Flow cytometric detection of MPL (CD110) as a diagnostic tool for differentiation of congenital thrombocytopenias

The aim of this study was to investigate the suitability of flow cytometric detection of the thrombopoietin (THPO) receptor MPL (CD110) as a diagnostic tool in congenital thrombocytopenia. MPL is expressed on early hematopoietic progenitors, megakaryocytes and platelets. Homozygous or compound heterozygous deleterious mutations in the MPL gene lead to congenital amegakaryocytic thrombocytopenia (CAMT).[1] The absence of MPL expression on platelets from CAMT patients has been described in a couple of case studies.[2,3] However, reduced expression of *MPL* on platelets has also been demonstrated in patients with other forms of congenital thrombocytopenia,[4] and in patients with inherited or acquired forms of thrombocytosis.[5-7] Here we show that MPL expression on platelets was low to undetectable in all conditions with high THPO plasma levels due to irreversible internalization of the receptor - irrespective of an underlying MPL defect. In contrast, levels of MPL on early $CD34^{+}CD38^{lo}$ hematopoietic progenitors were relatively independent of THPO levels, and therefore are appropriate as a diagnostic tool to help in identifying conditions with impaired expression of MPL.

Flow cytometric detection of platelet surface proteins is a routine method in the diagnosis of congenital platelet disorders like Bernard-Soulier syndrome or Glanzmann thrombasthenia.[8] The flow cytometric analysis of platelets, even in small samples of extremely thrombocytopenic individuals, is well established and makes this method ideal for a swift diagnosis on a molecular level. It seems obvious to use this method in the diagnosis of CAMT, which is characterized by a MPL defect. The applicability of this method in the diagnosis of inherited thrombocytopenia and its interference from external influences has not yet been evaluated. Furthermore, the poor availability of monoclonal antibodies in the past hindered its usage for diagnostic purposes in hematological laboratories: over the intervening years none of the commercially available monoclonal antibodies intended for the detection of MPL were found to be suitable, since they proved not to be specific for MPL.[9,10]

We used the monoclonal CD110 antibody clone 1.6[10] for flow cytometric detection of MPL on platelets and bone marrow cells from both healthy donors and patients with different forms of thrombocytopenia. Peripheral blood and bone marrow from healthy controls and from thrombocytopenic patients were obtained after informed consent was given in accordance with the Declaration of Helsinki. The study has been approved by the ethics committee of the Hannover Medical School. For experimental details see figure legends. For quantitative assessment we used cumulative subtraction[11] to determine the percentage of $CD110^{+}$ events relative to an isotype control in a univariate population comparison. It should be noted that "percentage posi-

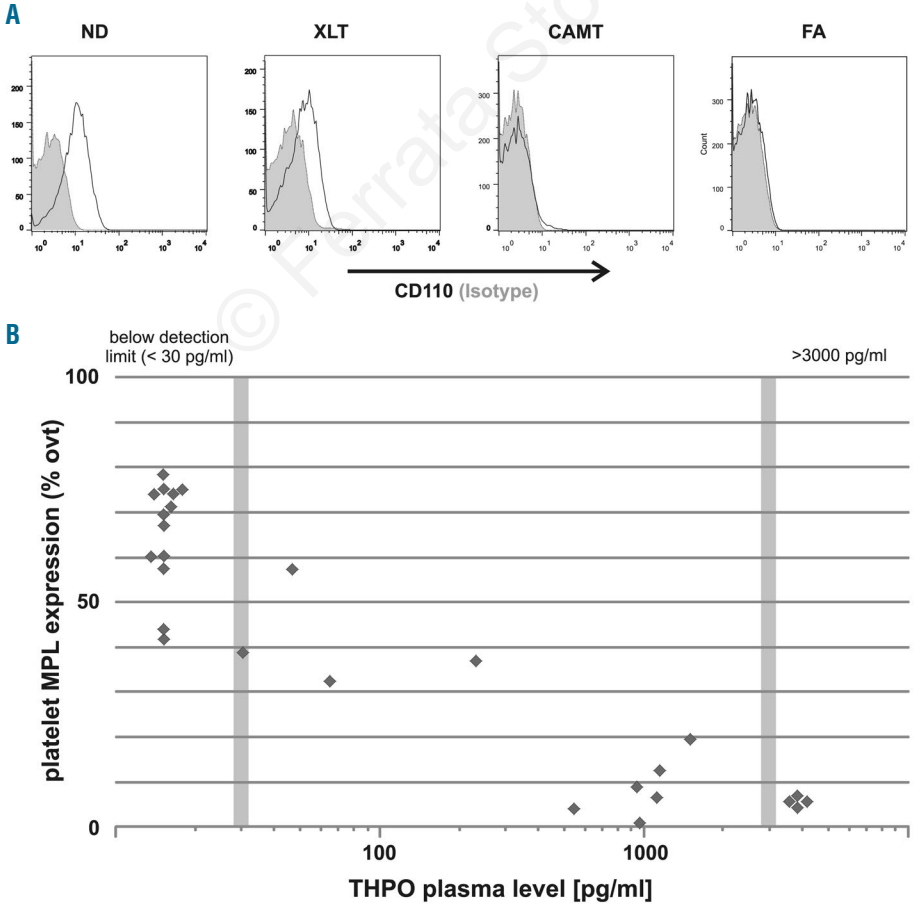

Figure 1. Detection of CD110 (MPL) on platelets. (A) Detection of MPL on platelets: Platelet rich plasma was prepared from anticoagulated peripheral blood. After short pre-incubation with human immunoglobulins (Gammagard, Baxter Healthcare Corporation, IL, USA) in order to block Fc receptors platelets were stained and analyzed on a flow cytometer (BD FACSCalibur, BD biosciences, San Jose, CA, USA). Histograms show the results from PE-fluorescence from platelets gated on scatter properties. Gray shaded histograms: isotype control (IgG2b-PE, Caltag Medsystems, UK); open histograms: anti CD110-PE (clone 1.6.1, BD biosciences, San Jose, CA, USA). ND: normal donor, XLT: x-linked thrombocytopenia, FA: Fanconi anemia. (B) MPL expression on platelets from thrombocytopenic and non-thrombocytopenic individuals calculated by cumulative subtraction[11] was plotted against THPO plasma levels as determined by ELISA (Quantikine, R&D Systems, MN, USA). Data corresponding to THPO concentrations below and above the detection range of the ELISA system were grouped left or right of the gray bars, respectively.

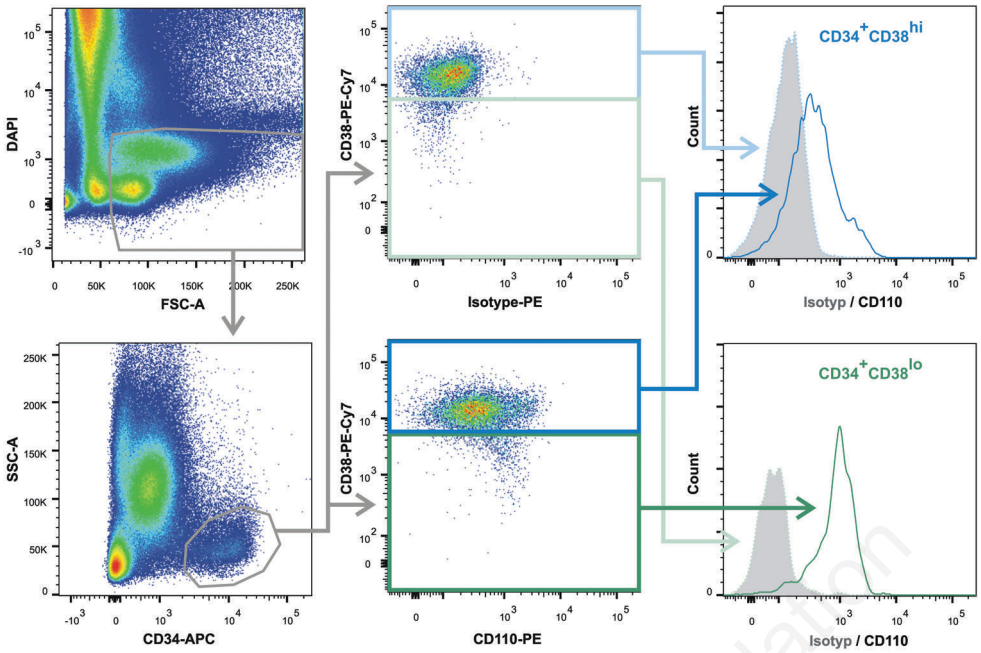

Figure 2. Detection of CD110 (MPL) on BM cells. Detection of MPL on hematopoietic progenitors from BM-gating strategy: BM mononuclear cells or total BM after erythrocyte-lysis were stained with CD34-APC (clone 8G12, BD biosciences), CD38-PECy7 (clone HIT2, eBiosciences) and CD110-PE or isotype control, respectively. DAPI was added immediately before flow cytometric analysis (LSR II, BD biosciences). The gating strategy is shown for a sample of frozen BM-MNC with a high percentage of dead cells and debris. Living cells were first gated on basis of DAPI exclusion and FSC (top left). Further analyses refer to $CD34^{+}SSC^{lo}$ cells (bottom left). Differentiation of CD34 subpopulations was done according to Terstappen *et al.*[13] with $CD34^{+}CD38^{hi}$ corresponding to populations PIII and PIV and $CD34^{+}CD38^{lo/-}$ to PI and PII. Specific binding of CD110 (top middle and open histograms) in comparison to an isotype control (bottom middle and grey histograms) is shown for $CD34^{+}CD38^{hi}$ (top right) and $CD34^{+}CD38^{lo}$ (bottom right) cells.

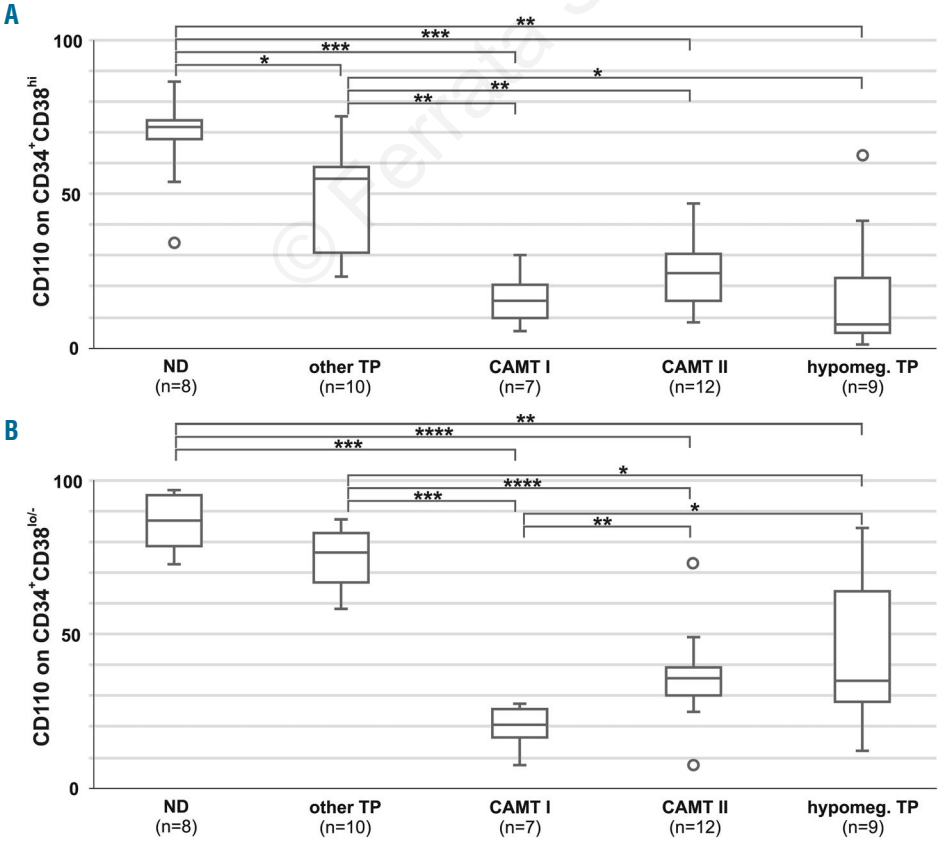

Figure 3. Detection of CD110 (MPL) on hematopoietic progenitors. Box plots summarizing all flow cytometric measurements of CD110 on $CD34^{+}CD38^{hi}$ (A), and $CD34^{+}CD38^{lo}$ (B) hematopoietic progenitors. The distribution of CD110 expression levels, calculated from cumulative subtraction[11] is plotted for healthy donors (ND) and different groups of thrombocytopenic patients (for explanation see text). Bottom and top of the boxes represent 25th and 75th percentiles, the band inside the median. The vertical lines protruding from the box extend to the minimum and the maximum values of the data set, as long as these values do not differ from the median by more than 1.58 times the interquartile range (outliers plotted as open circles). The non-parametric Wilcoxon-Mann-Whitney test was used for comparison between groups. *:$P \leq 0.05$, **:$P \leq 0.01$, ***: $P \leq 0.001$, ****:$P \leq 0.0001$

tive" is a calculated parameter which is affected by both percentage of positive cells and expression level, and has been chosen as a robust parameter which is relatively insensitive to slight variations in instrument settings and experimental conditions.

In initial experiments we could demonstrate the expression of MPL on platelets from healthy donors as well as from patients suffering from thrombocytopenia of different etiologies other than CAMT (e.g. idiopathic thrombocytopenia, autosomal dominant macrothrombocytopenia, Wiskott-Aldrich Syndrome). In contrast, but as expected, MPL either could not or could scarcely be measured on the surface of platelets from CAMT patients (Figure 1A).

We extended our screening to patients with an as yet undiagnosed hypomegakaryocytic thrombocytopenia which was suspicious for CAMT. Surprisingly, in a couple of patients we found a wild-type *MPL* gene despite a complete lack of MPL expression on platelets. Some of these turned out to have another form of congenital hypomegakaryocytic thrombocytopenia or pancytopenia like Fanconi anemia (Figure 1A). Since THPO plasma levels are extremely high in all hypomegakaryocytic forms of thrombocytopenia (up to several ng/ml in contrast to <30 pg/ml in healthy donors), we hypothesized that the lack of MPL expression was due to high THPO levels. Indeed, MPL was detectable neither on platelets from a patient with chemotherapy induced severe thrombocytopenia, nor on platelets from a healthy donor 24 h after transfusion to an aplastic anemia patient (*Online Supplementary Figure S1A*). Flow cytometric analysis of MPL on platelets was not able to differentiate between CAMT and other hypomegakaryocytic forms of thrombocytopenia. Levels of MPL detectable on platelets from normal donors or thrombocytopenic patients were inversely correlated with THPO serum levels (Figure 1B). These results can be explained by the results from studies with iodinated THPO which have shown that the binding of THPO to its receptor MPL on platelets leads to fast and irreversible internalization of the receptor-ligand complex, leaving not more than 20% of the total binding sites available on the platelet surface.[12] Most of the constitutively produced THPO is cleared from the circulation by this process. Elevated THPO serum levels can be a consequence of low MPL expression (e.g. in CAMT) but can also cause decreased levels of MPL on the platelet surface (in other forms of hypomegakaryocytic thrombocytopenia).

We subsequently tested the detection of MPL in the BM where it is mainly expressed on megakaryocytes and early hematopoietic progenitors. We didn't consider the analysis of megakaryocytes due to their rarity and heterogeneity in size and autofluorescence, which makes detection of faintly expressed surface molecules difficult. We analyzed CD110 expression on hematopoietic progenitors specified by the expression of CD34 and CD38. CD34 is expressed on all hematopoietic progenitors; the subpopulation of early multipotential progenitors and stem cells is characterized by low or absent CD38 expression.[13] Analyses were done either with total BM leukocytes from fresh BM samples after hypotonic lysis of erythrocytes, or with frozen mononuclear cells from BM or umbilical cord blood for retrospective analysis of cryopreserved patient samples.

Analysis of BM (n=5) and cord blood (n=3) from healthy donors revealed a measurable MPL expression on $CD34^+$ hematopoietic progenitors, which was significantly stronger on early $CD34^+CD38^{lo/-}$ cells compared to more mature $CD34^+CD38^{hi}$ cells (Figure 2).

Upon analyzing BM cells from thrombocytopenic patients we found different patterns of MPL expression: in thrombocytopenic patients with thrombocytopenia due to increased platelet destruction and normal THPO levels (i.e. idiopathic thrombocytopenia, x-linked thrombocytopenia, autosomal dominant macrothrombocytopenia; n=7) MPL expression was low but clearly above background on $CD34^+CD38^{hi}$ cells (Figure 3A, see "other TP"), and only slightly reduced compared to healthy donors on $CD34^+CD38^{lo/-}$ cells (Figure 3B). CAMT I patients (n=7) who are predicted to have a complete loss of the receptor function[14] demonstrated no measurable MPL expression on either $CD34^+CD38^{hi}$ or $CD34^+CD38^{lo/-}$ cells (Figure 3). In contrast, CAMT II patients (n=12) who are characterized by a transient amelioration of thrombocytopenia during the first year of life due to a residual function of the MPL receptor,[14] showed a heterogeneous pattern of MPL expression. MPL expression on committed $CD34^+CD38^{hi}$ progenitors was low to undetectable (Figure 3A). On early $CD34^+CD38^{lo/-}$ progenitors it was significantly higher than in CAMT I patients (Figure 3B), but the distribution of individual values ranged from non-detectable up to almost normal levels of CD110. This might be due to the different mutations and mechanisms which lead to the reduced receptor activity. For example, the patient with nearly normal CD110 expression out of the CAMT II group, depicted as an upper outlier in Figure 3B, has a MPL missense mutation in the transmembrane domain which seems to allow for an almost normal expression of the extracellular domain of the receptor which is recognized by the monoclonal antibody. Furthermore, apparently low CD110 expression in the CAMT II group could be a result of mutations affecting the binding site of the monoclonal antibody. The results for the group of hypomegakaryocytic thrombocytopenia (n=13) coming along with high plasma levels of THPO resembled those for the CAMT II group in some respects: MPL expression on $CD34^+CD38^{hi}$ cells was low to undetectable in most of the cases, whereas MPL expression on $CD34^+CD38^{lo/-}$ cells showed a large variability (Figure 3).

Comparing the results of MPL expression analysis on different cell types, we found that MPL expression measured on $CD34^+CD38^{lo/-}$ cells gave the best discrimination between patients with impaired MPL expression and patients with other forms of hypomegakaryocytic thrombocytopenia with high plasma levels of THPO (Figure 3 and *Online Supplementary Figure S1B*).

The comparison of flow cytometric detection of MPL on different cell types within the group of patients with high THPO plasma levels (>500 pg/ml), showed that in contrast to platelets MPL expression on $CD34^+CD38^{lo/-}$ early hematopoietic progenitors seems to be unaffected by THPO plasma levels (*Online Supplementary Figure S2*).

Our results suggest a mechanism of recycling of internalized receptors or *de novo* synthesis of MPL on early hematopoietic progenitors in contrast to irreversible internalization on platelets. This is in line with experiments showing a recycling of THPO receptor molecules to the cell surface after internalization in a murine hematopoietic cell line but not in platelets.[15] The results obtained with more mature $CD34^+CD38^{hi}$ progenitors lay between those from early progenitors and platelets, revealing a progressive loss of the capability to re-express the receptor after internalization during hematopoietic development.

In summary, flow cytometric measurement of MPL (CD110) on platelets is a suitable method to distinguish between hypomegakaryocytic thrombocytopenia with high THPO plasma levels and forms of thrombocytopenia due to accelerated destruction of platelets with THPO levels in a normal range, but not to recognize patients with a primary MPL defect. In contrast, although not completely discriminating between CAMT and other forms of hypomegakaryocytic thrombocytopenia, the level of

expression measured on early $CD34^{+}CD38^{lo/-}$ progenitors is relatively independent from THPO plasma levels and therefore is more appropriate to help in identifying and characterizing conditions with impaired expression of MPL.

Matthias Ballmaier,[1] Wolfgang Holter,[2] and Manuela Germeshausen[1]

[1]Dept. of Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Germany; and [2]Dept. of Pediatric Hematology and Oncology, University Hospital Erlangen, Germany, present address: St. Anna Children's Hospital, Vienna, Austria

Acknowledgments: the authors would like to thank all patients and families who participated in this study and the referring physicians. We also would like to acknowledge the excellent technical assistance of Sabine Deerberg, Yvonne Peter, and Christina Struckmann.

Funding: this work was supported in part by grants of the Federal Ministry of Education and Research (German Network on Congenital Bone Marrow Failure Syndromes).

Correspondence: Ballmaier.Matthias@mh-hannover.de
doi:10.3324/haematol.2015.125963

Key words: congenital thrombocytopenia, MPL, thrombopoietin, congenital amegakaryocytic thrombocytopenia, flow cytometry.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Ballmaier M, Germeshausen M. Advances in the understanding of congenital amegakaryocytic thrombocytopenia. Br J Haematol. 2009;146(1):3-16.
2. Ballmaier M, Germeshausen M, Schulze H, et al. c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. Blood. 2001;97(1):139-146.
3. Savoia A, Dufour C, Locatelli F, et al. Congenital amegakaryocytic thrombocytopenia: clinical and biological consequences of five novel mutations. Haematologica. 2007;92(9):1186-1193.
4. Heller PG, Glembotsky AC, Gandhi MJ, et al. Low Mpl receptor expression in a pedigree with familial platelet disorder with predisposition to acute myelogenous leukemia and a novel AML1 mutation. Blood. 2005;105(12):4664-4670.
5. Horikawa Y, Matsumura I, Hashimoto K, et al. Markedly reduced expression of platelet c-mpl receptor in essential thrombocythemia. Blood. 1997;90(10):4031-4038.
6. El-Harith el HA, Roesl C, Ballmaier M, et al. Familial thrombocytosis caused by the novel germ-line mutation p.Pro106Leu in the MPL gene. Br J Haematol. 2009;144(2):185-194.
7. Li J, Xia Y, Kuter DJ. The platelet thrombopoietin receptor number and function are markedly decreased in patients with essential thrombocythaemia. Br J Haematol. 2000;111(3):943-953.
8. Linden MD, Frelinger AL 3rd, Barnard MR, Przyklenk K, Furman MI, Michelson AD. Application of flow cytometry to platelet disorders. Semin Thromb Hemost. 2004;30(5):501-511.
9. de Haas M, van den Oudenrijn S, Baardman R, van der Schoot E, von dem Borne AEGK. Platelet antigens: CD110 or Tpo receptor. Cluster report. In: Mason D, Andre P, Bensussan A, et al., eds. Leukocyte Typing VII: White Cell Differentiation Antigens: Proceedings of the Seventh International Workshop and Conference held in Harrogate, United Kingdom. VII ed. New York, Oxford: University Press, Inc., 2002:558-561.
10. Abbott C, Huang G, Ellison AR, et al. Mouse monoclonal antibodies against human c-Mpl and characterization for flow cytometry applications. Hybridoma (Larchmt). 2010;29(2):103-113.
11. Overton WR. Modified histogram subtraction technique for analysis of flow cytometry data. Cytometry. 1988;9(6):619-626.
12. Li J, Xia Y, Kuter DJ. Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: binding, internalization, stability and pharmacokinetics. Br J Haematol. 1999;106(2):345-356.
13. Terstappen LW, Huang S, Safford M, Lansdorp PM, Loken MR. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. Blood. 1991;77(6):1218-1227.
14. Germeshausen M, Ballmaier M, Welte K. MPL mutations in 23 patients suffering from congenital amegakaryocytic thrombocytopenia: the type of mutation predicts the course of the disease. Hum Mutat. 2006;27(3):296.
15. Dahlen DD, Broudy VC, Drachman JG. Internalization of the thrombopoietin receptor is regulated by 2 cytoplasmic motifs. Blood. 2003; 102(1):102-108.