CD34+ gene expression profiling of individual children with very severe aplastic anemia indicates a pathogenic role of integrin receptors and the proapoptotic death ligand TRAIL

Ute Fischer,[1] Christian Ruckert,[2] Bernd Hubner,[1] Olaf Eckermann,[1] Vera Binder,[1] Tamam Bakchoul,[3] Friedhelm R. Schuster,[1] Sylvia Merk,[2] Hans-Ulrich Klein,[2] Monika Führer,[4] Martin Dugas,[2] and Arndt Borkhardt[1]

[1]University Children's Hospital, Department of Pediatric Oncology, Hematology and Clinical Immunology, Medical Faculty, Heinrich Heine University, Düsseldorf; [2]Institute of Medical Informatics, University of Münster, Münster; [3]Institute for Clinical Immunology and Transfusion Medicine, Medical Faculty, Justus Liebig University, Giessen; and [4]von Haunersches Children's Hospital, University of Munich, Germany

ABSTRACT

Background
Very severe aplastic anemia is characterized by a hypoplastic bone marrow due to destruction of CD34+ stem cells by autoreactive T cells. Investigation of the pathomechanism by patient-specific gene expression analysis of the attacked stem cells has previously been impractical because of the scarcity of these cells at diagnosis.

Design and Methods
Employing unbiased RNA amplification, patient-specific gene expression profiling was carried out for CD34+ cells from patients newly diagnosed with very severe aplastic anemia (n=13), refractory anemia (n=8) and healthy controls (n=10). These data were compared to profiles of myelodysplastic disease (n=55), including refractory anemia (n=18). To identify possible targets of autoimmune attack, presence of autoreactive antibodies was tested in pre-therapeutic sera of patients with very severe aplastic anemia (n=19).

Results
CD34+ gene expression profiling distinguished between healthy controls, children with aplastic or refractory anemia and clonal disease. Interferon stimulated genes such as the apoptosis inducing death ligand TRAIL were strongly up-regulated in CD34+ cells of patients with aplastic anemia, in particular in patients responding to immunosuppressive treatment. In contrast, mRNA expression of integrin GPVI and the integrin complexes GPIa/IIa, GPIIb/IIIa, GPIB/GPIX/GPV was significantly down-regulated and corresponding antibodies were detected in 7 of 11 profiled patients and in 11 of 19 aplastic anemia patients.

Conclusions
As a potential diagnostic tool, patient-specific gene expression profiling of CD34+ stem cells made it possible to make the difficult differential diagnosis of most patients with aplastic and refractory anemia. Profiling indicated a prognostic correlation of TRAIL expression and patient benefit from immunosuppressive therapy. Downregulation of integrin expression and concurrent presence of autoreactive anti-integrin-antibodies suggested a previously unrecognized pathological role of integrins in aplastic anemia.

Key words: very severe aplastic anemia, myelodysplastic syndrome, integrins, CD34+ stem cells, autoantibodies.

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Correspondence:
Arndt Borkhardt, Department of Pediatric Oncology, Hematology and Clinical Immunology, Center for Child and Adolescent Health, Heinrich Heine University, Moorenstr. 5, 40225 Düsseldorf, Germany.
Phone: international +0049-211.8117680.
Fax: international +0049.211.8116707.
E-mail: arndt.borkhardt@med.uni-duesseldorf.de

The online version of this article has a Supplementary Appendix.

Introduction

Severe aplastic anemia (SAA) is a potentially life threatening disorder that is characterized by bone marrow failure and resulting low blood cell counts.[1,2] Children typically present with paleness, bacterial infections or bleeding symptoms. In children with very severe aplastic anemia (vSAA, <200 granulocytes/μL blood) only few remaining hematopoietic stem cells can be found in the bone marrow at diagnosis.[2]

There is now overwhelming experimental evidence that SAA can be regarded as an autoimmune process mediated by oligoclonally expanded populations of autologous T cells that attack the patient's own hematopoietic stem cells.[2] The antigenic target on stem cells has not yet been defined and may vary between individuals. Autoantibodies against hnRNP K, kinectin, postmeiotic segregation increased 1 (PMS1), moesin and diazepam-binding inhibitor-related protein 1 (DRS-1) have been detected in patients with SAA.[3-6] In one patient, also platelet directed autoantibodies have been found.[7] Antiplatelet antibodies usually target membrane bound glycoproteins on platelets (GP), also termed integrins, that are known autoantigens in patients with systemic lupus erythematosus (SLE),[8,9] or bleeding disorders such as autoimmune thrombocytopenia (ITP)[10] and neonatal alloimmune thrombocytopenia (NAIT).[11] Integrins are highly expressed on hematopoietic stem cells (HSC).[12] They mediate adhesion of HSC to the extracellular matrix, and are important regulators of stem cell mobilization, homing and engraftment.[13] They share a common heterodimeric structure composed of alpha and beta subunits.[14] Given their widespread expression pattern and their role in autoimmunity, it appears plausible that GP autoantibodies may contribute to the pathogenesis of SAA.

Clinical symptoms of SAA do not appear until CD34$^+$ cells have fallen below 1% of normal counts. The low number of CD34$^+$ cells at diagnosis has meant that research is extremely challenging. Only CD34$^+$ cells pooled from several SAA patients have been analyzed by mRNA profiling. These expression profiles indicated a stressed and dying cell population.[15] Destruction of CD34$^+$ cells is likely induced by interferon-γ (IFN-γ) stimulated apoptotic cell death. Massive apoptosis of bone marrow stem and progenitor cells has been observed in patients with aplastic anemia indicating that apoptosis is a major mechanism of cell loss in this disease.[16,17] IFN-γ is frequently over-expressed and released by T cells in the bone marrow of SAA patients and induces a proapoptotic gene expression pattern in normal CD34$^+$ cells *in vitro*.[18,19] It transcriptionally stimulates the extrinsic apoptotic pathway that is mediated by activation of death receptors through binding of specific death ligands.[20] IFN-γ enhances expression of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). The effect of this death ligand on target cells is dependent on its interaction with a complex system of receptors: pro-apoptic death-receptors (TRAIL-R1/DR4 and TRAIL-2/DR5) and anti-apoptotic decoy receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2, and OPG).[21] Besides, IFN-γ also induces the death receptor Fas/CD95 and other apoptotic regulators.[22] These findings indicate that interferon signaling is involved in the pathophysiology of bone marrow failure in SAA patients, but expression analyses of individual SAA patients are still lacking. Pooled samples from heterogeneous patient cohorts may not reflect individual gene expression that differs strongly between subjects and critically influences the results of such analyses. Therefore, in the present study, we profiled and analyzed CD34$^+$ gene expression of 13 individual pediatric patients newly diagnosed with very severe aplastic anemia.

Design and Methods

Patients' samples and controls

Nineteen patients with vSAA diagnosed as previously described[23,24] were included in this study. After obtaining informed consent, all children were enrolled in the BFM-SAA 94 therapy study[25] approved by the local ethics committee. Immunosuppressive therapy (IST) and evaluation of the therapeutic response has been carried out as described.[24] Gene expression profiling was carried out for 13 of these patients. Eight children with refractory cytopenia were also included. Patients' samples were collected at diagnosis. The control group consisted of age-matched healthy children (n=10).

Separation of CD34$^+$ hematopoietic progenitor cells

Bone marrow derived mononuclear cells were separated by Ficoll density centrifugation and resuspended in phosphate buffered saline, 2% bovine serum albumin, 0.6% sodium-citrate. CD34$^+$ cells were isolated using the Dynal CD34 progenitor cell selection system (Invitrogen, Darmstadt, Germany) according to the manufacturer's recommendations; 1-2.5×10^5 CD34$^+$ cells with a purity of more than 95% were retrieved.

mRNA amplification and labeling

Total RNA of CD34$^+$ cells was extracted using TRIZOL®Reagent (Invitrogen) as recommended by the manufacturer. Linear RNA amplification and RNA target preparation for microarray analysis was performed as recommended ('Two-Cycle Target Labeling Protocol', Affymetrix, Santa Clara, CA, USA). Due to limited amounts of total RNA, an additional cycle of cDNA synthesis and *in vitro* transcription (IVT) amplification was performed. The protocol is described in the *Online Supplementary Appendix*.

Array hybridization and scanning

Array hybridization and scanning were performed at the Biomedical Research Center (BMFZ, University of Düsseldorf, Germany). According to the manufacturer's protocol, cRNA was hybridized on an Affymetrix GeneChip U133 Plus 2.0 array using the GeneChip® Hybridization Oven 640 (Affymetrix). Washing and staining was performed on a GeneChip® Fluidic station 450. Arrays were scanned on a GeneChip® Scanner3000 7G.

Array data analysis

All arrays passed the explanatory quality control methods implemented in the R-package ArrayQualityMetrics (version 2.2.1). Array data analysis has been carried out essentially as described.[26-29] A description of the analysis workflow is presented in the *Online Supplementary Appendix*.

Detection of platelet reactive antibodies

Patient sera were screened for platelet-reactive antibodies by platelet adhesion immunofluorescence (PAIF) and monoclonal antibody-specific immobilization of platelet antigen (MAIPA) tests, essentially as described previously[30,31] and in the *Online Supplementary Appendix*.

Results

Global gene expression of CD34+ stem cells from patients with very severe aplastic anemia reveals a signature of apoptotic cell death and evasion

In order to profile the transcriptome of rare CD34+ cells of individual vSAA patients, we used technological improvements allowing unbiased, high-quality mRNA amplification prior to array based gene expression analysis. We focused our investigation on 13 newly diagnosed children suffering from vSAA and compared their expression profile to CD34+ cells of 10 age-matched healthy children (Table 1 and Figure 1). Of 45,500 genes represented on the array, we identified 1,879 genes (4.1%) that were differentially expressed (adjusted $P<0.05$, *Online Supplementary Table S1*). With the exception of one sample (vSAA4), all vSAA patients were clustered together separately from healthy subjects when applying hierarchical clustering to the gene signature. The majority of these genes (1,177 genes) were down-regulated; 702 genes were increased in expression compared to controls. Genes that were up-regulated in vSAA patients belonged to the functional categories' cell death (Table 3 and *Online Supplementary Table S2*), immune and stress responses (Table 3), proliferation and signal transduction. Down-regulated genes were shown to be involved in cell division (e.g. BUB1), cell cycle control (e.g. CDK genes), DNA repair (e.g. BRCA2, FANCI, FANCG, FANCF), DNA binding (e.g. histones and HMGB proteins), differentiation (e.g. HOX genes, GATA1 and 2) and apoptosis. Unexpectedly, the expression of the main apoptosis executers caspase-3 (-1.06 logFC, adjusted $P=0.017$) and -6 (-2.17 logFC, adjusted $P=0.009$ and -1.19 logFC, adjusted $P=0.013$) was significantly down-regulated in vSAA cells compared to healthy controls, which may indicate an adaptive process of surviving CD34+ stem cells.

Interferon signature genes are significantly up-regulated in CD34+ cells from individual patients with severe aplastic anemia

We analyzed the individual expression of interferon sig-

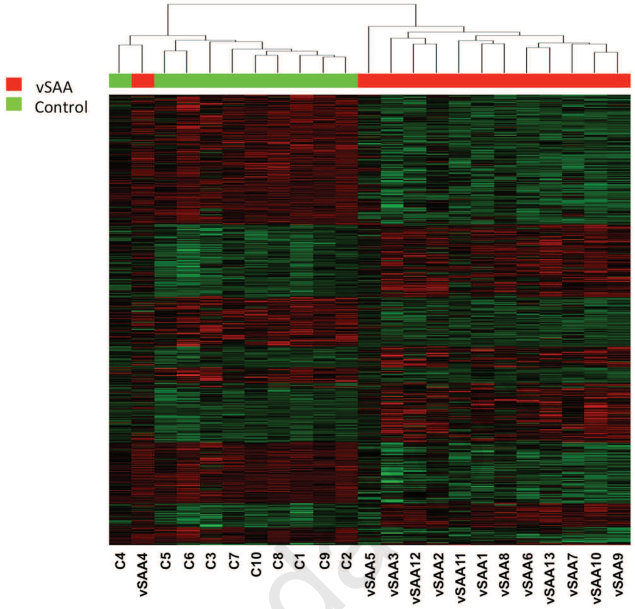

Figure 1. Gene expression differs significantly between vSAA patients and healthy controls. Heatmap of samples from the 13 vSAA patients (vSAA) presented in Table 1 and 10 age-matched healthy controls (C) analyzed by an Affymetrix GeneChip U133 Plus 2.0 array. Each column represents a patient or a normal control. Hierarchical clustering of 1,879 differentially expressed genes is shown. Relative gene expression is indicated by color. Significantly higher expression is shown in red and lower expression in green (normalized to the median).

Table 1. Characteristics of 13 pediatric vSAA patients enrolled in CD34+ cell expression profiling. All patients had idiopathic or hepatitis-induced vSAA. IST response was evaluated after six months (response M6) and at later time points.

Expression profiling	Age	Gender	Etiology	Response (M6)	Late response	BMT
vSAA1	8	male	idiopathic	NR	M12: NR, M30: PR	no
vSAA2	7	male	idiopathic	NR	nd	yes
vSAA3	10	female	idiopathic	NR	day 112: NR	yes
vSAA4	11	female	hepatitis	NR	nd	yes
vSAA5	15	female	hepatitis	NR	nd	yes
vSAA6	6	male	idiopathic	NR	nd	yes
vSAA7	13	female	idiopathic	NR	nd	yes
vSAA8	3	male	hepatitis	PR	M12: CR, M18: CR	no
vSAA9	11	female	idiopathic	no IST	nd	yes
vSAA10	15	male	idiopathic	CR	M12: PR	no
vSAA11	11	male	nd	CR	M12: CR, M46: relapse	no
vSAA12	11	female	nd	PR	M6: PR M12: NR	no
vSAA13	17	female	idiopathic	died, Day 19	na	no

NR: no response; PR: partial response; CR: complete response; nd: not determined; na: not applicable, BMT: bone marrow transplantation

Table 2. Platelet and HLA reactive autoantibodies were detected in sera of pediatric vSAA patients by PAIF or MAIPA.

Patients	Expression profiling	PAIF	GPIIb/IIIa	GPIa/IIa	GPIb/IX	HLA-I
1	vSAA1	-	-	-	-	-
2	vSAA2	+	-	+	+	+
3	vSAA3	-	-	-	-	-
4	vSAA4	+	+	-	-	-
5	vSAA5	-	-	-	-	-
6	vSAA6	+	+	+	-	-
7	vSAA9	+	-	-	-	-
8	vSAA10	+	-	-	-	+
9	vSAA11	-	-	-	-	-
10	vSAA12	+	-	+	+	+
11	vSAA13	+	+	+	-	+
12	np	-	-	-	-	-
13	np	+	-	-	-	-
14	np	-	-	-	-	-
15	np	+	+	-	+	+
16	np	-	-	-	-	-
17	np	+	+	+	-	-
18	np	+	-	-	-	-
19	np	-	-	-	-	-
n = 19 (100%)	11	11 (58%)	5 (26%)	5 (26%)	3 (16%)	5 (26%)

Sera from 19 newly diagnosed vSAA patients (11 patients also analyzed by gene expression profiling and 8 patients not profiled, np) were tested for the presence of autoantibodies; 11 patients had detectable platelet reactive antibodies (58%).

nature genes (ISG) in CD34+ cells derived from 13 vSAA patients and 10 age-matched controls (Figure 2).

In general, 12 of the 13 patients clustered separately from the analyzed controls. Genes previously shown to be stimulated by interferon[19] were also up-regulated in the investigated vSAA patient population. However, the expression level of specific genes clearly differed between subjects. Interferon induced genes that were overall significantly up-regulated in the vSAA cohort compared to control samples are shown in Table 3. These genes are involved in cell death signaling, immune responses, cell proliferation and homeostasis, as well as signal transduction. In our expression analysis, classical IFN-γ target genes such as *IFN-γ* itself, *ISG20*, *IRF1*, *IFIT2*, *IFIT5*, *IFITM1*, *IFITM3*, *SP100*, *GBP1*, and GBP2 were up-regulated. This confirms the reliability of the results gained in our study.

Three genes involved in immune response, cell proliferation, and DNA repair processes, respectively, previously reported to be up-regulated in IFN-γ treated CD34+ cells were down-modulated in our patient cohort: *FCER1A* (-2.8 logFC), *PRTN3* (-2.2 logFC), *BRCA2* (-0.6 logFC).

The interferon signature gene-restricted expression profile indicates the therapeutic response

In a recent study, flow cytometric detection of intracellular interferon in circulating T cells of untreated SAA patients was associated with a subsequent response to IST in 96% of cases.[32] We assumed that increased interferon activity could lead to a pronounced induction of interferon signature genes (ISG) in CD34+ cells. We hypothesized that ISG expression in CD34+ cells may present a molecular fingerprint of autoreactive T-cell activity and may be useful to select patients that are more likely to respond to IST. To this end, we analyzed ISG expression in responders and non-responders (Figure 3A). Eleven of the 13 patients were informative (one patient did not receive IST and one patient

Table 3. Interferon signature genes are up-regulated in vSAA CD34+ cells. List of 27 selected interferon signature genes up-regulated in vSAA CD34+ cells.

Gene function	Gene symbol	GenBank#	logFC	Adj. *P* value
Cell death and apoptosis	Fas	U89101	0.59	0.017
	TNFSF10	U37518	1.60	0.031
	TRADD	L41690	0.71	0.004
Immune response	IFNG	J00219	1.60	0.001
	IFIT2	M14660	1.16	0.006
	IFIT5	U34605	0.87	0.048
	IFITM1	J04164	1.89	<0.001
	IFITM3	X57352	0.91	0.011
	IRF1	L05072	1.67	<0.001
	GBP1	M55542	147	0.027
	GBP2	M55543	1.52	0.004
	ISG20	U88964	1.86	0.003
	SP100	U36501	1.26	0.007
	MR1	U22963	1.05	0.007
	HLA-DOB	X03066	1.76	0.004
	CASP1	U13697	1.07	0.048
Cell growth and maintenance	TAP1	X57522	1.12	<0.001
	RAB7L1	D84488	1.92	<0.001
Cytokines/chemokines and receptors	IL15	AF031167	2.35	<0.001
	IL15RA	U31628	0.84	0.048
	IL10RA	AY195619	1.68	0.009
	IL12RB1	U03187	1.52	0.006
	CCL5	M21121	1.43	0.048
Signal transduction	STAT1	M97935	1.38	0.011
	SOCS1	AB000734	0.72	0.018
	RGS1	S59049	1.23	0.001

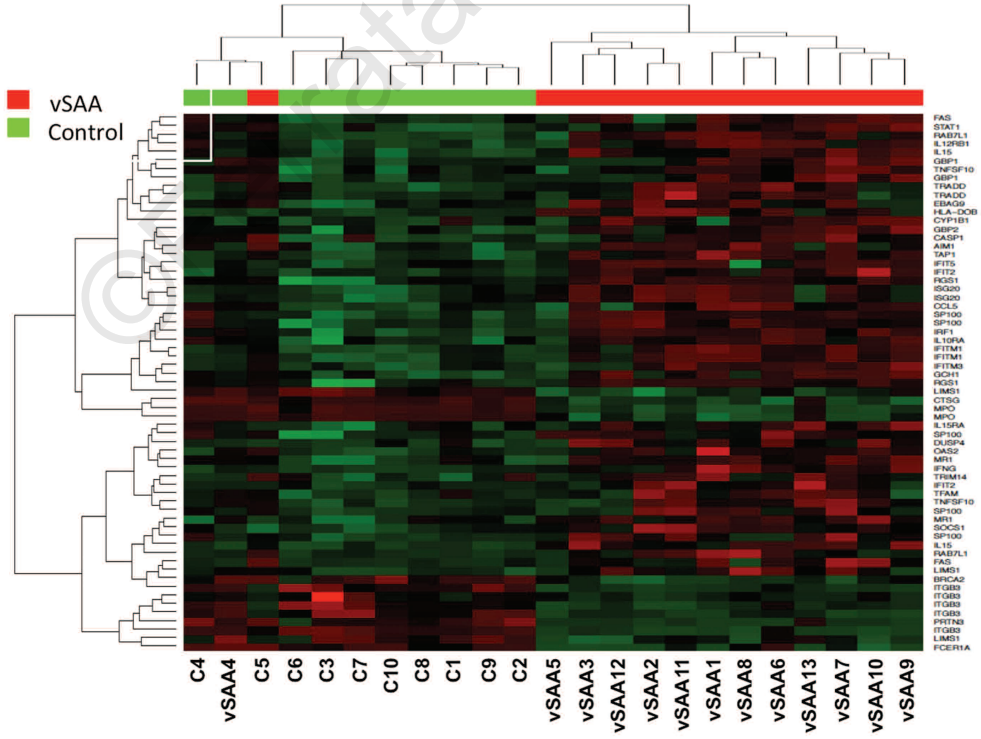

Figure 2. Heatmap of samples from 13 vSAA patients and 10 normal control persons analyzed and shown as in Table 1. Hierarchical clustering of 40 interferon signature genes differentially expressed between vSAA and control CD34+ cells.

died prior to IST). Two of the patients not responding to therapy had an expression pattern similar to the analyzed control group showing no upregulation of ISG, this may be indicative of an absence of this cytokine in the bone marrow or a defective interferon signaling in these cells. In contrast, none of the responders showed an expression pattern without strong induction of ISG. Nevertheless, there was no significant difference in overall ISG signature between the remaining 5 non-responders and the 4 responders. Therefore, analysis of overall ISG expression may identify some non-responders but is not sufficient as a predictive marker. Interestingly, in these analyses we observed a high expression of the death ligand TRAIL (synonymous TNFSF10) in CD34$^+$ cells of all patients responding to IST (Figure 3B). TRAIL expression was lower in control samples taken from healthy bone marrow donors as well as in CD34$^+$ cells from 3 of 7 non-responders. Of the remaining 4 non-responders, 3 cases had lower TRAIL expression than responding patients and only one case showed a similar upregulation. CD34$^+$ cells from vSAA patients did not express the death-inducing TRAIL receptors TRAIL-R1 and -R2. However, we found a high upregulation of the decoy receptor TRAIL-R3 (synonymous DcR1/TNFRSF10c;1.6 logFC, adjusted P=0.027) in CD34$^+$ cells of vSAA patients.

Gene expression profiling of CD34$^+$ cells distinguishes between aplastic or refractory anemia

Myelodysplastic disease initially often manifests as refractory cytopenia (RC). Evaluation of bone marrow biopsies from children presenting with vSAA or RC are histologically challenging and can sometimes lead to misclassification.

To identify gene expression signatures that distinguish between these children, we compared expression profiles of our cohort of 13 vSAA patients with 8 samples collected from patients with RC (*Online Supplementary Figure S1*). Only one vSAA patient sample (vSAA8) had a profile more similar to RC than to the other vSAAs, and 2 samples of RC (RC6, RC7) were grouped together with the vSAA cohort. The remaining samples of vSAA or RC clustered separately and had a clearly distinct profile with respect to the analyzed gene set. We carefully rechecked the clinical, laboratory and pathological findings of the patients, but found no obvious differences between patients whose samples clustered according to the established diagnosis and those whose samples showed atypical clustering.

We further compared our data set with 66 previously published[1] profiles of 55 patients with myelodysplastic syndromes, including RC (n=18), refractory anemia with ringed sideroblasts (RARS, n=19), refractory anemia with excess blasts (RAEB, n=18) and 11 healthy controls (*Online Supplementary Figure S1*). Hierarchical clustering of the 66 most differentially expressed genes between the five subgroups revealed distinct signatures between vSAA patients and children suffering from RC and other subtypes of MDS. Only one vSAA sample (vSAA4) clustered with the RC

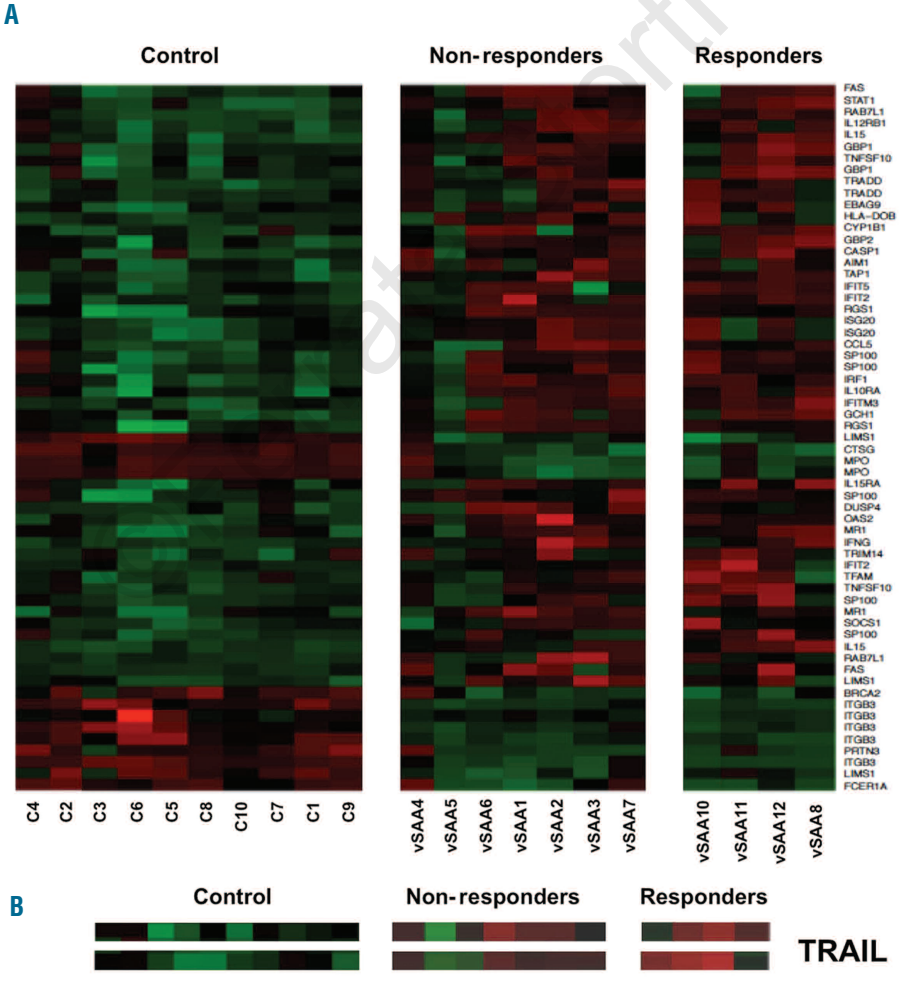

Figure 3. Response to immunosuppressive therapy. (A) Heatmap of the 40 known interferon stimulated genes presented in Table 3 that were differentially expressed in CD34$^+$ cells of vSAA patients and healthy controls. Columns representing individual patients are ordered corresponding to IST response. (B) Heatmap of samples from 13 vSAA patients (7 non-responders, 4 responders) and 10 normal controls. Two probes detecting TNFSF12/TRAIL are shown.

cohort. CD34+ gene expression of this patient resembled healthy controls (Figure 1) and also had a different profile in the ISG analysis (Figure 2). Similar to our prior analysis (*Online Supplementary Figure S1*), the only RC sample clustering together with the vSAA cohort was RC6. Thus, in most cases, expression distinguished between RC and SAA, and may provide a sensitive diagnostic tool for those cases that are difficult to interpret by histopathology.

Integrins on CD34+ cells as a potential target of autoimmune T-cell attack in severe aplastic anemia

The bone marrow failure syndrome paroxysmal nocturnal hemoglobinuria (PNH) can develop as a long-term complication in SAA.[33] PNH CD34+ cells are deficient in glycosyl phosphatidyl inositol (GPI), a common anchor for proteins expressed on plasma membranes. PNH clones are probably selected over time due to the absence of cell surface proteins that act as targets for autoimmune attack by CD8+ cytotoxic T cells or NK cells. Therefore, we hypothesized that CD34+ cells still surviving in patients with vSAA could use similar mechanisms to evade the immune system. To this end, we first checked whether the mRNA expression of the *PIG-A* gene, responsible for lack of surface protein expression in PNH cells, was altered in vSAA samples. However, PIG-A expression was not significantly changed in the vSAA cohort. Therefore, loss of GPI anchorage of proteins as a broad mechanism to reduce antigenic targets on CD34+ did not seem to play a role in our cohort of vSAA cells. Next, we analyzed mRNA expression of other known cell surface proteins. Interestingly, we found a significant downregulation of major integrin genes or GP (*Online Supplementary Table S3* and *Online Supplementary Figure S2*). These integrins are known autoantigens in patients with SLE or various bleeding disorders (*Online Supplementary Table S3*). The gene *ITGA2B* encodes the alpha subunit of the GPIIb/GPIIIa complex, glycoprotein IIb. The beta subunit GPIIIa is encoded by the *ITGB3* gene. mRNA expression of both genes was significantly down-regulated in CD34+ cells from vSAA patients with log ratios of -2.47 to -4.23 (ITGA2B) and -1.28 to -4.02 (ITGB3). There was also a decrease in mRNA expression of the gene *ITGA2* encoding integrin alpha-2 (GPIa), a subunit of the GPIa/IIa complex. All components of the trimeric GPIB/GPIX/GPV complex, as well as GP6, the receptor for collagen on platelets, were markedly down-regulated.

In addition to downregulation of these cell surface receptors, ITGB3BP, that interacts intracellularly with the cytoplasmic tail of ITGB3,[34] was also down-regulated in vSAA samples. Binding of ITGB3BP is essential for ITGB3 signaling.[34]

As the identified cell surface receptors are acknowledged targets of autoantibodies, we analyzed serum from 11 of these 13 vSAA patients that had been collected before any blood transfusions were given. Using PAIF and MAIPA, we detected autoantibodies against those integrins in 7 of 11 patients (Table 2). Consistently, we found a decrease in the vSAA patients in the antibody-binding sites of GPIIb/IIIa for 4 kinds of antibodies on circulating platelets ($P<0.05$). To test these findings in an extended patient cohort, we analyzed peripheral blood from a further 8 patients with vSAA from whom we had stored blood samples prior to therapy but who were not included in the transcriptome analysis. Four of these 8 patients had autoantibodies against platelet membrane proteins. In total, 11 (58%) of 19 patient samples were positive for glycoprotein directed autoantibodies.

Discussion

Because of autoimmune attack, CD34+ cells are extremely scarce in the bone marrow of children with vSAA. Previously, samples from different patients had to be pooled in order to obtain enough RNA for a transcriptome analysis.[15] An important drawback of this approach is the lack of patient specific information. Furthermore, the analyzed cohorts were heterogeneous with respect to age (range 9-74 years), and severity of disease (moderate to severe cases of aplastic anemia were included). In the present study, we profiled CD34+ gene expression of 13 individual, newly diagnosed, pediatric vSAA patients by performing an mRNA amplification prior to expression array analysis.

Transcriptome analysis could clearly differentiate healthy controls from samples of vSAA patients. The CD34+ gene expression signature of most, but not all patients was characteristic of a stressed and dying cell population. Crucial apoptotic mediators were highly up-regulated in the patient samples, such as components of the death receptor-induced (e.g. FAS and TRAIL), the mitochondria-triggered (e.g. BCL2L12/BIM) or the perforin- and granzyme-mediated pathways. This agrees with a previous report.[15] However, in surviving CD34+ cells, we also observed a pronounced decrease in expression of the main executing enzymes of apoptotic cell death: caspase-3 and caspase-6. This may indicate a selection of apoptosis resistant bone marrow stem cells in the course of the disease.

Autoimmune suppression of hematopoiesis has been thought to play a major role in the pathogenesis of aplastic anemia (AA) based on the high response rates achieved by immunosuppressive therapy in these patients. In the bone marrow, antigen-specific T cells are expanded that probably mediate cytotoxicity for CD34+ hematopoietic stem cells. Previously, it was shown that IFN-γ was up-regulated in bone marrow derived T cells of a proportion of AA patients.[18] We, therefore, carried out a targeted analysis of IFN-γ stimulated genes in CD34+ cells from individual patients. Overall ISG expression of vSAA patients revealed signatures that may be correlated to a lack of response to IST. However, only the analysis of specific genes in individual patients showed a probable association of high TRAIL expression with IST response.

Previous flow cytometry studies showed that CD34+ cells normally lack detectable expression of TRAIL and TRAIL receptors and *in vitro* recombinant TRAIL has no cytotoxic effect on primary CD34+ cells.[35] However, in CD34+ cells of all vSAA patients responding to IST, we observed a strong upregulation of the death ligand TRAIL, whereas TRAIL expression was lower in samples from non-responders and healthy volunteers. Consistently, TRAIL mRNA expression was seen to be up-regulated in IFN-γ treated, but not in untreated or freshly isolated CD34+ cells.[36] Furthermore, in a previous study, elevated TRAIL mRNA expression in the CD34+ cell population was found in AA samples only in active disease (n=3) not in remission (n=2), although only a small number of patients were analyzed.[36] Taken together, these findings may indicate that high TRAIL expression identifies a patient population that is characterized by active autoimmune disease and that may respond better to IST.

In our study, CD34+ cells from vSAA patients showed no altered expression of the death-inducing TRAIL receptors TRAIL-R1 and -R2 when compared to healthy controls. In contrast, the anti-apoptotic receptor TRAIL-R3 was up-reg-

ulated in CD34+ cells of vSAA patients. TRAIL-R3 is a plasma membrane-bound high affinity receptor for TRAIL. In contrast to the death receptors TRAIL-R1 and -R2, it lacks the cytoplasmic region that contains the death-inducing protein domain. Consequently, TRAIL-R3 is not capable of mediating apoptosis. It serves as a decoy receptor and protects cells from TRAIL-induced cytotoxicity by competing with other TRAIL receptors for ligand binding.[37,38] We could speculate that upregulation of a decoy receptor for TRAIL ligand may enable CD34+ vSAA stem cells to kill infiltrating effector lymphocytes by secretion of this death ligand while remaining unharmed themselves. This is reminiscent of protective mechanisms observed in some solid tumors that induce massive death of cytotoxic T cells by producing soluble and membrane-bound TRAIL.[39-43] Similar mechanisms of immune evasion have been described for the closely related death ligand FasL.[44]

TRAIL also exhibits broad tumor-selective cell killing,[45] thus high TRAIL concentrations in the bone marrow may protect against clonal disease. Interestingly, CD34+ cells transduced with adenovirus expressing TRAIL exhibited tumor-specific cytotoxic activity on a wide variety of different tumor cell types *in vitro* and *in vivo*.[46,47] Nevertheless, expression of TRAIL-R3, as well as downregulation of crucial apoptotic mediators, such as caspases, may counteract this effect and protect stem cell-derived tumor cells from cytotoxic TRAIL effects.

TRAIL may also participate in the pathological suppression of erythropoiesis in SAA patients, because immature erythroblasts express both TRAIL-R1 and TRAIL-R2.[48] During normal differentiation of immature erythroid precursors, apoptotic pathways are stimulated. Therefore, these precursors are sensitized and very vulnerable to apoptotic triggering mediated by activation of death receptors. Increased levels of TRAIL in the bone marrow may also be implicated in the pathogenesis of other bone marrow failure syndromes, such as Fanconi's anemia[49] and myelodysplastic syndrome (MDS).[50] Thus, taken together, our findings support previous reports indicating a pathological role for TRAIL in SAA.

In this study, we were also able to demonstrate that the transcription of major integrins was dramatically down-regulated in the few remaining CD34+ bone marrow cells from children with vSAA. These integrins are the main targets of autoimmune attack against platelets in patients with a wide variety of bleeding disorders, such as immune thrombocytopenic purpura (ITP). Downregulation of cell surface integrins may indicate a selection process and a mechanism of evading autoimmune attack. Thus, we analyzed sera of vSAA patients that were preserved prior to receipt of blood transfusions for the presence of platelet reactive antibodies. Reminiscent of the situation in ITP, we detected autoantibodies against these cell surface proteins in 58% and 48% of the vSAA patient cohort employing PAIF and MAIPA, respectively. The MAIPA assay is highly specific for ITP diagnosis (78-100%), but its sensitivity is comparatively low (38-46%). Thus, the number of patients harboring autoantibodies in our study may actually be even higher.[51] Recently, a pediatric SAA patient was reported to express platelet reactive antibodies amongst a variety of other autoreactive immunoglobulins.[7] The clinical significance of this finding is not yet clear. There is convincing evidence in ITP patients that platelets are not only destroyed, but also produced in smaller numbers. *In vitro* megakaryocyte production and differentiation were demonstrated to be inhibited by platelet reactive antibodies and morphological studies revealed obvious megakaryocyte cell damage already in immature stages.[52-54] Consistently, treatment with thrombopoietin mimetics leads to increased platelet counts in many ITP patients.[55] Therefore, it may be speculated that anti-integrin antibodies could either play an active role in the destruction of early progenitors or stem cells in vSAA patients, or at least perturb the delicate integrin signaling network that regulates HSC homeostasis, proliferation and migration. It would be interesting to analyze the impact of these antibodies in animal models, e.g. the NOD/SCID mouse model receiving human CD34+ cells. But the major function of the attacked integrin receptors is platelet aggregation. It appears plausible that both the decrease in platelet count and the decrease in antibody-binding epitopes of these proteins on platelets may promote bleeding symptoms in vSAA patients.

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