Janus Kinase 3 is Expressed in Erythrocytes, Phosphorylated Upon Energy Depletion and Involved in the Regulation of Suicidal Erythrocyte Death

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Key Words

Cell volume • Annexin • Eryptosis • Janus kinase 3 • Phosphatidylserine

Abstract

Janus kinase 3, a tyrosine kinase expressed in haematopoietic tissues, plays a decisive role in T-lymphocyte survival. JAK3 deficiency leads to (Severe) Combined Immunodeficiency (SCID) resulting from enhanced lymphocyte apoptosis. JAK3 is activated by phosphorylation. Nothing is known about expression of JAK3 in erythrocytes, which may undergo apoptosis-like cell death (eryptosis) characterized by cell membrane scrambling with phosphatidylserine exposure and cell shrinkage. Triggers of eryptosis include energy depletion. The present study utilized immunohistochemistry and confocal microscopy to test for JAK3 expression and phosphorylation, and FACS analysis to determine phosphatidylserine exposure (annexin binding) and cell volume (forward scatter). As a result, JAK3 was expressed in erythrocytes and phosphorylated following 24h and 48h glucose depletion. Forward scatter was slightly but significantly smaller in erythrocytes from JAK3-deficient mice (*jak3^{-/-}*) than

in erythrocytes from wild type mice (*jak3^{+/+}*). Annexin V binding was similarly low in both genotypes. The JAK3 inhibitors WHI-P131/JANEX-1 (4-(4'-Hydroxyphenyl)amino-6,7-dimethoxyquinazoline, 156 μ M) and WHI-P154 (4-[(3'-Bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline, 11.2 μ M) did not significantly modify annexin V binding or forward scatter. Glucose depletion increased annexin V binding, an effect significantly blunted in *jak3^{-/-}* erythrocytes and in the presence of the JAK3 inhibitors. The observations disclose a completely novel role of Janus kinase 3, i.e. the triggering of cell membrane scrambling in energy depleted erythrocytes.

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Introduction

The Janus kinase 3 (JAK3) is a nonreceptor tyrosine kinase involved in the signalling of cytokine receptors of hematopoietic cells [1-5]. In lymphocytes and tumor cells JAK3 participates in the stimulation of cell proliferation and confers protection against apoptosis [6-8]. Along those lines, JAK3 inhibitors have been shown to foster apoptosis of those cells [9, 10]. In contrast, expression levels of

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pro-apoptotic proteins is decreased and the survival is increased in JAK3-deficient (*jak3^{-/-}*) dendritic cells [11]. In those cells JAK3 thus fosters suicidal cell death.

Similar to nucleated cells, erythrocytes may undergo suicidal death or eryptosis [12, 13]. Similar to apoptosis of nucleated cells eryptosis is paralleled by cell shrinkage, cell membrane blebbing and cell membrane scrambling resulting in exposure of phosphatidylserine at the erythrocyte surface [12-14]. Eryptosis is triggered by anemia-causing xenobiotics and endogenous mediators [12, 15-21] and accelerated eryptosis has been observed in several clinical disorders [12, 13], including iron deficiency [22], phosphate depletion [23], hemolytic uremic syndrome [24], sepsis [25], malaria [13, 26-29] or Wilson's disease [30]. Eryptosis is further triggered by energy depletion following glucose withdrawal [31] and is inhibited by the energy sensing kinase AMPK [32].

Whether JAK3 is expressed in erythrocytes and whether the kinase plays a role in the regulation of erythrocyte survival has never been tested. The present study thus explored, whether JAK3 is expressed in erythrocytes and plays a role in the regulation of suicidal erythrocyte death upon energy depletion.

Materials and Methods

Human erythrocytes

Leukocyte-depleted erythrocytes from concentrates provided by the blood bank of the University of Tübingen were used. In erythrocyte concentrates leukocytes are depleted by a factor of at least 2000 in respect of whole blood [33]. The volunteers providing erythrocytes gave informed consent. The study has been approved by the Ethical commission of the University of Tübingen.

Mice

Experiments were performed in 6-12 week old male and female JAK3-deficient mice (*jak3^{-/-}*) and corresponding wild type mice (*jak3^{+/+}*) purchased from Charles River Laboratories, Sulzfeld, Germany. The *jak3^{-/-}* mice have been described previously [34]. All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and were approved by local authorities.

Blood chemistry, blood count and isolation of murine erythrocytes

For all experiments except for the blood count heparin blood was retrieved from the mice. The plasma concentrations of erythropoietin were determined using an immunoassay kit according to the manufacturer's instructions (R&D systems, Wiesbaden, Germany). For blood count EDTA blood was analyzed using an electronic hematology particle counter (scil

Vet abc, Weinheim, Germany). Murine erythrocytes were isolated by washing two times with Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 HEPES/NaOH (pH 7.4), 5 glucose, 1 CaCl₂.

Western blotting

To examine the expression of JAK3 in human or murine erythrocytes 150 µl erythrocyte pellet were lysed in 50 ml of 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4). Ghost membranes were pelleted (15,000 x g for 20 min at 4°C) and lysed in 200 µl lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM Na₃VO₄, 0.4% β-mercaptoethanol) containing protease inhibitor cocktail (Sigma). For determination of the total erythrocyte protein, 300 µl erythrocyte from each sample were lysed in 300 µl ice-cold lysis buffer. In all cases 50 µg of protein were solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 (TBST) at room temperature for 1h. Then, the membrane was incubated with affinity purified rabbit anti-JAK3 antibody (1:1000; 62 kDa, Cell Signaling, Germany), at 4°C overnight. After washing 3 times with TBST (10 min each) the blots were incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody (1:2000; Cell Signaling) for 1h at room temperature. After washing antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). Antibody-binding was quantified with Quantity One Software (Biorad, Germany).

Immunoprecipitation and Western blotting

To detect phospho-tyrosine Jak3 1500 µg of total protein from erythrocytes were immunoprecipitated with 6 µg (2 µg/500 µg total protein) of p-Tyr (PY-20) (sc-508; Santa Cruz Biotech) using the Dynabeads Protein G immunoprecipitation kit from Invitrogen following the manufacturer's protocol. Then the subsequent western blotting with affinity purified rabbit anti-JAK3 antibody (1:1000; 62 kDa, Cell Signaling, Germany) was performed. The total JAK3 protein also detected in same samples by western blotting with JAK3 antibody

Confocal microscopy and immunofluorescence

For the detection of JAK3 erythrocytes were resuspended in PBS at 5 × 10⁷ cells/ml, and 20 µl were smeared onto a glass slide. After air drying for 30 min and fixation with methanol for 2 min the slide was rinsed 4 times over 10 min with PBS. Then, the slide was blocked with 10% normal goat serum (Invitrogen, Paisley, UK) for 10 min and again rinsed 3 times with PBS for 5 min each. After incubation with rabbit anti-JAK3 antibody (1:200, Cell signaling) or rabbit anti-phospho- JAK3 (tyr 980) (1:50, Santa Cruz Biotech), diluted in PBS at 4 °C overnight, the slide was again rinsed 3 times with PBS for 5 min each. Subsequently, the slide was incubated with FITC-conjugated goat anti-rabbit antibody (1:500, Invitrogen), diluted in PBS for 1.5h at room temperature. For control, the slide was incubated only with FITC-conjugated goat anti-rabbit antibody. Then, the slide was rinsed 3 times with PBS for 10 min each and

Fig. 1. Expression and phosphorylation of JAK3 in human erythrocytes. A. Original Western blots demonstrating the expression of total JAK3 protein in total lysate and in membrane preparations of erythrocytes. B. Confocal microscopy of FITC-dependent fluorescence of human erythrocytes stained with a primary anti-JAK3 antibody and a secondary FITC-conjugated anti rabbit antibody. C. Original Western blot of tyrosine phosphorylated JAK3 protein in erythrocytes upon incubation in Ringer and following 24h and 48h energy depletion (by incubation in glucose-free Ringer) determined by immunoprecipitation of erythrocyte total cell lysate with phospho-tyrosine antibody followed by western blotting with JAK3 antibody. D. Confocal microscopy of FITC-dependent fluorescence of human erythrocytes stained with a primary anti-phospho-⁹⁸⁰tyr-JAK3 antibody and a secondary FITC-conjugated anti rabbit antibody, erythrocytes were energy depleted by incubation in glucose-free Ringer for 24h and 48h. E. Confocal microscopy of FITC-dependent fluorescence of human erythrocytes stained with the secondary rabbit antibody alone.

mounted with Prolong® Gold antifade reagent (Invitrogen). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Incubations and solutions

For the *in vitro* experiments on suicidal death of erythrocytes incubations were carried out at 37°C in Ringer solution at a hematocrit of 0.4% in a total volume of 200 µl. Where indicated, glucose-free Ringer was used. JAK3 inhibitors WHI-P131/JANEX-1 (4-(4'-Hydroxyphenyl)amino-6,7-dimethoxyquinazoline, 156 µM) and WHI-P154 (4-[(3'-Bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline, 11.2 µM) and WHI-P97 (22 µM) (all from Calbiochem, Bad Soden, Germany) were added to the respective solutions, where indicated.

Determination of the osmotic resistance

Two µl of blood were added to 200 µl of PBS solutions with decreasing osmolarity. After centrifugation for 5 min at 500 x g the supernatant was transferred to a 96 well plate and the absorption at 405 nm was determined as a measure of hemolysis. Absorption of the supernatant of erythrocytes lysed in pure distilled water was defined as 100% hemolysis.

Phosphatidylserine exposure and forward scatter

For FACS analysis erythrocytes were washed after incubation once in Ringer solution containing 5 mM CaCl₂ [35].

The cells were then stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular ATP concentration

For determination of erythrocyte ATP, 90 µl of erythrocyte pellets were incubated for 48h at 37°C in Ringer solution and glucose-free Ringer solution with or without addition of JAK3 inhibitors WHI-P131/JANEX-1 (4-(4'-Hydroxyphenyl)amino-6,7-dimethoxyquinazoline, 156 µM) and WHI-P154 (4-[(3'-Bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline, 11.2 µM) (final hematocrit 5%). All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO₄ (5%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated KHCO₃ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin-luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l erythrocyte cytosol.

Fig. 2. Forward scatter with or without glucose depletion in the presence and absence of JAK3 inhibitors. A. Histogram of forward scatter in a representative experiment of erythrocytes exposed for 48h to Ringer (1, black line), or glucose-depleted Ringer in the absence (2, red line) or presence (3, green line) of JAK3 inhibitor WHI-P131/JANEX-1 (156 μM). B. Arithmetic means $\pm$ SEM ($n = 8$) of the forward scatter of erythrocytes exposed for 48h to glucose-containing Ringer without (open bars) or with (closed bars) WHI-P131/JANEX-1 (156 μM) or WHI-P154 (11.2 μM) or WHI-P97 (22μM). C. Arithmetic means $\pm$ SEM ($n = 8$) of the forward scatter of erythrocytes exposed for 48h to glucose-containing (+glu, left white bar) or glucose-depleted (mGLU = minus glucose ringer), right bars) Ringer without (black bar) or with (grey bars) WHI-P131/JANEX-1 (156 μM) or WHI-P154 (11.2 μM). ### indicates significant ($p < 0.001$) difference from glucose-containing Ringer, * indicates significant ($p < 0.05$) difference from absence of JAK3 inhibitors (ANOVA).

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by paired or unpaired t-test, by U-test, or by ANOVA, as appropriate, $p < 0.05$ was considered as statistically significant.

Results

According to Western blotting, human erythrocytes express the JAK3 protein that is localized mainly in the cytoplasmic fraction (Fig. 1A). Confocal microscopy confirmed the expression of JAK3 in human erythrocytes (Fig. 1B), while staining of human erythrocytes with the secondary FITC-conjugated antibody without the primary antibody did not result in detectable fluorescence (Fig. 1E). According to confocal microscopy of immunofluorescence utilizing a phospho JAK3 (tyr 980) antibody a 24h and 48h glucose depletion increased the tyrosine-phosphorylated JAK3 (Fig. 1D). JAK3 phosphorylation was further detected by immunoprecipitation with phospho-tyrosine antibody followed by immunoblotting with Jak3 antibody. The abundance of phospho-JAK3 was $122 \pm 15\%$ ($n = 4$) higher in erythrocytes exposed for 24h to glucose depleted Ringer than in erythrocytes exposed for 24h to glucose containing Ringer (Fig. 1C).

Additional experiments were performed to determine, whether JAK3 is involved in the regulation of suicidal erythrocyte death or eryptosis following energy

depletion. Hallmarks of eryptosis include cell shrinkage. Thus, forward scatter was employed to estimate alterations of cell volume. Glucose withdrawal (mGlu) indeed decreased forward scatter significantly (Fig. 2). The addition of WHI-P131/JANEX-1 (156μM) or WHI-P154 (11.2μM) or WHI-P97 (22 μM) did not significantly modify the forward scatter in the presence of glucose and did not significantly blunt the decrease of forward scatter following glucose depletion (Fig. 2).

Another hallmark of eryptosis is triggering of cell membrane scrambling resulting in exposure of phosphatidylserine at the erythrocyte surface. Phosphatidylserine exposing erythrocytes were identified by determination of annexin V-binding. Glucose withdrawal (mGlu) was followed by the expected significant increase in annexin V-binding (Fig. 3). Addition of WHI-P131/JANEX-1 (156μM) or WHI-P154 (11.2μM) or WHI-P97 (22 μM) did not significantly modify the percentage of annexin V binding erythrocytes in the presence of glucose but significantly blunted the increase of annexin V binding following glucose withdrawal (Fig. 3). A dose response curve for WHI-P154 reveals maximal inhibition at a concentration of approximately 5 μM (Fig. 3).

To determine, whether JAK3 influences the decline of intracellular ATP concentration, cytosolic ATP was determined after 48h incubation upon glucose withdrawal with or without presence of WHI-P131/JANEX-1 (156 μM) or WHI-P154 (11.2 μM). Energy depletion caused a significant reduction in intracellular ATP concentration

Fig. 3. Annexin V binding with or without glucose depletion in the presence and absence of JAK3 inhibitors. A. Histogram of annexin V binding in a representative experiment of erythrocytes exposed for 48h to Ringer (1, black line), or glucose-depleted Ringer in the absence (2, red line) or presence (3, green line) of JAK3 inhibitor WHI-P131/JANEX-1 (156 μ M), M is the marker indicating annexin positive cells. B. Arithmetic means $\pm$ SEM (n = 8) of the percent Annexin V binding of erythrocytes exposed for 48h to glucose-containing (+glu, left white bar) or glucose-depleted (mGLU, right bars) Ringer without (black bar) or with (grey bars) WHI-P131/JANEX-1 (156 μ M) or WHI-P154 (11.2 μ M) or WHI-P97 (22 μ M). ### indicates significant (p<0.001) difference from glucose-containing Ringer, *** (p<0.001), ** (p<0.01), * (p<0.05) indicates significant difference from the absence of JAK3 inhibitors (ANOVA). C. Arithmetic means $\pm$ SEM (n = 8) of the percent Annexin V binding of erythrocytes exposed for 48h to glucose-containing Ringer without (open bars) or with (closed bars) WHI-P131/JANEX-1 (156 μ M), WHI-P154 (11.2 μ M) and WHI-P97 (22 μ M). D. Dose response curve of the antiscrambling effect of WHI-P154 during energy depletion. Arithmetic means $\pm$ SEM (n = 3) of the % annexinV binding cells as a function of inhibitor concentration.

in erythrocytes. Addition of neither WHI-P131/JANEX-1 (156 μ M) nor WHI-P154 (11.2 μ M) significantly modified the effect of energy depletion on the intracellular ATP concentration (Fig. 4).

Further experiments were performed in gene targeted mice lacking functional JAK3 (*jak3*^{-/-}) and in the corresponding wild type mice (*jak3*^{+/+}). Similar to human erythrocytes, wild type murine erythrocytes expressed JAK3 protein, while no band for JAK3 was detectable in western blot in erythrocytes from JAK3-deficient mice (*jak3*^{-/-}) (Fig. 5A).

As shown in Fig. 5C, the reticulocyte number was not significantly different in *jak3*^{-/-} mice than in *jak3*^{+/+} mice. In addition, the plasma concentration of erythropoietin was also not different in *jak3*^{-/-} mice than in *jak3*^{+/+} mice. The analysis of peripheral blood did not reveal any significant difference of red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB) concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) between *jak3*^{-/-} mice than in *jak3*^{+/+} mice (Fig. 5D). The osmotic resistance was also similar in both genotypes (Fig. 5E).

FACS analysis revealed that in the presence of glucose forward scatter was slightly but significantly smaller in *jak3*^{-/-} than in *jak3*^{+/+} erythrocytes (Fig. 6A and 6B), whereas annexin V binding was not significant

Fig. 4. Effect of glucose depletion on intracellular ATP content in the presence and absence of JAK3 inhibitors. Arithmetic means $\pm$ SEM (n = 4) of the intracellular ATP content of erythrocytes exposed for 48h to glucose-containing (Ringer, left white bar) or glucose-depleted (right bars) Ringer without (black bar) or with (grey bars) WHI-P131/JANEX-1 (156 μ M) and WHI-P154 (11.2 μ M). ### indicates significant (p<0.001) difference from glucose-containing Ringer.

different between *jak3*^{-/-} and *jak3*^{+/+} erythrocytes (Fig. 6C and 6D). To assess, whether genetic JAK3 deficiency modifies the eryptotic effect of energy depletion, *jak3*^{-/-} and *jak3*^{+/+} erythrocytes were incubated for 16h in the presence and absence of glucose. Energy-depletion

Fig. 5. Erythrocyte parameters in JAK3-deficient mice. A. Original Western blots demonstrating the expression of JAK3 protein in erythrocytes from two different JAK3-deficient mice (*jak3*^{-/-}) and the corresponding wild type mice (*jak3*^{+/+}). B. Confocal microscopy of FITC-dependent fluorescence of murine erythrocytes from JAK3-deficient mice (*jak3*^{-/-}) and corresponding wild type mice (*jak3*^{+/+}) stained with a primary anti-JAK3 antibody and a secondary FITC-conjugated anti rabbit antibody (upper panel) and stained with a primary anti-phospho-⁹⁸⁰tyr-JAK3 antibody and a secondary FITC-conjugated anti rabbit antibody. Erythrocytes were energy depleted by incubation in zero glucose Ringer for 24h (lower panel). C. Arithmetic means $\pm$ SEM of the plasma erythropoietin concentration ($n = 4$) of JAK3-deficient mice (*jak3*^{-/-}, black bar) and the wild type mice (*jak3*^{+/+}, white bar). D. Arithmetic means $\pm$ SEM ($n = 4$) of erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC) and reticulocyte count of JAK3-deficient mice (*jak3*^{-/-}, black bars) and the corresponding wild type mice (*jak3*^{+/+}, white bars). E. Osmotic resistance of erythrocytes from JAK3-deficient mice (*jak3*^{-/-}, closed symbols) and the corresponding wild type mice (*jak3*^{+/+}, open symbols).

resulted in cell shrinkage an effect not significantly different in *jak3*^{-/-} and in *jak3*^{+/+} erythrocytes (Fig. 6A and 6B). Most importantly, glucose depletion was followed by an increase in annexin V binding, an effect significantly less pronounced in *jak3*^{-/-} than in *jak3*^{+/+} erythrocytes (Fig. 6C and 6D).

As the inhibitors are not specific [36], we performed additional experiments to elucidate whether the antiscrambling effect of the inhibitors was related to their effect on Jak3. To this end, we evaluated the effect of the inhibitor WHI-P131/JANEX-1 (156 μ M) in *jak3*^{-/-} and *jak3*^{+/+} erythrocytes upon energy depletion. As a result, the inhibitor significantly blunted the annexin V binding in glucose depleted *jak3*^{+/+} erythrocytes but did not significantly modify annexin V binding in energy depleted *jak3*^{-/-} erythrocytes (Fig. 6E). WHI-P154

tended to decrease annexin V binding in glucose depleted *jak3*^{+/+} erythrocytes but not in energy depleted *jak3*^{-/-} erythrocytes (Fig. 6E). For both substances the inhibitory effect was significantly less pronounced in *jak3*^{-/-} than in *jak3*^{+/+} erythrocytes. Accordingly, WHI-P131/JANEX-1 and presumably WHI-P154 inhibits eryptosis at least partially by inhibiting Jak3.

Discussion

The present observations disclose a completely novel role of Janus kinase 3 JAK3. They demonstrate for the first time expression of JAK3 in erythrocytes. Moreover, they reveal that JAK3 is phosphorylated upon energy depletion. The antibody used detects phosphorylation at

Fig. 6. Decreased eryptosis of energy depleted erythrocytes from JAK3-deficient mice. **A.** Histogram of forward scatter as a measure of cell volume in a representative experiment of erythrocytes from JAK3-deficient mice (*jak3^{-/-}*) (green line) and the corresponding wild mice (*jak3^{+/+}*) (black line) exposed for 16h to glucose-depleted Ringer. **B.** Arithmetic means $\pm$ SEM ($n = 4$) of forward scatter of erythrocytes from JAK3-deficient mice (*jak3^{-/-}*, black bars) and the corresponding wild type mice (*jak3^{+/+}*, white bars) spontaneous cell size at the beginning of the incubation (left bars) or exposed for 16h to glucose-depleted Ringer (right bars). ** indicates significant difference ($p < 0.05$) from wild type mice (ANOVA). **C.** Histogram of annexin V-binding reflecting phosphatidylserine exposure in a representative experiment of erythrocytes from JAK3-deficient mice (*jak3^{-/-}*) (green line) and the corresponding wild type mice (*jak3^{+/+}*) (black line) exposed for 16h to glucose-depleted Ringer. **D.** Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin V-binding erythrocytes from JAK3-deficient mice (*jak3^{-/-}*, black bars) and the corresponding wild type mice (*jak3^{+/+}*, white bars) prior to (left bars) or following (right bars) exposure for 16h to glucose-depleted Ringer. ### indicates significant ($p < 0.001$) difference from spontaneous annexin V-binding, *** indicates significant ($p < 0.001$) difference from wild type mice (ANOVA). **E.** Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin V-binding erythrocytes from JAK3-deficient mice (*jak3^{-/-}*, black bars) and the corresponding wild type mice (*jak3^{+/+}*, white bars) exposed for 16h to glucose-depleted Ringer in the presence or absence of Jak3 inhibitor WHI-P131/JANEX-1 (156 μ M) or WHIP154 (11.2 μ M). ** indicates significant ($p < 0.05$) difference from the absence of JAK3 inhibitors.

tyrosine 980, a phosphorylation site previously shown to activate JAK3 [37]. The present observations further show that JAK3 is a completely novel regulator of erythrocyte survival, which is activated following glucose withdrawal and contributes to the triggering of cell membrane scrambling during energy depletion. At first glance, a stimulating effect of JAK3 on suicidal erythrocyte death or eryptosis upon energy depletion appears surprising in view of its rather antiapoptotic effects in lymphocytes [6-8]. In contrast to their apoptosis stimulating effects in lymphocytes [9, 10], JAK3 inhibitors rather blunt the eryptosis following energy depletion. The role of JAK3 in the regulation of erythrocyte survival is comparable to its role in the regulation of dendritic cell survival. Dendritic cells from JAK3 deficient mice exhibited enhanced survival upon cytokine withdrawal, and thus, JAK3 appeared to be an important negative regulator of dendritic cell survival [11].

Inhibition of JAK3 in human erythrocytes and genetic knockout of JAK3 do not appreciably modify cell membrane scrambling of unstressed erythrocytes. Presumably the kinase is largely inactive and does not play a major role in the absence of cell stress. Nevertheless, the erythrocyte volume appears to be slightly smaller in gene targeted mice lacking functional JAK3 (*jak3^{-/-}*) than in the corresponding wild type mice (*jak3^{+/+}*). JAK3 is apparently activated upon energy depletion and contributes to the stimulation of cell membrane scrambling in energy depleted erythrocytes. The effect of pharmacological or genetic knockout is, however, modest, indicating that other, JAK independent, mechanisms participate in the triggering of eryptosis during energy depletion. As demonstrated earlier [31], energy depletion by glucose withdrawal stimulates protein kinase C and leads to increase of cytosolic Ca²⁺ concentration in erythrocytes. The cellular energy

status is further sensed by the AMP-activated protein kinase (AMPK), which is activated upon increase in cytosolic AMP/ATP concentration ratio [38, 39], an increase in cytosolic Ca^{2+} concentration [38] and by nitric oxide [40]. AMPK stimulates cellular glucose uptake, glycolysis, stimulation of fatty acid oxidation and expression of enzymes required for ATP production [39, 41-43]. The kinase downregulates energy-utilizing mechanisms including protein synthesis, gluconeogenesis and lipogenesis [39, 41, 44]. Thus, AMPK enhances the capacity of the cell to generate ATP [44] and to survive energy depletion [44, 45]. In analogy to its antiapoptotic effect in nucleated cells [45], AMPK protects against the suicidal erythrocyte death [32]. At least in theory, AMPK could contribute to the powerful inhibitory effect of NO on Ca^{2+} -induced cell membrane scrambling of erythrocytes [46]. Even though it is similarly activated by energy depletion, JAK3 does not protect against but stimulates erythrocyte death.

The effect of glucose depletion and its sensitivity to JAK3 is qualitatively similar but not quantitatively identical in human and murine erythrocytes. The murine erythrocytes are obviously more sensitive to glucose depletion. Murine erythrocytes further appear to be less sensitive to the JAK3 inhibitors than human erythrocytes. In both species, however, JAK3 fosters cell membrane scrambling.

JAK3-sensitive erythrocyte survival may be relevant for the life span of circulating erythrocytes. Phosphatidylserine-exposing cells are bound to [47] and subsequently engulfed by [48] macrophages. They are thus rapidly cleared from circulating blood [22]. Accordingly, accelerated eryptosis may lead to anemia [12]. However, as indicated above, in energy replete conditions JAK3 does not appreciably modify eryptosis. Along those lines, the blood count is not significantly different between *jak3*^{-/-} mice and *jak3*^{+/+} mice. The difference between *jak3*^{-/-} mice and *jak3*^{+/+} mice may

become only apparent under conditions of energy depletion.

Eryptosis may further affect the microcirculation. Phosphatidylserine-exposing erythrocytes may bind to the vascular wall [49-53] and foster the assembly of prothrombinase and tenase, the generation of thrombin and thus blood clotting [49, 54, 55]. For instance, enhanced trapping of eryptotic erythrocytes has previously been observed in renal medulla following ischemia [56]. Accordingly, suicidal erythrocytes have thus been proposed to participate in vascular injury of metabolic syndrome [57]. An influence of JAK3 on adhesion to the vascular wall is, however, only expected during energy depletion of circulating erythrocytes.

In view of the present observations, JAK3 may be similarly activated upon energy depletion of nucleated cells. Energy dependent JAK3 activity may have significant implications in the development and progression of tumors, as activating JAK3 mutations have been linked to the development of human lymphoid cancer cells [58-60] as well as the development and progression of solid tumors [61, 62].

In conclusion, the present observations reveal that erythrocytes express JAK3, which is activated by and participates in the cell membrane scrambling effect of energy depletion. The present observations thus reveal a novel regulator of eryptosis and a novel regulation and function of JAK3.

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