Two patterns of thrombopoietin signaling suggest no coupling between platelet production and thrombopoietin reactivity in thrombocytopenia-absent radii syndrome

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ABSTRACT

Background
Thrombocytopenia with absent radii syndrome is defined by bilateral radius aplasia and thrombocytopenia. Due to impaired thrombopoietin signaling there are only few bone marrow megakaryocytes and these are immature; the resulting platelet production defect improves somewhat over time. A microdeletion on chromosome 1q21 is present in all patients but is not sufficient to form thrombocytopenia with absent radii syndrome. We aimed to refine the signaling defect in this syndrome.

Design and Methods
We report an extended study of 23 pediatric and adult patients suffering from thrombocytopenia with absent radii syndrome in order to scrutinize thrombopoietin signal transduction by immunoblotting and gel electrophoretic shift assays. In addition, platelet immunotyping and reactivity were analyzed by flow cytometry. Results were correlated with clinical data including age and platelet counts.

Results
Two distinct signaling patterns were identified. Juvenile patients showed abrogated thrombopoietin signaling (pattern #1), which is restored in adults (pattern #2). Phosphorylated Jak2 was indicative of activation of STAT1, 3 and 5, Tyk2, ERK, and Akt, showing its pivotal role in distinct thrombopoietin-dependent pathways. Jak2 cDNA was not mutated and the thrombopoietin receptor was present on platelets. All platelets of patients expressed normal levels of CD41/61, CD49b, and CD49f receptors, while CD42a/b and CD29 were slightly reduced and the fibronectin receptor CD49e markedly reduced. Lysosomal granule release in response to thrombin receptor activating peptide was diminished.

Conclusions
We show a combined defect of platelet production and function in thrombocytopenia with absent radii syndrome. The rise in platelets that most patients have during the first years of life preceded the restored thrombopoietin signaling detected at a much later age, implying that these events are uncoupled and that an unknown factor mediates the improvement of platelet production.

Key words: thrombocytopenia absent radii syndrome, thrombopoietin, platelets, therapy.

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Introduction

In 1969, Judith Hall summarized clinical data from 40 patients presenting with radius aplasia and thrombocytopenia, coining the name "Thrombocytopenia absent radii syndrome (TAR)".[1] Children born with this rare congenital disorder have low platelet counts (<150×10^9/L) resulting in petechiae and increased bruising. At birth, blood counts might also reveal leukocytosis with additional eosinophilia, but the changes in the white cell lineage typically normalize quickly. Although it has been described that platelet counts increase around the age of 2 years, they usually remain below the lower reference value. The thrombocytopenia has been attributed to a platelet production defect with few megakaryocytes in an otherwise normocellular bone marrow.[2,3] Letestu *et al.* demonstrated that megakaryocytes from TAR patients have a maturation arrest at the CD41/CD42 level which allows only few megakaryocytes to mature fully and release platelets.[4] TAR syndrome has thus frequently been considered a unilineage bone marrow failure syndrome.[5] Thrombopoietin (TPO) reactivity is impaired resulting in abrogated megakaryocyte progenitor growth and absent synergism with platelet agonists such as ADP. These findings correlate with reduced tyrosine phosphorylation of platelet proteins after TPO stimulation,[6] especially Jak2.[7] Sequence analysis excluded mutations in the Hox genes *A10*, *A11*, and *D11*[8] and the *MPL* gene encoding the TPO receptor c-Mpl.[4,9]

Although some pedigrees with several affected family members suggested an autosomal recessive inheritance,[1,10] the ratio of affected to unaffected children was less than expected for this trait and TAR does not occur more frequently in consanguineous families.[1,11] These facts argue for a complex or compound pattern of inheritance.[12] Recently, we demonstrated a heterozygous microdeletion on chromosome 1q21 in 30 patients with TAR syndrome.[13] In 75% of cases, the deletion was inherited from one of the unaffected parents (here referred to as carrier) and some families revealed several carriers over three generations, suggesting that TAR syndrome is at least a digenic disorder. The minimal microdeletion spans 120 kb and comprises about 12 annotated genes among which protein inhibitor of activated STAT3 (*PIAS3*) encodes for a negative regulator of STAT3, a key regulator of TPO signaling. These results prompted us to revisit the TPO-dependent signal transduction in TAR syndrome with respect to carriers and healthy donors.

Design and Methods

Patients

Twenty-three patients of Caucasian descent with TAR syndrome (clinically diagnosed by bilateral radius aplasia and platelet counts <150×10^9/L) reported to the Charité Hospital between January 2007 and October 2010. Clinical data of these and eight additional patients are summarized in Table 1. The microdeletion was detected as previously described.[13] Apart from one family with two affected children all patients were unrelated. Blood was withdrawn from patients and parents after written informed consent to a study approved by the local ethic review board according to the Helsinki declaration, as well as from 50 healthy donors (including 20 children).

Platelet isolation, stimulation and immunoblotting

Platelets were isolated from citrated blood as described elsewhere[6] and stimulated with 50 ng/mL TPO (R&D Systems, Wiesbaden, Germany) for the indicated times (10 and 30 min) prior to stopping by addition of 2x sample buffer. For the inhibitor experiments platelet suspension was pre-incubated with WP 1066 (Sigma, Hamburg, Germany), Jak2 inhibitor II (Calbiochem), or AG-490 (Merck, Darmstadt, Germany) at the indicated concentrations and times, prior to TPO stimulation. Solubilized proteins were subjected to gel electrophoresis, transferred to PVDF membranes (0.45 µm, Roche Diagnostics, Mannheim, Germany) and analyzed for expression of proteins. Antibodies against pAkt (D9E), Akt (C67E7), pJak2 (C80C3), Jak2 (D2E12), pSTAT1 (58D6), pSTAT3 (#9131), STAT3 (#9132), pSTAT5 (14H2), pTyk2 (#9321), Tyk2 (#9312), pmTor (S2448), mTor were purchased from Cell Signaling (Frankfurt/M, Germany); pERK (E-14), ERK (K23), PIAS3

Table 1. Characteristics and platelet counts of patients with TAR syndrome.

ID	Sex	Inheritance	pJak2	n#	Age (years)	Platelets (x10^9/L)
01[§]	f	maternal	#2	3	29	114
02[§]	f	maternal	#2	1	20	146
03[†,§]	f	maternal	#1 #2	5	17 21	34 59
04[§]	f	maternal	---	1	0	20
08[§]	m	ND	---	12	1	19
10[†,§]	f	paternal	---	17	4	64
11[†,§]	f	*de novo*	#2	7	4	41
12[§]	m	*de novo*	---	10	0.4	70
14[†,§]	m	*de novo*	---	3	0.2	25
15[§]	m	ND	#2	1	26	163
17[†,§]	f	maternal	---	3	15	26
21[§]	f	ND	---	1	0	11
25[§]	m	maternal	#1	7	26	118
26[§]	m	maternal	#2	6	22	155
27[§]	f	maternal	#2	9	33	108
31	m	*de novo*	---	1	1	87
32	f	paternal	#1	4	0.4	140
34	f	paternal	#1	6	18	64
35	m	non-maternal	#2	11	39	114
36	m	maternal	#1	9	2	79
37	f	ND	#2	2	36	136
38	f	*de novo*	#1	1	0.2	167
39	f	paternal	#1	1	4	34
40	m	*de novo*	#1	1	8	88
41	m	maternal	#1	8	1.5	26
42	m	ND	#1	14	6	100
43	f	*de novo*	#1	9	4	29
44	f	maternal	#1	20	18	42
46	f	ND	#2	3	20	139
47	f	ND	#2	3	18	100
51	f	maternal	#1	1	0.8	136

ID: patient identity number and if previously described in ref 13[§] or 6[†]; m: male; f:females; ND: not determined; pJak2 describes the pattern identified in figure 2A; n# describes the number of platelet counts displayed in Figure 1A; – indicates not available.

(C-12) from Santa Cruz Biotechnology (Heidelberg, Germany), GAPDH (6C5) from Abcam (Cambridge, UK), pS6K (AB-207), S6K (AB-241) from Advanced Targeting Systems (San Diego, USA) and c-Mpl (Clone 167639) from R&D System.

Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and chemoluminescence reagent Super Signal West Dura (Fisher Scientific, Nidderau, Germany). Secondary re-probing was performed according to the manufacturers' instructions.

Densitometric analysis was carried out after scanning non-saturated films using ImageJ software (Wayne Rasband, NIH, USA: http://rsb.info.nih.gov/ij) using the values of 30 min TPO stimulation and arbitrarily setting the healthy control present on the same film as 100%.

Electrophoretic mobility shift assay

Stimulation of platelets with TPO was terminated using electrophoretic mobility shift assay (EMSA) lysis buffer as described previously.[14] Ten micrograms of protein were incubated for 30 min at 30°C in the presence of one volume of 10x buffer and a double-strand probe for pSTAT1/3 (5'TTCATTTCCCGTAAATCCCTA) in the presence or absence of supershift antibodies (Cell Signaling). Probes were labeled using T4-polynucleotide kinase in the presence of ^{32}P-γATP (3000 Ci/mmol). Complexes were separated by 5% non-denaturating acrylamide gel electrophoresis. Films were exposed for autoradiographic analysis for 1 to 7 days.

Quantitative mRNA expression in lymphocytic cell lines

Patient-derived B-lymphocytes were transduced with Epstein-Barr virus and immortalized cells grown in RPMI-medium supplemented with 15% fetal bovine serum and megakaryocytes were differentiated from human CD34$^+$ cells in the presence of TPO and interleukin-1β (R&D systems). RNA was isolated from washed and pelleted cells with standard procedures and transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time mRNA expression was performed using PCR master mix/SYBR green (Applied Biosystems, Darmstadt, Germany) amplifying approximately 100 bp amplicons with a primer efficiency of at least 95% in a real time-PCR machine (Applied Biosystems). Samples were analyzed using the $\Delta\Delta C_T$ method. All experiments were performed twice using triplicate samples from two controls, six patients, three carriers and two unaffected parents.

Flow cytometric analysis

Expression of platelet surface receptors in resting and stimulated platelets was analyzed in whole blood. All antibodies used for flow cytometry (beside anti-c-Mpl) were directly fluorophore-labeled and purchased from BD Biosciences (R&D Systems, Heidelberg, Germany). Tubes were loaded with 50 μL of whole blood (diluted 1:10 with Hepes Tyrodes buffer) and antibodies were added. Incubation was stopped after 15 min by adding 1.5 mL PBS and measured within the next hour. For stimulation analyses, platelets were incubated for 5 min with agonists at the indicated concentrations before adding antibodies. For c-Mpl, platelet-rich plasma was produced by centrifugation for 3 min at 1200 rpm and by using a secondary goat-anti-mouse-antibody added for 15 min. Samples were evaluated on a FACS-Canto II (BD) and the data obtained were analyzed using FlowJo software (Ashland, OR, USA).

Statistical analysis

Statistical significance was evaluated using a two-sided Student's t-test and significance indicated as $^*P<0.05$), $^{**}P<0.01$, or $^{***}P<0.001$. A two-tailed Mann-Whitney rank test was used for pattern attribution and the significance level was determined.

Results

Platelet counts in patients with thrombocytopenia with absent radii syndrome

Patients with TAR syndrome are born with thrombocytopenia, with platelet counts typically even below 100×10^9/L. Although it has been reported that platelet counts increase around the age of 2 years, we found that the data supporting this statement are unexpectedly rare.[1,15,16] We, therefore, analyzed 180 platelet counts from 31 patients over time. Overall platelet counts were very low in children, while adults had higher counts, but hardly ever reached the lower reference value (Figure 1A). In about 55% of cases we saw an increase in platelet counts within the first 2 years of life, which is considered a typical course (Figure 1B). However, in some patients platelet counts remained very low ($<100\times10^9$/L), even into adulthood (Figure 1C). A small fraction of patients had almost normal platelet counts from birth onwards, although the counts always remained beneath the lower reference value (*data not shown*). Taken together, our time course analysis implies that platelet biogenesis changes during the first 2 years of life in most TAR patients, but also that not all patients react uniformly.

Thrombopoietin signal transduction is only abrogated in pediatric patients

The thrombocytopenia in TAR syndrome corresponds to a low number of megakaryocytic progenitors in the bone marrow.[3] Colony growth is markedly impaired although TPO levels are elevated and its receptor is overall expressed[6] and does not harbor mutations in the coding exons.[4,9] As bone marrow biopsies are typically not available we analyzed TPO signal transduction in platelets as a surrogate for the responsiveness of precursors to determine whether signaling is age-dependent or correlates with an increase in platelets. We confirmed our results originally achieved by immunoprecipitation experiments showing that in pediatric TAR patients Jak2 kinase, although expressed in normal amounts, does not become phosphorylated when platelets are stimulated with TPO (pattern #1, Figure 2A). This could be demonstrated in 12/13 patients below 18 years old. In 5/11 patients there was no phosphorylation of Jak2 targets STAT1, STAT3, and STAT5 (#1A), while in 6/11 patients we found some residual pSTAT3 (#1B) which made up to half of the signal found in healthy controls. Unexpectedly, in 10/11 samples from adult patients we readily detected phosphorylated Jak2 kinase as well as STAT1, 3 and 5 (pattern #2, Figure 2A). Jak2, STAT1, 3, and 5 proteins were expressed in similar amounts in platelets from TAR patients (*data not shown*). We found a highly significant correlation of pJak2 status (pattern #1 *versus* #2) with both platelet count ($P<0.004$) and age ($P<0.0002$), suggesting that there are two types of TPO-responsiveness indicated by the presence or absence of pJak2 (Figure 2B). In one patient we observed a transition from pattern #1 (age 17) to #2 (age 21). In contrast, we found no correlation with the sex of patients, pattern of inheritance, or severity of skeletal alterations.

There could be several explanations for the detection of residual STAT3 phosphorylation in the absence of pJak2. To our knowledge TPO signaling has not been analyzed in healthy children, typically, sick children are compared to healthy adult controls. As TPO reactivity alters between neonates and adults[17,18] we sought to exclude the possibility that TPO signaling in platelets of children might differ with

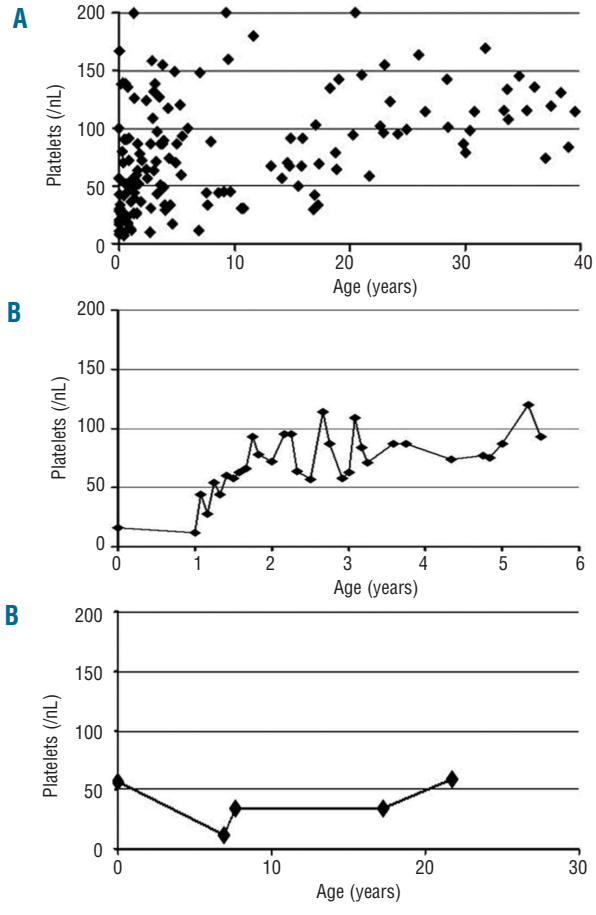

Figure 1. Platelet counts and time courses in TAR patients. (A) 180 platelet counts from 31 patients over time indicating an overall increase in platelets during the first years of life. No more than one value per 3 months is depicted. (B) Example of a typical time course shows an increase in peripheral platelets around the age of 2 years, without reaching the lower norm (TAR-10). (C) Typical time course of a patient with continued low platelet counts over time (TAR-03).

age. We found inconspicuous signaling in 20 children, from age 3 months to 18 years (Figure 2C), confirming that the unexpected change in TPO signaling in patients with TAR is genuine. Second, as mutations in the JH2 pseudo-kinase domain of Jak2 have been described to cause myeloproliferative disorders[19] we sequenced Jak2 mRNA from two patients, but no mutation was found in the coding exons (*data not shown*). To further explore the possibility that the degree of Jak2 phosphorylation improves gradually over time, which could result in a switch to pattern #2, we scanned films with non-saturating signals and subjected them to densitometric analysis calculating the fraction of pJak2 to Jak2 compared to healthy controls. pJak2 was virtually absent in all patients with pattern #1 (Figure 2D) and did not increase with age. Although Jak2 protein is expressed in platelets of TAR patients, the amount could be altered to some degree, which would bias our results. However, when Jak2 expression was normalized to GAPDH, there was no substantial change in the pJak2/Jak2 ratio (*data not shown*). We next sought to analyze the influence of Jak2 inhibition by using three different Jak2 inhibitors. High doses of WP1060 resulted in complete inhibition of Jak2 phosphorylation and pSTAT3 was not detectable (Figure 2E). Suboptimal Jak2 inhibition by WP1060, Jak2 inhibitor, or AG-490 led to markedly reduced pJak2 detection, while the pSTAT3 signal was reduced to a lesser extent. This could be explained so far by a more sensitive antibody for pSTAT3 compared to pJak2 or by the fact that the residual Jak2 kinase activity is sufficient to phosphorylate some of the STAT3 present in the platelet within 10 to 30 min. In the light of this result we conclude that absence of pSTAT3 in pattern #1A as well as a reduced amount of detectable pSTAT3 in pattern #1B both reflect impaired TPO-dependent Jak2 phosphorylation in TAR after binding of TPO to its cognate receptor c-Mpl. While previous studies excluded mutations in the *MPL* gene,[9] Letestu *et al.* reported reduced c-Mpl protein expression in platelets of TAR patients.[4] We analyzed c-Mpl expression by immunoblotting or flow cytometry in 14 patients (Figure 2F). Although c-Mpl was slightly reduced in some patients with pattern #1 compared to in those with pattern #2 or controls, there was no significant correlation with age or platelet counts. This makes it unlikely that an altered level of c-Mpl expression causes the change in TPO signaling. Taken these data together, we report an unexpected TPO-dependent signaling defect in 13 of 23 patients with TAR syndrome, correlating with age and platelet count. The change in signaling occurred around the age of 18 - 20 years and was, therefore, much later than the increase in platelets observed after the first years of life. It is worth emphasizing that despite normal pJak2 levels in platelets of older TAR patients (pattern #2), the platelet counts remain below the lower reference value, implying that inconspicuous TPO signaling in TAR syndrome does not reconstitute normal platelet biogenesis.

Extended analysis of thrombopoietin signaling in thrombocytopenia with absent radii syndrome

Although the Jak-STAT pathway is the best studied TPO-signaling cascade, additional signaling cascades are activated. A current overview is depicted in Figure 3A. Of the four Jak kinase family members only Tyk2 is expressed next to Jak2 in the megakaryocytic lineage.[20,21] We, therefore, extended our analysis to Tyk2 (Figure 3B), the MAPK ERK1/2 pathway[14] (Figure 3C) and the Akt-mTOR-S6K pathway[22] (Figure 3D). We found the same two activation patterns for Tyk2, ERK2, Akt, and S6K as for the Jak2-STAT pathway. There was no pTyk2 in three patients with pattern #1B, which could account for the residual pSTAT3. Akt and mTOR were already constitutively phosphorylated (*data not shown*). We found some TPO-induced phosphorylation of S6K in patients with pattern #1, suggesting that some signaling is transduced even in those patients with virtual absence of a pJak2 signal. In the presence of WP-1066 or Jak2-inhibitor II phosphorylation of Tyk2, Akt, and Erk1/2 was also reduced in platelets of healthy controls (Figure 3E), supporting our hypothesis that Jak2 remains a key regulator for distinct TPO-dependent signaling cascades. Taken together, we present several lines of evidence showing that thrombocytopenia in TAR syndrome is reflected by absent or markedly reduced Jak2 phosphorylation indicating that activation of several TPO pathways depends strictly on pJak2.

Contribution of the microdeletion for thrombocytopenia in thrombocytopendia-absent radii syndrome

A microdeletion on chromosome 1q21 was detected in over 80 TAR patients (*data not shown*), but was also found in

healthy parents (carriers). *PIAS3* and *RBM8A* are genes within the microdeleted region for which an effect on STAT3 expression or function has been reported. We, therefore, first sought to exclude a Jak2-STAT signaling defect in carriers and the non-carrier parents. As shown in Figure 4A TPO-responsiveness was normal for pJak2 and pSTAT3 induction compared to that in controls. This is in agreement with the normal platelet counts found in both groups (*data not shown*). We next used real-time PCR analysis to determine mRNA expression levels of the 12 annotated genes located within the minimal microdeletion region in patients with patterns #1 and #2, in carriers and unaffected parents and in healthy controls. As bone marrow megakaryocytes were not available from patients, we used lymphoblastic cell lines generated from B cells of TAR patients, their parents, and controls. First, we determined whether the expression of these genes in lymphoblastic cell lines is comparable to that of primary megakaryocytes differentiated from $CD34^+$ cells. *HFE2*, a gene encoding the iron regulatory protein hemojuvelin, *ITGA10* encoding the integrin α10-chain, and *ANKRD35* were undetectable in lymphoblastic cell lines while *GNRHR2* is a pseudogene.[23] The remaining eight genes showed expression levels comparable to those in megakaryocytes, confirming that our approach is, overall, biologically meaningful (Figure 4B). Interestingly, the expression of thioredoxin-interacting protein TXNIP, an enzyme with a described role in metabolic maintenance, was up-regulated 8-fold in differentiated megakaryocytes. Overall, the microdeletion led to reduced mRNA expression in six patients and three carriers compared to two unaffected parents and two controls. One representative experiment of two is depicted in Figure 4C. The gene set includes *PIAS3*, a negative regulator of the Jak2-STAT pathway. Immunoblot analysis of PIAS3 protein in platelets of TAR patients and carriers showed reduced expression (Figure 4D), which was confirmed densitometrically to be between 50% and 70% of normal (*data not shown*), suggesting that reduced PIAS3 expression alone does not correlate with altered TPO signaling. Although platelets are anucleate cellular fragments, the activated STAT transcription factors are still able to bind DNA.[14] We sought to identify whether any STAT-complexes are formed in TAR patients. STAT1/1 and STAT3/3 complexes were readily identified in healthy donors and confirmed by supershift experiments (Figure 4E, left panel). In TAR patients with pattern #1B very weak bands were detectable (middle panel), while an almost normal reactivity was shown in pattern #2 patients (right panel) as well as in carriers (*data not shown*). Interestingly, we found a faster migrating complex that increased in intensity after TPO-stimulation and which

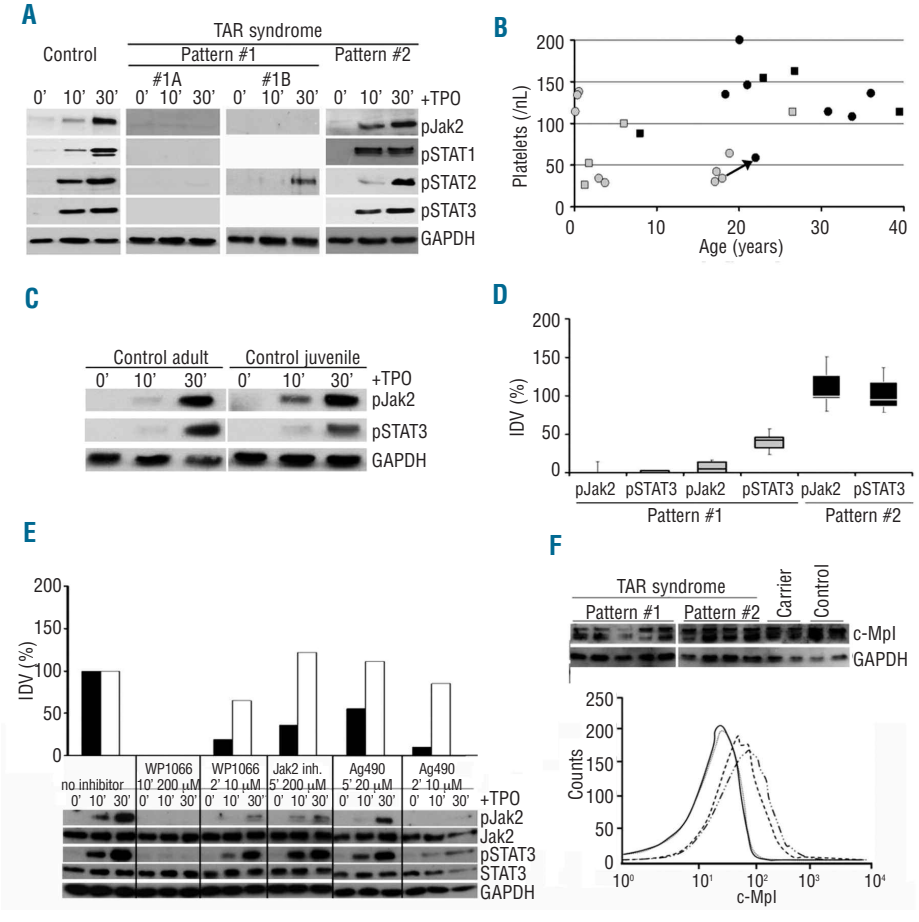

Figure 2. Two patterns of TPO-signaling in TAR. (A) Platelets from TAR patients and controls were stimulated with TPO for 10 or 30 min and subjected to immunoblotting using phospho-specific antibodies for Jak2, STAT1, STAT3 and STAT5. Two phosphorylation patterns for Jak2 (#1; #2: TAR-02) and a subgroup for pSTAT3 (#1A: TAR32; #1B: TAR-25) were identified. GAPDH was the loading control. (B) Correlation of pJak2 status with age and platelet count indicates that patients with pattern #1 (n=13, open symbols: TAR-11, 25, 32, 34, 36, 38, 39, 41, 42, 43, 44, 51) are younger with very low platelets, while patients with pattern #2 (n= 11, filled symbols: TAR-01, 02, 03, 15, 26, 27, 35, 37, 40, 46, 47) are typically older than 18 and have sub-normal platelet counts. The change in patterning found in one patient is indicated by an arrow. Squares represent males, circles represent females. (C) Platelets from healthy children have a normal response to TPO as shown by pJak2 and pSTAT3 immunoblotting. (D) Densitometric analysis of immunoblots reveals normal pJak2 and pSTAT3 in patients with pattern #2 (n=8, black columns: TAR-01, 02, 15, 26, 27, 35, 37, 40), while in patients with pattern #1 no pJak2 and pSTAT3 (n=5, #1A: TAR-32, 34, 36, 39, 44) or some residual pSTAT3 (n=6, #1B: TAR-03, 26, 38, 41, 42, 43) was detectable.

IDV indicates individual densitometry value in %. (E) Platelets were preincubated with Jak2 inhibitors, as indicated, prior to TPO-stimulation for 10 or 30 min. Levels of expression of pJak2, Jak2, pSTAT3, STAT3 and GAPDH were analyzed by immunoblotting. pJak2 (black bars) and pSTAT3 level (white bars) were compared to those in controls. (F) c-Mpl expression in platelets by immunoblot (upper panel) shows similar expression in patients with pattern #1 (TAR-25, 36, 38, 39, 44), and pattern #2 (TAR-02, 15, 26, 35), and in carriers and controls. GAPDH expression was the loading control. Flow cytometric analysis (lower panel) confirms comparable expression levels in TAR patients (TAR-15, 39, 40, 41, 42, 43, 44) (striped curve) and controls (dotted curve) compared to secondary antibody controls (black & gray curves).

could be partly shifted with antibodies against STAT3 and PIAS3, but not against STAT1. This complex was much more intense in patients with pattern #2 and even more profound in pattern #1. In summary, the real-time analysis in lymphoblastic cell lines showed that both TAR patients and carriers have about half the mRNA levels found in unaffected parents and controls. Altered PIAS3-DNA complexes found in TAR platelets, however, cannot rule out the possibility that these genes contribute to the TAR phenotype.

Impaired platelet receptor expression and function

Increased bleeding in TAR syndrome has been attributed to the low platelet count while some reports suggest that platelets in TAR also have functional defects.[24,25] Here, we performed an extended study of semi-quantitative platelet immunotyping (n=7) and function analysis (n=13) in whole blood by flow cytometry in patients with both signaling patterns. CD41/61 was normally expressed on the platelet surface while CD42a and CD42b were reduced. CD49b and CD49f, encoding the α-integrin chains for collagen and laminin receptors respectively, were present in normal amounts. CD49e, which forms the α-chain of fibronectin receptor, was markedly reduced, especially in two patients with pattern #1 (Figure 5A). CD29, encoding the common β1 integrin chain, was slightly reduced. Reactivity of platelets toward suboptimal and optimal concentrations of ADP and TRAP6 was normal with respect to αIIbβ3 integrin activation (Figure 5B, left panel), α-granule release (middle panel) and P-selectin. CD63 was slightly elevated in resting and ADP-stimulated platelets, while even high concentrations of TRAP6 did not lead to maximal release (right panel). Age, platelet count, and phosphorylation patterns had no impact on platelet reactivity. Our data thus suggest that TAR patients harbor a combined defect in platelet production and impaired function.

Discussion

TAR syndrome is a rare disorder and progress in the understanding of the underlying pathogenesis has been slow.[16] As mature megakaryocytes are scarce in patients, TAR syndrome is assigned to the unilineage bone marrow failure syndromes. However, in these syndromes blood counts typically deteriorate over time, gradually affecting more than one lineage and patients have an increased incidence of development of myelodysplastic syndrome or leukemia.[5] In contrast, platelet counts typically rise in TAR patients within the first 2 years of life and no other lineages are affected in older patients. There is no evidence for increased cases of leukemia, although there are four case reports of TAR patients developing acute myeloid leukemia[26-28] or acute lymphoid leukemia.[29] Nevertheless, as a sign of disturbed bone marrow composition a transient increase in leukocytes at birth may be observed in some patients.

Given the very few bone marrow biopsies there is no information available on changes of megakaryopoiesis over age. We found that impaired TPO responsiveness in children with TAR is not present in older patients. So far we have only a single patient in whom we have been able to observe a transition from pattern #1 to #2, as an indicator that this could be a general mechanism. It is worth noting that this patient (TAR-03) still has very low platelet counts. It has been reported that a megakaryocyte maturation defect occurs at the level of CD42 cells, that only a fraction of all bone marrow megakaryocytes will mature completely and that the platelets found in circulation are those derived from the few fully differentiated megakaryocytes.[4] In contrast, we found a reduced CD42 receptor density on the platelet surface, most likely due to our semi-quantitative approach. Additionally, the β1 integrin receptors, especially the CD29/CD49e complex that binds to fibronectin, were

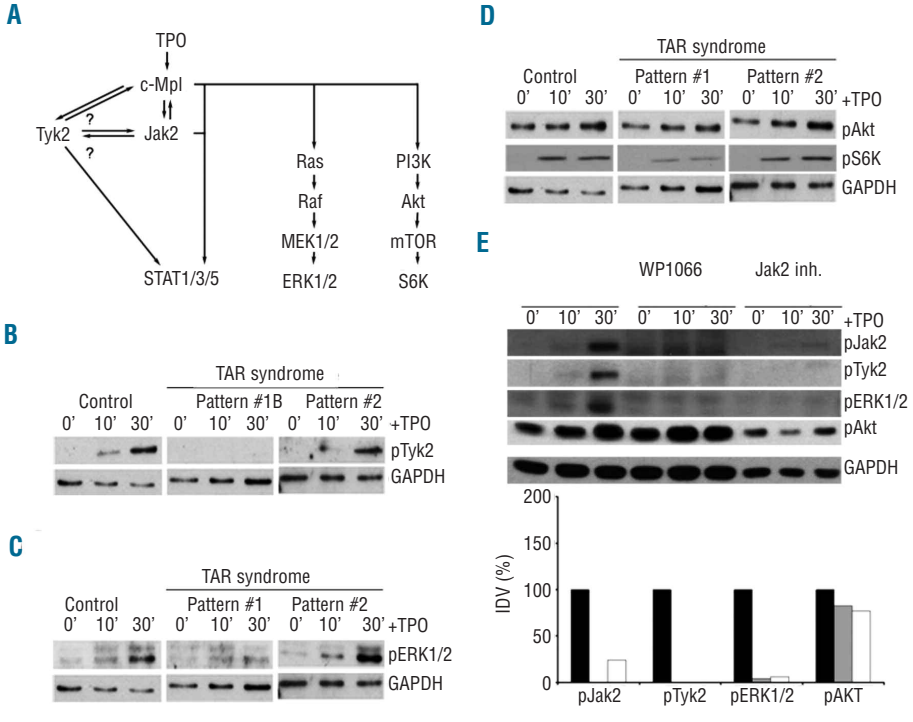

Figure 3. Extended analysis of TPO signal transduction in TAR patients. (A) Overview indicating the three most important signaling pathways after TPO stimulation. (B-D) Immunoblot analysis of phosphorylated (B) Tyk2 (#1: TAR-25, #2: TAR-02), (C) ERK2 (#1: TAR-32, #2: TAR-02), and (D) Akt and S6K proteins (#1: TAR-32, #2: TAR-02) in TPO-stimulated platelets indicate the same patterns of responsiveness as shown for pJak2. GAPDH protein is shown as a loading control. (E) Platelets were pre-incubated with Jak2 inhibitors prior to TPO incubation. Expression of pTyk, pERK1/2, and pAkt was subsequently analyzed by immunoblotting and quantified by densitometry.

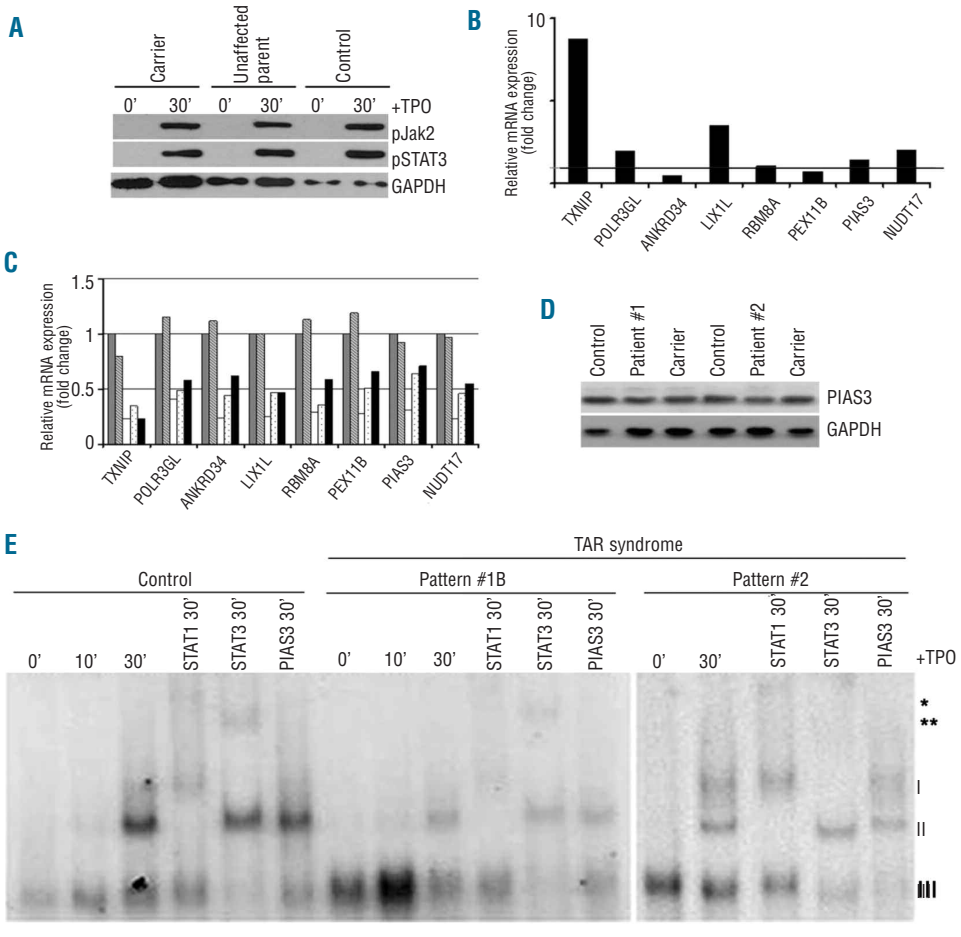

Figure 4. Influence of the microdeletion on TPO responsiveness and mRNA expression of microdeleted genes. (A) Platelets from carriers and non-affected parents show normal phosphorylation of Jak2 and STAT3 by immunoblotting. (B) lymphoblastoid cells were harvested in the logarithmic phase prior to RNA isolation. mRNA expression levels were compared with those of megakaryocytes cultured from CD34$^+$ cells indicating comparable expression for seven of eight genes, while *TXNIP* expression was increased 8-fold in megakaryocytes. One representative experiment of two is shown. (C) Real-time PCR analysis of microdeleted gene expression in lymphoblastoid cell lines showed that all genes analyzed had about 50% reduced expression levels in patient TAR-25 with pattern #1 (white columns), patient TAR-26 with pattern #2 (dotted columns) and carriers (black columns), while unaffected parents (gray hatched columns) had normal levels when compared to unrelated controls (gray columns). (D) Patients TAR-32 (pattern #1), TAR-27 (pattern #2), and carriers had similar PIAS3 protein in platelet lysates compared to controls. (E) Gel mobility shift assays of TPO-stimulated platelets revealed the presence of activated STAT dimers in TAR patients. Residual STAT3/3 dimers (I), STAT1/1 dimers (II) were present in lysates of a patient with pattern #1 (TAR-25) and normal complexes were found in a pattern #2 patient (TAR-37). A complex containing STAT3 and PIAS3 is faster migrating (III). Supershift was performed with the indicated antibodies. * indicates STAT1 supershift and ** STAT3 supershift.

markedly reduced. There was no difference between patients with pattern #1 or #2 suggesting that megakaryocytes in TAR syndrome harbor a general maturation defect in the bone marrow and that TAR platelets have both an altered surface receptor composition and a slightly impaired reactivity which might contribute to prolonged bleeding episodes in some patients. Recently, a mutation in the promoter region of the TGF-β2 gene was described in four TAR patients, although gene expression was not affected. The authors also report on a mesenchymal stem cell defect in TAR, as they failed to detect endoglin (CD105) on adherent bone marrow cells by flow cytometry.[30] The consequences of this finding do, however, remain unknown so far.

Several questions remain open. For example, why are there more females affected than males (1:1.6 in our cohort compared to 1:1.8 [1] and 1:3.8 [31])? Even more enigmatic is the switch in TPO signal transduction described here, many years after the initial increase in platelet counts (Figures 1A and 2B). Fetal and neonatal megakaryocytes are less sensitive to elevated TPO concentrations than are megakaryocytes isolated from adult bone marrow.[32,33] As megakaryocytes are rare cells and their numbers are even more reduced in TAR syndrome, we decided to use platelets from TAR patients as a surrogate to allow a uniform analysis of TPO signaling. However, platelets lack a nucleus and negative feedback loops to limit cytokine signaling require the *de novo* transcription of inhibitors. Thus, we assume that TPO signaling in bone marrow megakaryocytes differs from our results found in platelets. The analysis of megakaryocytes derived from CD34-positive cells or induced pluripotent stem cells of patients might bridge this technical gap in the future.

One report described reduced c-Mpl protein in platelets of four TAR patients, although two of them showed almost normal levels without any correlation with age. c-Mpl protein was found to be reduced in cord blood platelets[4] whereas others found increased protein on megakaryocytes derived from cord blood.[18] These discrepancies could be attributed to different technical approaches. The combined immunoblot and flow cytometric analysis in this study suggest partly reduced c-Mpl expression in platelets from young TAR patients.

Our data show for the first time that failure to phosphorylate Jak2 in human platelets affects all downstream signaling pathways. In a subgroup of patients with pattern #1 patients we found residual STAT3 phosphorylation in response to TPO without any detectable pJak2 (Figure 2A). This could be explained by indirect signaling involving Akt and mTOR (Figure 3D and *data not shown*), as described for BaF3-cells expressing a c-Mpl receptor mutant that lacks the membrane proximal domain essential for Jak2 activation.[34] Cells transfected with this mutant still proliferate in

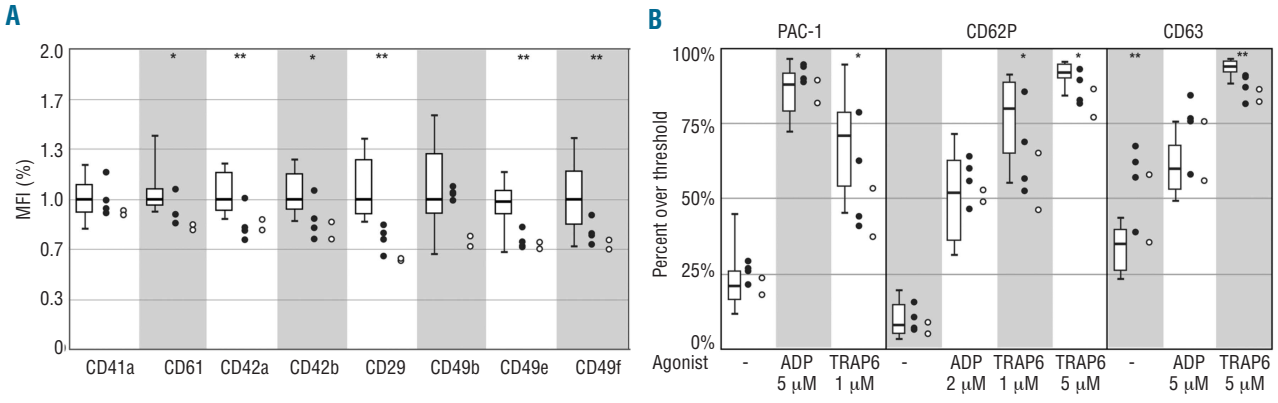

Figure 5. Impaired platelet surface receptor expression and reactivity in TAR patients (TAR-11, 51, 3, 37, 46, 47). (A) Platelet receptors were measured by flow cytometry and expression level compared to that of a cohort of healthy controls. Fibrinogen receptor expression (CD41a and CD61) was normal, whereas fibronectin receptor (CD29/CD49e) and the GPIb/IX/V complex (CD42a/b) were reduced in TAR patients. (B) Platelets from TAR patients (TAR-3, 11, 15, 17, 36, 37, 38, 39, 40, 41, 46, 47, 51) and controls were stimulated with ADP or TRAP6 and expression of activated integrin αIIbβ3 (PAC-1). α-granule release (CD62P) and lysosomal granule release (CD63) measured by flow cytometry. Integrin activation and α-granule release were comparable to those of controls, while CD63 activation was slightly elevated in resting platelets but could not be fully released in TRAP-treated platelets from TAR patients. White dots represent patients with pattern #1, black dots patients with pattern #2.

response to TPO. Tyk2 phosphorylation mimicked the pattern we found for Jak2, supporting the observation that it plays a subordinated function for TPO signaling.[21] Another paradoxical finding was the presence of PIAS3 within the microdeleted region: PIAS3 was originally found to regulate STAT3 negatively by inhibiting the binding of activated dimers to DNA[35] and reduced PIAS3 expression should thus augment the STAT3 signal. In our gel shift experiments we found a protein complex bound to a STAT1/3-responsive DNA element in resting platelets which was more profound in lysates from TAR patients and which could be supershifted with antibodies directed against STAT3 or PIAS3 (Figure 4E). We cannot, therefore, completely rule out that PIAS3 contributes somehow to the diminished platelet counts in TAR.

The microdeletion on chromosome 1q21 has been found in all patients analyzed so far, but also in unaffected family members. While the coding exons of the 12 annotated genes within this region did not harbor any mutation, the non-coding regions including promoters or regulatory elements have not yet been analyzed. We found the same levels of gene expression in lymphoblastoid cell lines derived from patients and carriers, which makes a general imprinting mechanism, i.e. due to an altered insulating chromatin element present in the centromeric region of chromosome 1, unlikely. Also, no sister chromatid adhesion was observed in TAR syndrome.[31] Hemojuvelin protein expression was normal in platelets from TAR patients compared to that in controls (*data not shown*). *RBM8A* encodes Y14, an RNA binding protein that interacts with the C-terminal domain of STAT3 and RNA interference causes its down-regulation in Hep3B cells incubated with interleukin-6.[36] Although, we did not observe altered expression in lymphoblastoid cell lines, it remains a candidate gene. Interestingly, *TXNIP* mRNA expression is increased 8-fold in megakaryocytes compared to in lymphoblastoid cell lines. Mice lacking TXNIP have a decreased long-term hematopoietic stem cell pool that becomes exhausted over time.[37] TXNIP-deficiency reduced SDF1α- and osteopontin-mediated interactions between stem cells and the bone marrow niche, causing impaired homing and retention in the osteoblastic niche. Plasma levels of SDF1α and FGF-4, both cytokines mediating TPO-independent thrombopoiesis,[38] were normal in our TAR patients (*data not shown*). However, it cannot be excluded that haploinsufficiency of *TXNIP* contributes to the changes in bone marrow composition over time, whereas a causative role for megakaryocyte paucity seems unlikely.

Besides thrombocytopenia, we also found reduced expression of GPIb/V/IX and fibronectin receptors on platelets and a diminished CD63-exposure in response to TRAP6 in all TAR patients (Figure 5). We conclude that altered platelet function might contribute to the overall increased bleeding tendency. However, it remains unclear why only a subgroup of patients suffer from severe bleeding problems. In our cohort, one patient died at the age of 4 due to bleeding and two more patients had recurrent and life-threatening bleeds after the age of 2. The thrombocytopenia in TAR has so far been treated with cytokines such as recombinant human erythropoietin[39] or interleukin-6[40] without much success, while severe bleeding has been successfully managed with activated factor VII.[41] Overall, the defect in platelet production seems intrinsic to the hematopoietic system as bone marrow transplantation was successful in a 2-year old boy.[42] Second-generation thrombomimetics such as eltrombopag or romiplostim are available to increase low platelet counts in chronic idiopathic thrombocytopenic purpura and have been used for the treatment of thrombocytopenia due to mutations in *MYH9*.[43] These drugs act on c-Mpl-expressing hematopoietic stem cells and megakaryocytes to increase the pool of immediate platelet progenitors. Although megakaryocytes are extremely scarce in the bone marrow of TAR patients, these thrombomimetics might overcome the maturation defect causing extremely low platelet counts during the first years of life. We hope that our new observations of TPO signaling in TAR syndrome open new therapy approaches for this enigmatic and challenging disorder.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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