



Enhanced procoagulant activity of monocytic leukemia cell lines and microparticles

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Background and aims

Patients with hematologic malignancies have a strong association with venous thromboembolism and in an even larger number of patients hypercoagulability remains subclinical. The expression of hemostatic proteins by tumor cells e.g. tissue factor (TF), microparticle (MP) formation as well as chemotherapy may contribute to the enhanced procoagulant activity. In acute myeloid leukemia (AML), the malignantly transformed promylecytic cells in AML M3 are well known for their procoagulant activity, however a lot less is known about the monocytic leukemias. In AML, the FLT3 (fms-like tyrosine kinase receptor-3) internal tandem duplication (ITD) has been described in 20-25% of adults with with the highest frequency in the monocytic lineage. The mutation results in constitutive dimerization of the receptor and auto-phosphorylation of tyrosine residues that accounts for poor prognosis. In these studies we investigated monocytic leukemia cell lines by antigenic and functional assays and related their procoagulant potential with the FLT3-ITD mutational status.

Samples and methods

Separation of normal monocytes

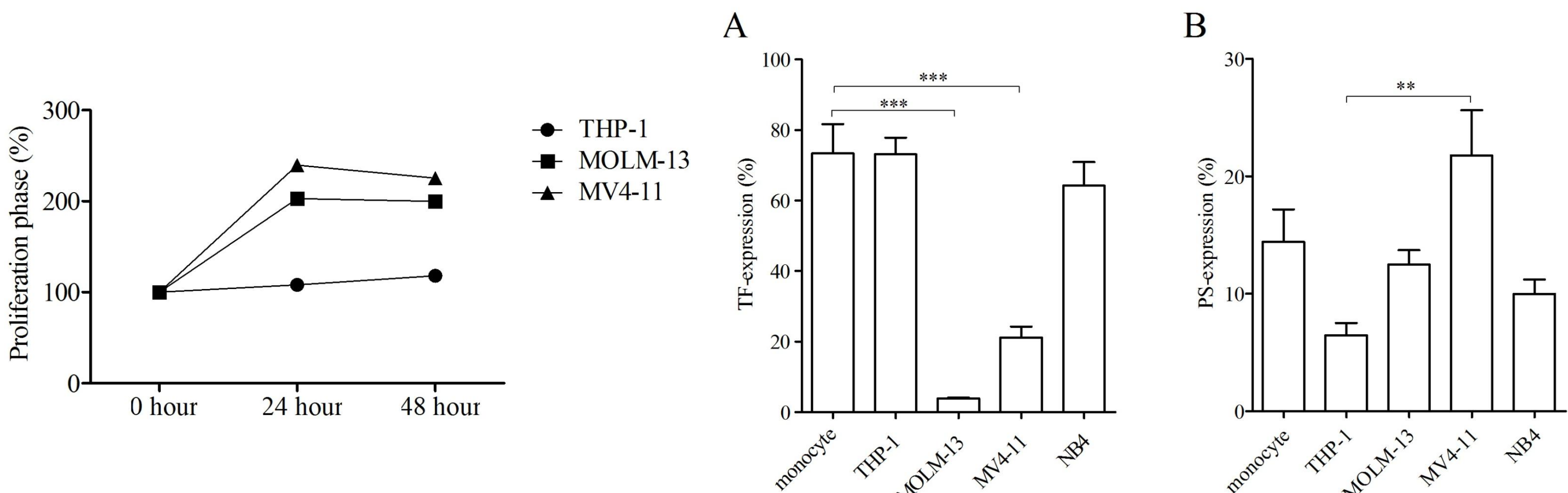
Ca. 160 mL EDTA anticoagulated peripheral blood → PBMC separation → magnetic monocyte separation by negative selection (Invitrogen, Untouched Human Monocytes).

	THP-1	MOLM-13	MV4-11
FLT3-ITD mutation	wt/wt	wt/FLT3-ITD ⁺	FLT3-ITD ⁺ /FLT3-ITD ⁺
Doubling time	50 hours	50 hours	50 hours
Phenotype	CD13, CD15, CD33	CD4, CD15, CD33	CD4, CD15, CD33
Cytogenetic status	Complex cytogenetic abnormalities		
Molecular alterations	MLL-AF9	MLL-AF9	MLL-AF4
Cell culturing	RPMI-1640 medium supplemented with 15% FBS, 37° C, 5% CO ₂		

As a positive control, an acute promyelocytic leukemia cell line (NB4) was used.

Results

Flow cytometry



The FLT3 mutated cell lines (MOLM-13 and MV4-11 displayed a high proliferation rate and showed shorter doubling times. These cell lines expressed significantly lower surface TF (panel A) but more PS (panel B) than the wild type THP1 cell line.

Functional assays

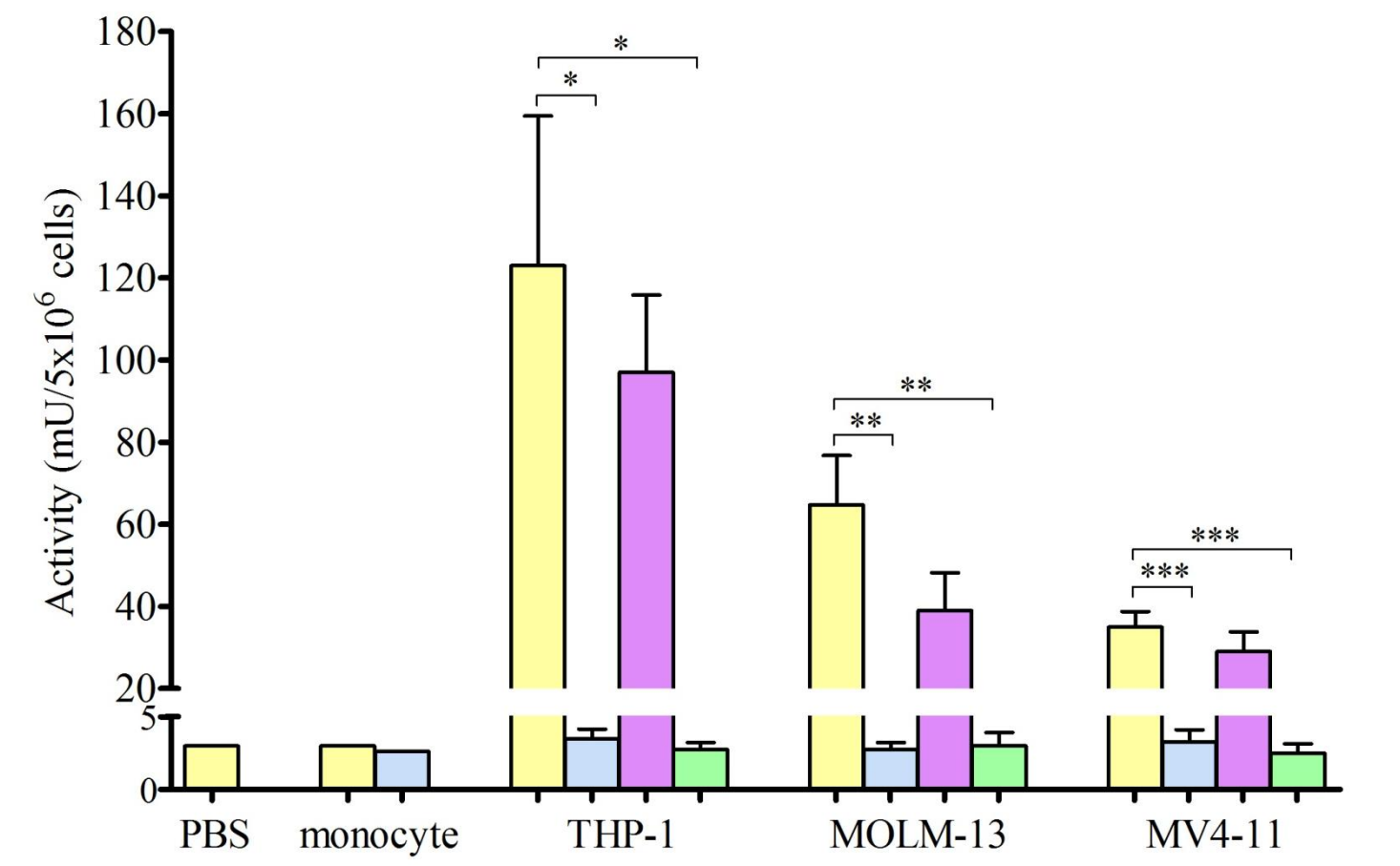
- Procoagulant activity (PCA) measured by a one-stage clotting assay on a KC1 coagulometer.
- Clotting times were converted to PCA by a thromboplastin dilution curve.
- Thrombin generation test (TGT) measure on a Fluoroscan Ascent fluorimeter.
- Data were evaluated by the Thromboscope software.

Flow cytometric assays

- Cell cycle studies by propidium iodide staining (PI)
- TF antigen detection by direct conjugated monoclonal antibodies
- Measurement of phosphatidylserine (PS) expression by annexin V binding
- Microparticle separation was performed with multiple high-speed centrifugation.
- The number of MPs were determined according to their PS expression by flow cytometry.

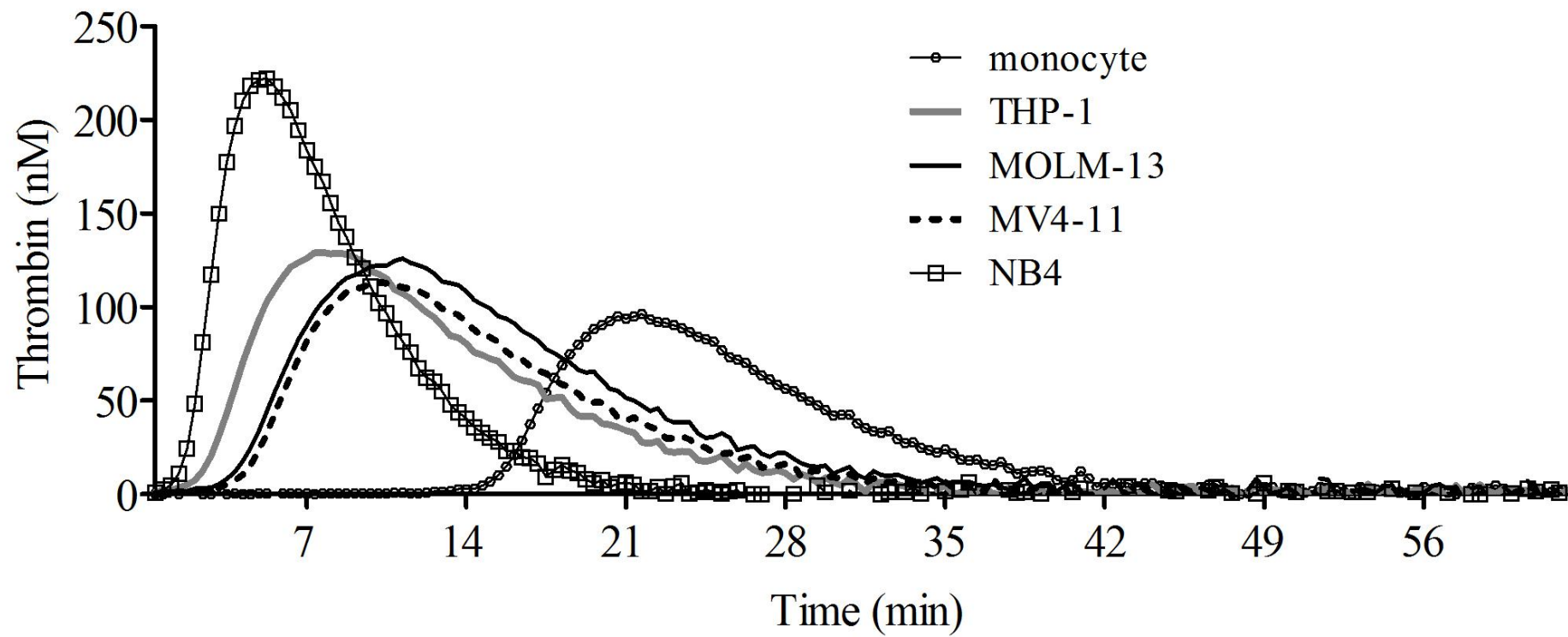
TF, PS, and PI measurements were done on a Beckman Coulter FC500 equipment and MP measurements were carried out on Becton Dickinson, Canto II flow cytometer.

Procoagulant activity

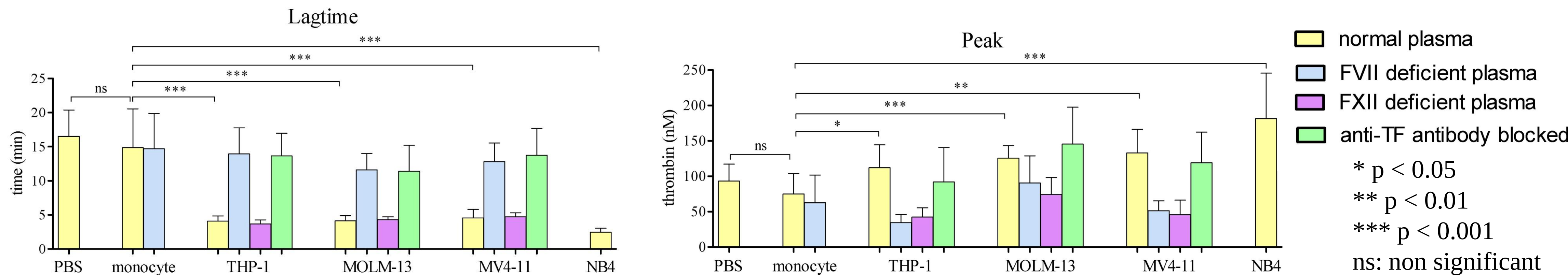


Contrary to monocytes, all thee cell lines induced considerable PCA in a clotting assay that was dependent on TF and FVII but not FXII.

Thrombin generation

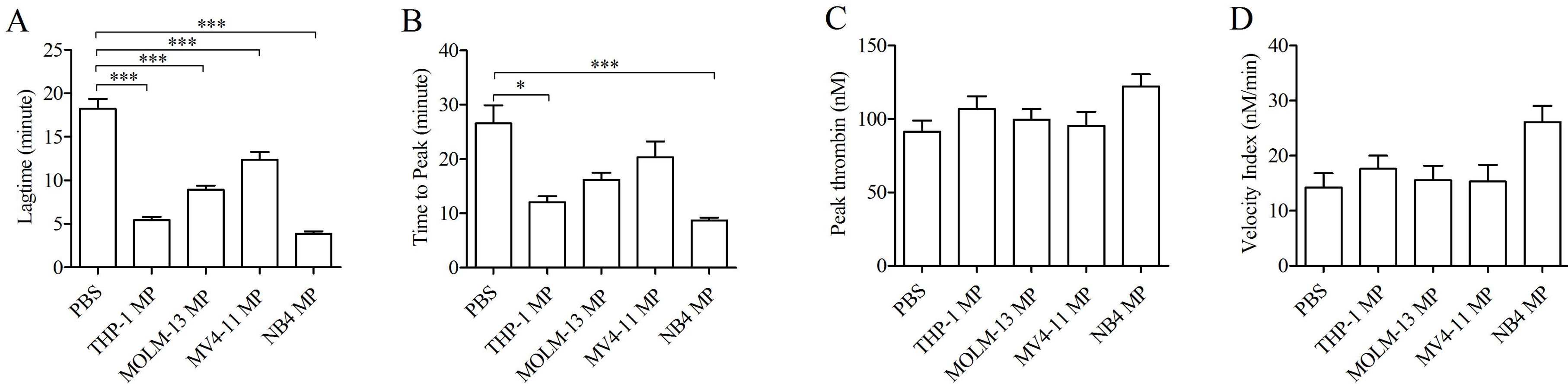


Representative TGT curves show that all leukemic cell lines induced thrombin generation at a similar rate. FVII deficient plasma and anti-TF blocking antibody significantly delayed thrombin formation and decreased peak thrombin values. FXII deficient plasma did not affect the speed of thrombin generation but resulted in an attenuated thrombin formation. Isolated human monocytes did not display any thrombin generation when compared to buffer control.



Thrombin generation of microparticles

In addition to native cells, MPs generated by these cells also contribute to the procoagulant activity by shortening the times for thrombin formation (panels A, B), but with no effect on thrombin quantity (panels C, D). Similarly to the parent cell lines the strongest effect was shown by NB4 followed by THP-1, MOLM-13 and MV4-11 MPs.



Thrombin generation of resistant MOLM-13 cell lines

Sunitinib and CEP-701 (Lestaurtinib)

- Sunitinib is a targeted tyrosine kinase inhibitor able to inhibit members of the receptor tyrosine kinase families.
- CEP-701 is a selective FLT3 tyrosine kinase inhibitor.
- MOLM-13 cell line was treated with medium containing at a final concentration of 100 nM Sunitinib or 30 nM CEP-701 for 2 months.
- The presence of resistance for these inhibitors was evaluated by the Presto Blue Assay.

* p < 0.05
** p < 0.01
*** p < 0.001
ns: non significant

TGT parameters	vs.	MOLM-13	CEP-701 resistant	Sunitinib resistant
Lagtime (min)	monocyte	*** ↓	** ↓	** ↓
Peak (nM)		*** ↑	ns	ns
Lagtime (min)	MOLM-13		*** ↑	ns
Peak (nM)			* ↓	ns
Lagtime (min)	CEP-701 resistant			** ↓
Peak (nM)				ns

Conclusions

- Monocytic leukemia cell lines exhibit significantly higher procoagulant activity compared to normal monocytes and this effect is primarily TF dependent.
- The FLT3-ITD mutation status is accompanied by higher PS expression and this modulates the procoagulant properties.
- Microparticles released by monocytic leukemia cells also contribute to the procoagulant activity by shortening the time required for thrombin formation.
- Sunitinib resistant MOLM-13 cell line displayed similar procoagulant effect as non-resistant MOLM-13, while CEP resistance cell line showed decreased procoagulant activity.