Unraveling apoptosis and autophagy mediated mechanisms contributing to the pathogenesis of pediatric Immune Thrombocytopenia

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Experimental Hematology / Oncology

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Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder with **poor response treatment** in some pediatric patients. Abnormalities in platelets produced by their precursors, the megakaryocytes (MKs) in the bone marrow (BM), are implicated in ITP pathophysiology. Previous studies, including those from our group[1-3], have demonstrated a role of **platelet apoptosis in ITP**, while the contribution of **autophagy** remains elusive.

Aims of the study

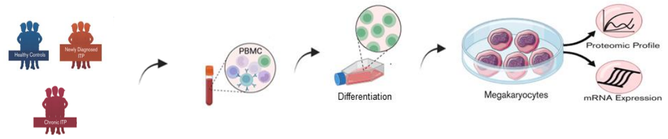
1) To investigate the autophagy flux and the autophagy-mediated effects in MKs in ITP disease

2) To examine the mechanistic molecular interplay between apoptosis and autophagy in pediatric ITP disorder

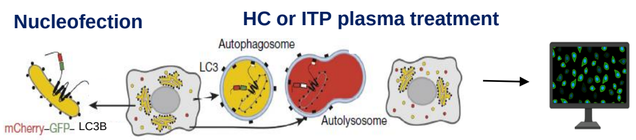
Methods

We used the MEG-01 cell line and MKs derived from peripheral blood mononuclear cells (PBMCs) of healthy controls (HCs) and age-matched pediatric ITP patients. Differentiation of CD34⁺ selected PBMCs into platelet-producing-MKs was assessed by flow cytometry using antibodies against CD61 and CD42a..

We investigated the expression of **apoptosis and autophagy** at mRNA level by qRT-PCR and at protein level by Western blot and confocal microscopy.



Autophagic flux was assessed with **chloroquine** treatment by immunofluorescence and also with nucleofection with the **LC3B-GFP-mCherry** reporter in MEG-01 and CD34⁺ HCMKs incubated either with control or ITP derived plasma.



We further performed **siRNA knockdown** of CASP3 and BAX apoptosis markers and examined mechanistic roles in ITP disorder.

Results

1) Effects of ITP plasma on apoptosis and autophagy in MEG-01

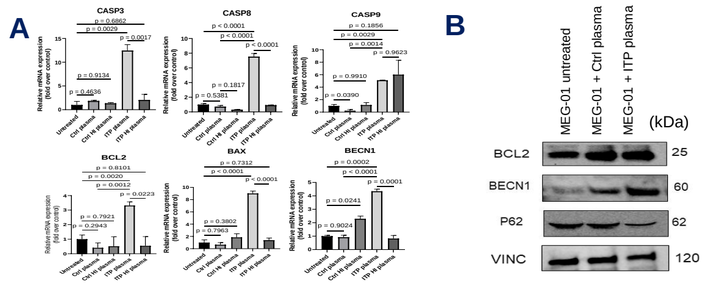


Figure 1. Effects of heat inactivation (HI) of control or ITP plasma plasma on apoptosis and autophagy in MEG-01 cell line. **(A)** Relative mRNA levels of apoptosis and autophagy genes were determined by qRT- PCR. Statistical analysis was performed using one-way ANOVA followed by multiple comparisons tests to compare the mean ranks between the groups. Error bars show SD. Significance is shown as $p < 0.05$. **(B)** Western blot for apoptosis and autophagy markers in the MEG-01 cell line. Vinculin served as a loading control.

2) Effects of ITP plasma on P62 expression in the MEG-01 cell line and in CD34⁺ derived MKs

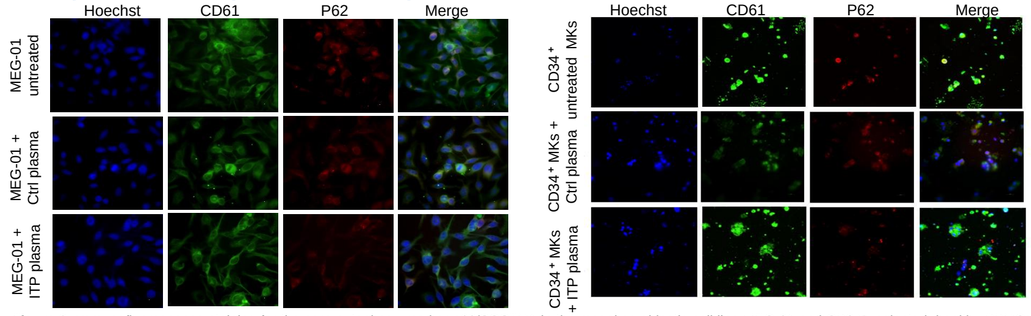


Figure 2. Immunofluorescence staining for the core autophagy marker p62/SQSTM1 in the megakaryoblastic cell line MEG-01 and CD34⁺ selected healthy PBMCs incubated with plasma derived either from healthy donors or pediatric age-matched ITP patients. Cell nuclei was assessed with Hoechst staining (blue), whereas CD61 (green) was used as a marker of megakaryocyte/platelet lineage and p62/SQSTM1 (red) for the autophagy cargo receptor.

3) Assessment of the autophagy flux in the megakaryoblastic MEG-01 cell line and CD34⁺ differentiated MKs from health derived donors

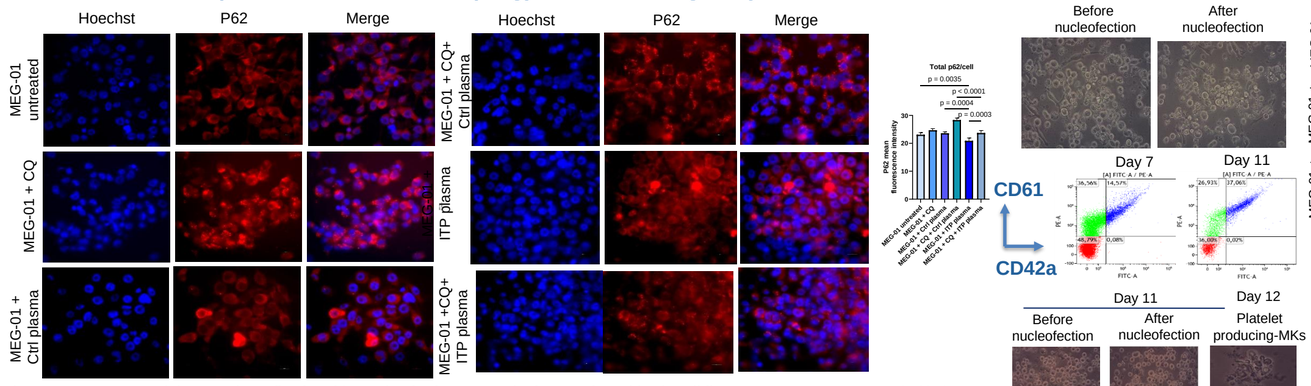


Figure 3. Assessment of the autophagy flux by Chloroquine (CQ) treatment in the megakaryoblastic cell line MEG-01. MKs were treated with the lysosomal inhibitor CQ (C=100uM) for 6h and plasma isolated either from healthy individuals or pediatric ITP patients (N=3). Cell nuclei were visualized with Hoechst staining (blue) and the core autophagy marker p62 was detected by immunofluorescence (red). Images were captured at 20× magnification by Leica SP8 Inverted microscope.

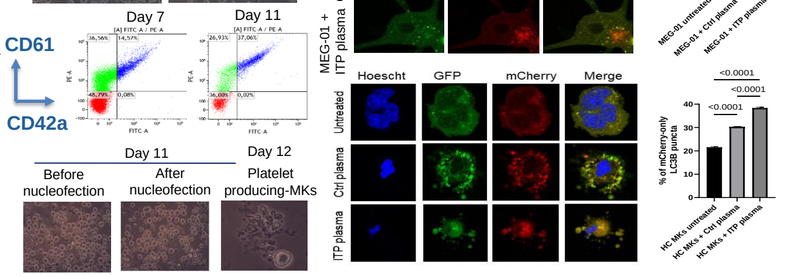


Figure 4. Assessment of the autophagy flux in the MEG-01 cell line and mature megakaryocytes differentiated from CD34⁺ selected PBMCs derived from adult healthy donors. Healthy derived MKs were nucleofected with the LC3B-GFP-mCherry reporter on Day 11 of differentiation and were coated on fibrinogen coated coverslips 24 h post transfection prior to treatment with healthy control or pediatric ITP derived plasma for 3h. Representative confocal images were acquired by the Leica SP8 microscope.

4) Effect of siRNA knockdown of CASP3 and BAX in HC and ITP-derived MKs on autophagy

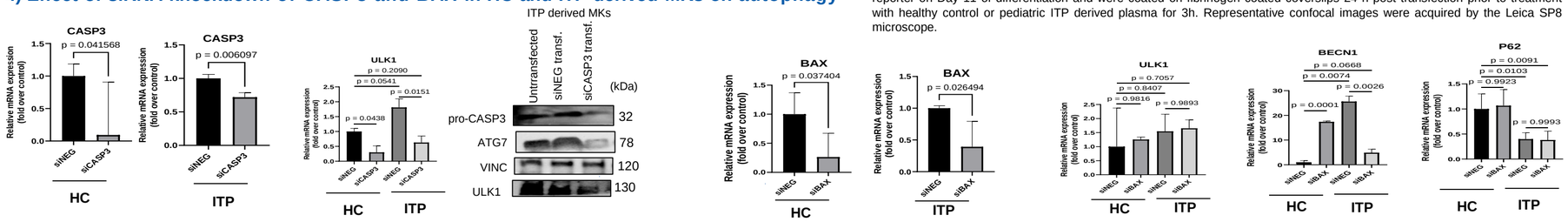


Figure 5. Effects of siRNA knockdown of the apoptotic markers BAX and CASP3 on specific autophagy markers in MKs differentiated from PBMCs of healthy controls or ITP patients. Relative mRNA levels were determined by qRT- PCR. Statistical analysis was performed using one-way ANOVA followed by multiple comparisons tests to compare the mean ranks between the groups. Error bars show SD. Significance is shown as $p < 0.05$. Western blot for ITP derived MKs transfected with siRNA targeting CASP3. Vinculin served as an internal loading control.

Conclusions

1) Pediatric ITP plasma can induce **caspase-dependent-apoptosis** and its heat inactivation reversed this effect

2) Both MEG-01 cells and CD34⁺ MKs transfected with the LC3B-GFP-mCherry reporter and treated with ITP plasma showed increased LC3B puncta compared to the control plasma treated and untreated cells by confocal and downregulation of P62 at protein level suggesting an enhanced autophagy flux in MKs.

3) Suppression of CASP3 downregulated the autophagy effectors ULK1 and ATG7 in ITP derived MKs.

References

1) Stein T, Bitsina C, Seiller M, Schmugge M, Franzoso FD, Clusterin can mediate apoptosis-induced molecular mechanisms in immune thrombocytopenia, *Blood VTH*, 2024 ; 100012

2) Schmugge M, Franzoso FD, Winkler J, Kroiss S, Seiler M, Speer O, Rand ML. IVIg treatment increases thrombin activation of platelets and thrombin generation in paediatric patients with immune thrombocytopenia. *Br J Haematol* 2023 Jun;201(6):1209-1219.

3) Goelz N, Eekels JJM, Pantic M, Kamber CT, Speer O, Franzoso FD, Schmugge M.,Platelets express adaptor proteins of the extrinsic apoptosis pathway and can activate caspase-8.*PLoS One*. 2021; 16(1):e0244848.

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