1). This was consistent with a moderate activation of mTOR (phosphorylation at S2448) and increased phosphorylation of mTOR substrate eukaryotic initiation factor 4 E binding protein 1 (eIF4E-BP1). Increased TNF- $\alpha$  secretion took place in both cases. Neither process observed was influenced by the presence of anti-Tim-3 antibody, suggesting that the effects observed are Tim-3-independent.

Pre-treatment of THP-1 cells for 1 h with 2  $\mu$ g/ml neutralising antibodies directed against TLR2, TLR4 and RAGE



**Figure 1.** Differential receptors are involved in HMGB1-induced biological responses of human AML cells. Total levels of the immune receptor Tim-3 and its surface presence were characterised in THP-1 and primary human AML-PB001F cells by in-cell Western (ICW) and in-cell (on-cell) assay (ICA) respectively (see Materials and Methods for details). Both cell types were exposed to 1  $\mu$ g/ml HMGB1 for 4 h with or without 1 h pre-treatment with single chain anti-Tim-3 antibody (aTim-3 (-)) followed by Western blot analysis of phospho-S65 vs total eIF4E-BP1, HIF-1 $\alpha$  and RAGE expression as well as by detection of phospho-S2448 mTOR, release of TNF- $\alpha$  and VEGF using ELISA. PI-3 K activity was monitored by colorimetric assay. Images are from one experiment representative of five which gave similar results. Data is shown as mean values  $\pm$  SEM of five independent experiments. \*  $p < 0.05$ ; \*\*  $p < 0.01$  and \*\*\* when  $p < 0.001$  vs control; <sup>a</sup>  $p < 0.05$ ; <sup>aa</sup>  $p < 0.01$  vs HMGB1.

followed by 4 h of exposure to 1  $\mu\text{g/ml}$  HMGB1 showed that TLRs 2 and 4, but not RAGE, are involved in HMGB1-induced TNF- $\alpha$  secretion (Supplementary Fig. 1). However, it does not rule out the fact that during long-term exposure RAGE might contribute to HMGB1-induced intracellular TNF- $\alpha$  expression which can be upregulated by RAGE ligands<sup>30</sup>

Consequently, we detected the activation of HIF-1 $\alpha$  accumulation in THP-1 cells but not in AML cells which had high background levels of HIF-1 $\alpha$  and thus probably did not respond to HMGB1 treatment (Fig. 1). In both cell types, however, we observed a significant increase in VEGF secretion (Fig. 1) which was significantly reduced (but not HIF-1 $\alpha$  accumulation as seen from either Western blot data or intracellular VEGF levels (this was verified by ELISA performed on the cell lysates – data not shown)) by the presence of anti-Tim-3 antibody. The level of downregulation was proportional to the amount of Tim-3 present on the surface of each cell type. THP-1 and primary AML cells express TLRs 2 and 4. They also express high levels of RAGE, as verified by Western blot analysis (Fig. 1), which suggests that HMGB1 induces activation of the PI-3K/mTOR pathway and HIF-1 $\alpha$  accumulation as well as TNF- $\alpha$  secretion through classic immune receptors like TLRs2/4, and RAGE, while secretion of VEGF is a Tim-3-dependent process.

### Characterisation of HMGB1-Tim-3 interactions *in vitro*

We then sought to obtain confirmation of direct interactions between HMGB1 and Tim-3 using the recombinant, purified Ig-like V-type domain of human Tim-3 (residues 22–124) and human HMGB1. We employed SRCD spectroscopy for both qualitative and quantitative binding assays. Titration of 200 nM HMGB1 with increasing amounts of Tim-3 (Fig. 2A) indicated a high nanomolar binding affinity ( $K_d = 10^{-7}$  M). The SRCD spectrum of a 1:1 HMGB1-Tim-3 complex appeared different from the sum of the SRCD spectra of the individual components (Fig. 2B), suggesting the presence of conformational rearrangements upon formation of the complex. Far UV thermal denaturation studies were also performed on the

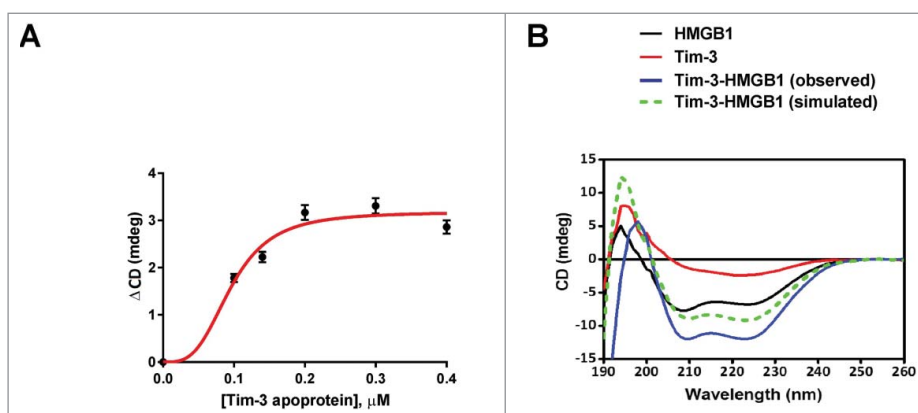
complex, further supporting the above data (Supplementary Figs. 2 and 3).

### HMGB1 induces TNF- $\alpha$ secretion by human AML leading to upregulation of SCF production

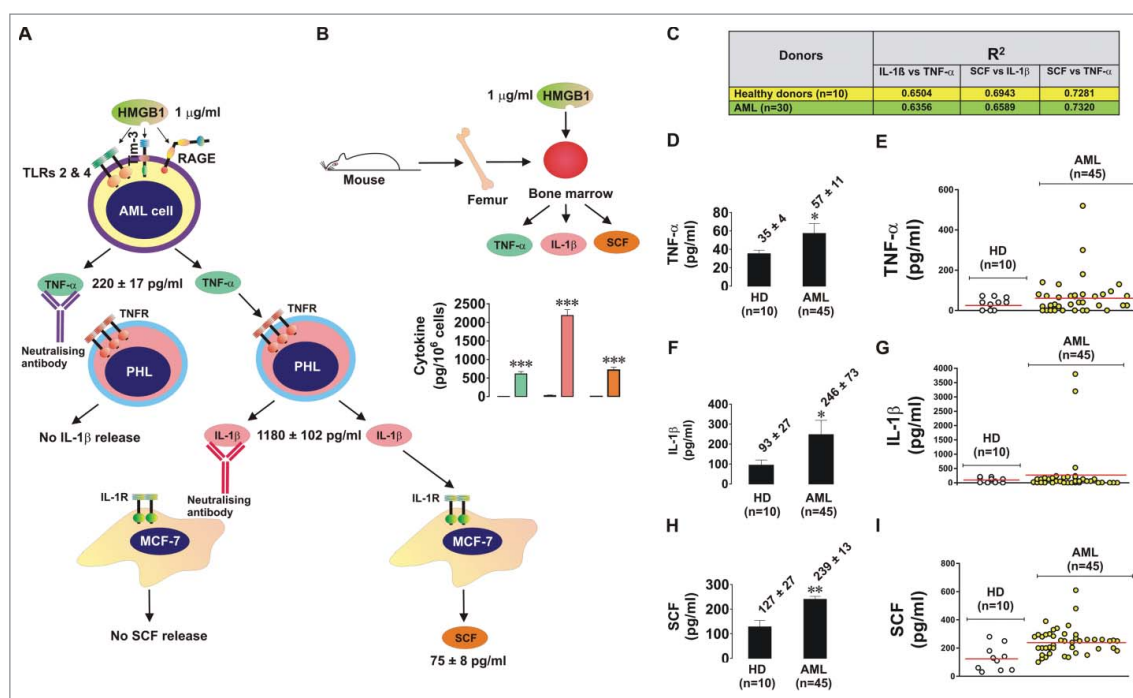
Since it was obvious that both the AML cell line (THP-1) and primary AML cells secreted TNF- $\alpha$  in response to stimulation with HMGB1, we studied the effects of released TNF- $\alpha$  on the production of IL-1 $\beta$  by primary healthy human leukocytes (PHL). Cell culture medium obtained after stimulation of THP-1 cells with 1  $\mu\text{g/ml}$  HMGB1 was used to treat primary healthy leukocytes for 4 h with or without 1 h pre-treatment with 2  $\mu\text{g/ml}$  TNF- $\alpha$ -neutralising antibody. We found that in the absence of TNF- $\alpha$ -neutralising antibody, PHL released IL-1 $\beta$ , while in the presence of TNF- $\alpha$ -neutralising antibody PHL did not release detectable amounts of IL-1 $\beta$ . Medium containing IL-1 $\beta$  was used to culture MCF-7 breast cancer epithelial cells (these cells express IL-1 receptor type 1 and are capable of releasing stem cell factor (SCF)) for 24 h in the absence or presence of 2  $\mu\text{g/ml}$  of IL-1 $\beta$ -neutralising antibody. We found that, in the presence of IL-1 $\beta$ -neutralising antibody, MCF-7 did not release detectable amounts of SCF, while in the absence of it SCF release was clearly detectable. These results (all shown in Fig. 3A) suggest that HMGB1 induces the release of TNF- $\alpha$  by AML cells. TNF- $\alpha$  induces IL-1 $\beta$  secretion by PHL. Released IL-1 $\beta$  stimulates the production of SCF by endothelial/epithelial cells. SCF is required for proliferation of AML cells and thus supports leukaemia progression.

We sought to obtain confirmation of this biological test using mouse bone marrow cells *ex vivo*. Mouse bone marrow cells were exposed to 1  $\mu\text{g/ml}$  HMGB1 for 24 h. We observed that the levels of secreted TNF- $\alpha$ , IL-1 $\beta$  and SCF were significantly increased compared to non-treated bone marrow cells (Fig. 3B) and that the ratio between these cytokines was similar to that observed in the biological test shown in Fig. 3A.

We then measured the levels of TNF- $\alpha$ , IL-1 $\beta$  and SCF in the blood plasma of 10 healthy human donors and 45 AML patients. We found that the levels of all three factors were significantly increased (Fig. 3C – I). There was a clear evidence of correlation between IL-1 $\beta$  vs TNF- $\alpha$ , SCF vs IL-1 $\beta$  and SCF vs



**Figure 2.** Interaction of HMGB1 and the immune receptor Tim-3. Interaction of HMGB1 protein with Tim-3 was analysed using SRCD spectroscopy-based titration which was conducted in the far UV region using 0.2  $\mu\text{M}$  HMGB1 and increasing stoichiometric concentrations of Tim-3 (A). Changes in CD signal monitored at 222 nm were plotted against Tim 3 concentration using Hill function. Qualitative binding was verified by analysis of interactions of equimolar concentrations of Tim-3 and HMGB1 using SRCD spectroscopy (B).



**Figure 3.** HMGB1 induces an intercellular signalling cascade leading to SCF secretion. (A) Primary human AML cells (AML-PB-001F) were exposed for 4 h to HMGB1 followed by collection of the culture medium (detection of TNF- $\alpha$  was performed in this medium using ELISA), which was used to culture primary human healthy leukocytes for 4 h in the absence or presence of TNF- $\alpha$ -neutralising antibody. Medium was collected (levels of IL-1 $\beta$  were measured by ELISA) and used to culture MCF-7 breast cancer epithelial cells for 4 h in the absence or presence of IL-1 $\beta$ -neutralising antibody. Following this exposure, medium was collected and SCF was measured in it by ELISA. (B) Primary mouse bone marrow cells ( $10^6$  cells per 3 ml medium) were exposed for 24 h to 1  $\mu$ g/ml HMGB1 followed by detection of TNF- $\alpha$ , IL-1 $\beta$  and SCF by ELISA. (C – I). Levels of TNF- $\alpha$ , IL-1 $\beta$  and SCF were measured in the blood plasma of healthy donors and AML patients by ELISA. Mean values  $\pm$  SEM are presented as well as levels of each protein in blood plasma of each analysed donor/patient. \* $p < 0.05$ ; \*\* $p < 0.01$  vs control.

TNF- $\alpha$  (Fig. 3C and Supplementary Fig. 4) in the blood plasma of both healthy donors and AML patients which contained detectable amounts of all the studied cytokines/SCF suggesting that there is a link between these three factors regardless of the presence of HMGB1. But the increase in all three factors suggests that HMGB1 activates this intercellular cascade in order to increase the levels of secreted SCF thus supporting leukaemia progression.

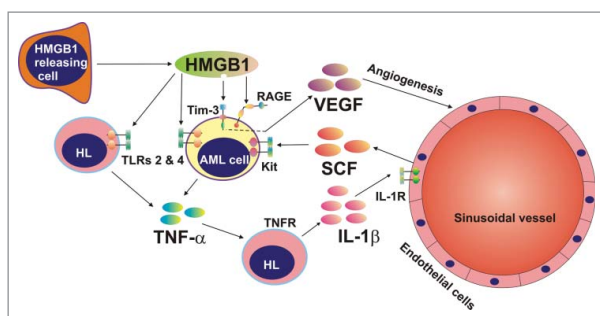
## Discussion

HMGB1 is an “alarmin” which can be secreted by stressed and dying cells as well as cancer and immune cells, and thus was suggested to play a role in leukaemia progression.<sup>1–7</sup> Recent evidence demonstrated that HMGB1 can also act as a ligand of the immune receptor Tim-3 which is highly expressed on the surface of human AML cells.<sup>1,2</sup> However, the signalling activity of HMGB1 in AML cells has not been elucidated and was thus the main aim of our work.

In both, a human AML cell line (THP-1 cells) and primary AML cells (AML-PB001F) we demonstrated that HMGB1 upregulates the activity of the PI-3 K/mTOR pathway, thus leading to increased TNF- $\alpha$  secretion and accumulation of HIF-1 $\alpha$  as well as VEGF release (Fig. 1). However, except VEGF secretion (not HIF-1 $\alpha$ -dependent expression), these effects were not Tim-3-mediated since Tim-3 neutralising single-chain antibody did not affect any of the processes described above (except for VEGF release). Importantly, non-differentiated THP-1 cells express moderate levels of Tim-3 on their surface – this process has to be induced by

activation of PKC $\alpha$  (for example PMA or latrophilin 1 ligands).<sup>15</sup> Primary AML cells which use Tim-3/galectin-9 pathway in order to escape immune attack express much higher levels of Tim-3 protein on their surface. This difference was proportional to that in HMGB1-induced VEGF release in THP-1 and primary AML cells. Furthermore, neutralisation of Tim-3 led to attenuation of HMGB1-induced VEGF secretion in both cases suggesting that this is a Tim-3-mediated process.

Highly sensitive SRCD spectroscopic analysis of HMGB1-Tim-3 interactions confirmed that these two proteins interact with each other specifically, but the apparent affinity was moderately high ( $K_d = 10^{-7}$  M). This affinity, however, can be increased by glycosides which normally bind to Tim-3 (the protein used in the studies was sugar-free). All these results suggest that this interaction is probably rather more secretory than a signal transduction event *per se*. Interestingly, the formation of the protein complex was accompanied by an increase in  $\alpha$ -helical content at the expense of the  $\beta$ -strand presumably arising mainly from the Tim-3 protein. Biological tests demonstrated that TNF- $\alpha$  released from primary human AML cells in an HMGB1-dependent manner is capable of inducing IL-1 $\beta$  secretion by primary human healthy leukocytes. This is in line with recent observations suggesting upregulation of both TNF- $\alpha$  and IL-1 $\beta$  secretion in response to stimulation with HMGB1.<sup>5,6</sup> This reaction is a very important step in AML progression since IL-1 $\beta$  interacts with IL-1 receptor type I and induces production and secretion of SCF required for proliferation of AML cells. Human AML cells express high levels of Kit receptor, which recognises SCF, and



**Figure 4.** HMGB1 induces SCF and VEGF production *via* interaction with differential signalling receptors. The scheme shows that secreted HMGB1 is capable of inducing TNF- $\alpha$  secretion by living AML cells (and possibly healthy leukocytes, based on results obtained in the experiments with mouse bone marrow samples). Secreted TNF- $\alpha$  induces IL-1 $\beta$  production by healthy leukocytes which then induces SCF release in endothelial cells. These processes are Tim-3-independent. HMGB1 also induces VEGF secretion by AML cells in Tim-3-dependent manner.

this haematopoietic factor thus becomes highly oncogenic since AML cells are capable of inducing SCF production by healthy cells. The same effect was seen in primary mouse bone marrow cells. When exposed to HMGB1, levels of TNF- $\alpha$ , IL-1 $\beta$  and SCF increased suggesting that HMGB1 can, in principle, induce this effect in bone marrow too. However, production of HMGB1 is not observed in healthy bone marrow<sup>1,2</sup> since there are not enough stressed/dying or injured cells in order to produce it (in our samples over 95% of cells were viable as determined by trypan blue exclusion, data not shown). However, in leukaemic bone marrow this process is likely to take place due to lack of oxygen and increased HMGB1 expression in transformed cells.

Finally, we found that in blood plasma of AML patients, the levels of TNF- $\alpha$ , IL-1 $\beta$  and SCF were significantly higher compared to blood plasma from healthy donors.

We therefore concluded that in human bone marrow affected by AML, and respectively, by hypoxic conditions, cells release HMGB1 which induces TNF- $\alpha$  production and subsequent secretion of IL-1 $\beta$  which stimulates SCF production/secretion by endothelial cells. This SCF is used to further stimulate the survival/proliferation of AML cells. While these processes are Tim-3-independent, HMGB-1 interacts with Tim-3 and induces VEGF secretion, which is required to induce angiogenesis in bone marrow so that hypoxic conditions caused by increasing AML cell numbers can be relieved. This mechanism is summarised in the Fig. 4 (a more detailed scheme illustrating the possible interactions in the bone marrow is shown in Supplementary Fig. 5).

HMGB1 has already been considered as a possible therapeutic target for leukaemia treatment.<sup>7</sup> Furthermore, targeting HMGB1 has recently been shown to increase drug sensitivity in AML.<sup>30</sup> Our findings demonstrate additional insights that HMGB1 could be considered as a possible therapeutic target in AML and further confirm the efficiency of targeting Tim-3 (here to specifically block AML-induced angiogenesis) in anti-AML therapy.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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