Bone marrow immunophenotyping by flow cytometry in refractory cytopenia of childhood

Anna M. Aalbers,[1,2] Marry M. van den Heuvel-Eibrink,[2] Irith Baumann,[3] Michael Dworzak,[4] Henrik Hasle,[5] Franco Locatelli,[6] Barbara De Moerloose,[7] Markus Schmugge,[8] Ester Mejstrikova,[9] Michaela Nováková,[9] Marco Zecca,[10] C. Michel Zwaan,[2] Jeroen G. te Marvelde,[1] Anton W. Langerak,[1] Jacques J.M. van Dongen,[1] Rob Pieters,[2] Charlotte M. Niemeyer,[11] and Vincent H.J. van der Velden[1]

[1]Department of Immunology, Erasmus MC, Erasmus University Medical Center, Rotterdam, The Netherlands; [2]Department of Pediatric Oncology/Hematology, Sophia Children's Hospital - Erasmus University Medical Center, Rotterdam, The Netherlands; [3]Department of Pathology, Clinical Center South West, Böblingen Clinics, Germany; [4]St. Anna Children's Hospital and Children's Cancer Research Institute, Department of Pediatrics, Medical University of Vienna, Austria; [5]Department of Pediatrics, Aarhus University Hospital Skejby, Aarhus, Denmark; [6]Department of Pediatric Hematology-Oncology, IRCCS Ospedale Bambino Gesù, Rome, University of Pavia, Italy; [7]Department of Pediatric Hematology/Oncology, Ghent University Hospital, Ghent, Belgium; [8]Department of Hematology, University Children's Hospital, Zurich, Switzerland; [9]Department of Pediatric Hematology/Oncology, Charles University and University Hospital Motol, Prague, Czech Republic; [10]Pediatric Hematology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; and [11]Department of Pediatrics and Adolescent Medicine, Division of Pediatric Hematology and Oncology, University of Freiburg, Germany

 doi:10.3324/haematol.2014.107706
Manuscript received on March 23, 2014. Manuscript accepted on November 13, 2014.
Correspondence: v.h.j.vandervelden@erasmusmc.nl

Supplemental Information

Supplemental Methods

Patients and controls

Data were reported to the coordinating study center of the EWOG-MDS study group through standardized data collection forms. Patients were HLA-typed for human leukocyte antigen (HLA)-A, -B, -C, -DR and -DQ by serological or molecular methods in the participating study centers. RCC patients included for analysis were either followed with a watch-and-wait strategy (n=17), or were treated with immunosuppressive therapy (IST) (n=24, of which n=22 with a hypocellular bone marrow), or received a hematopoietic stem cell transplantation (HSCT) (n=39; in n=1 no information on treatment was available), based on a treatment algorithm according to EWOG-MDS RC06 as previously described.[1] Details on treatment with IST and treatment response have been described previously.[1]

Gating strategies and cell population definitions

After exclusion of debris and dead cells based on scatter and CD45 expression, cell populations were identified using CD45 expression and forward or sideward scatter (FSC/SSC) properties, and additional markers when indicated. Granulocytes were defined as $CD45^{dim/bright}/SSC^{int/high}$ cells, and $CD33^{dim}$ when indicated; monocytes as $CD45^{dim/bright}/SSC^{int}$, and $CD64^{+}$ when indicated; immature erythroid cells as $CD45^{dim/neg}$, SSC^{low} cells, excluding $CD71^{neg}$ and $CD235^{neg}$ cells (other cells) and excluding $CD71^{neg}$ and $CD235^{dim}$ cells (unlyzed erythrocytes). Myeloid blast cells were defined as $CD45^{dim}/SSC^{low/int}/CD34^{+}$ cells, and $CD117^{+}$ when indicated; promyelocytes as $CD45^{dim}/SSC^{low/int/high}/CD34^{-}/CD117^{+}/CD13.33^{+}$ cells. $CD34^{+}$ B-cell precursors were defined as $CD45^{dim}/SSC^{low}/CD34^{+}/CD19^{+}$ cells. Other cell types that were identified were lymphocytes, defined as $CD45^{bright}/SSC^{low}$ cells; B cells as $SSC^{low}/CD19^{+}$ cells; mature B cells as $SSC^{low}/CD19^{+}/CD10^{-}$; B-cell precursors as $SSC^{low}/CD19^{+}/CD10^{+}$ (irrespective of CD34 positivity); T cells as $SSC^{low}/CD3^{+}$; NK cells as $CD3^{-}/CD16.56^{+}$; basophils as $CD123^{+}/HLA\text{-}DR^{-}$, and plasmacytoid

dendritic cells (DCs) as $CD123^{+}$/HLA-DR^{+}. Unless otherwise indicated, percentages of cell populations indicate proportions within all nucleated cells.

Statistical analyses

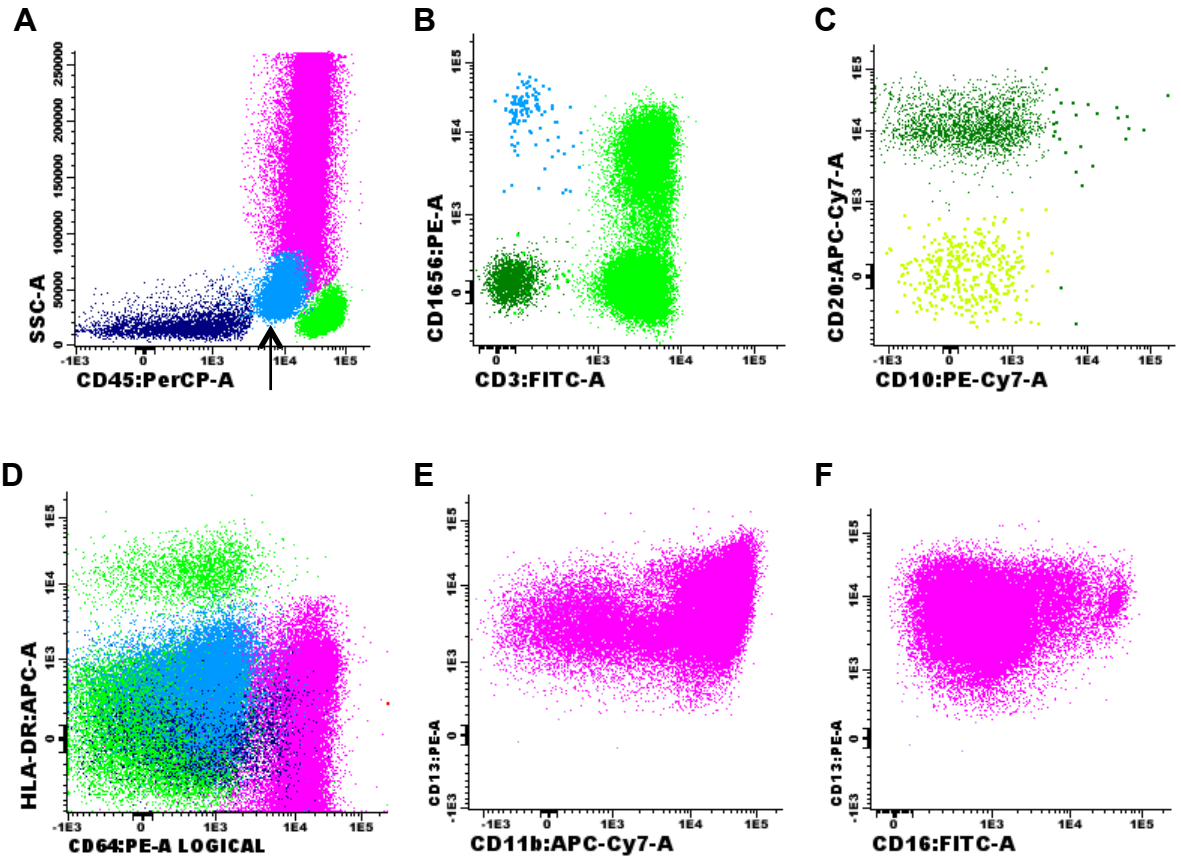
Statistical analyses were performed with SPSS 20 (IBM, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Categorical variables were compared using the Chi-square test or Fisher's exact test. Continuous variables were compared using the Mann-Whitney-U test or the Kruskal-Wallis test when more than two groups were compared. All reported *P*-values are two-sided and were considered statistically significant when <0.05; *P*-values >0.1 were reported as non-significant (NS), whereas those between 0.05 and 0.1 were reported in detail.

Supplemental References

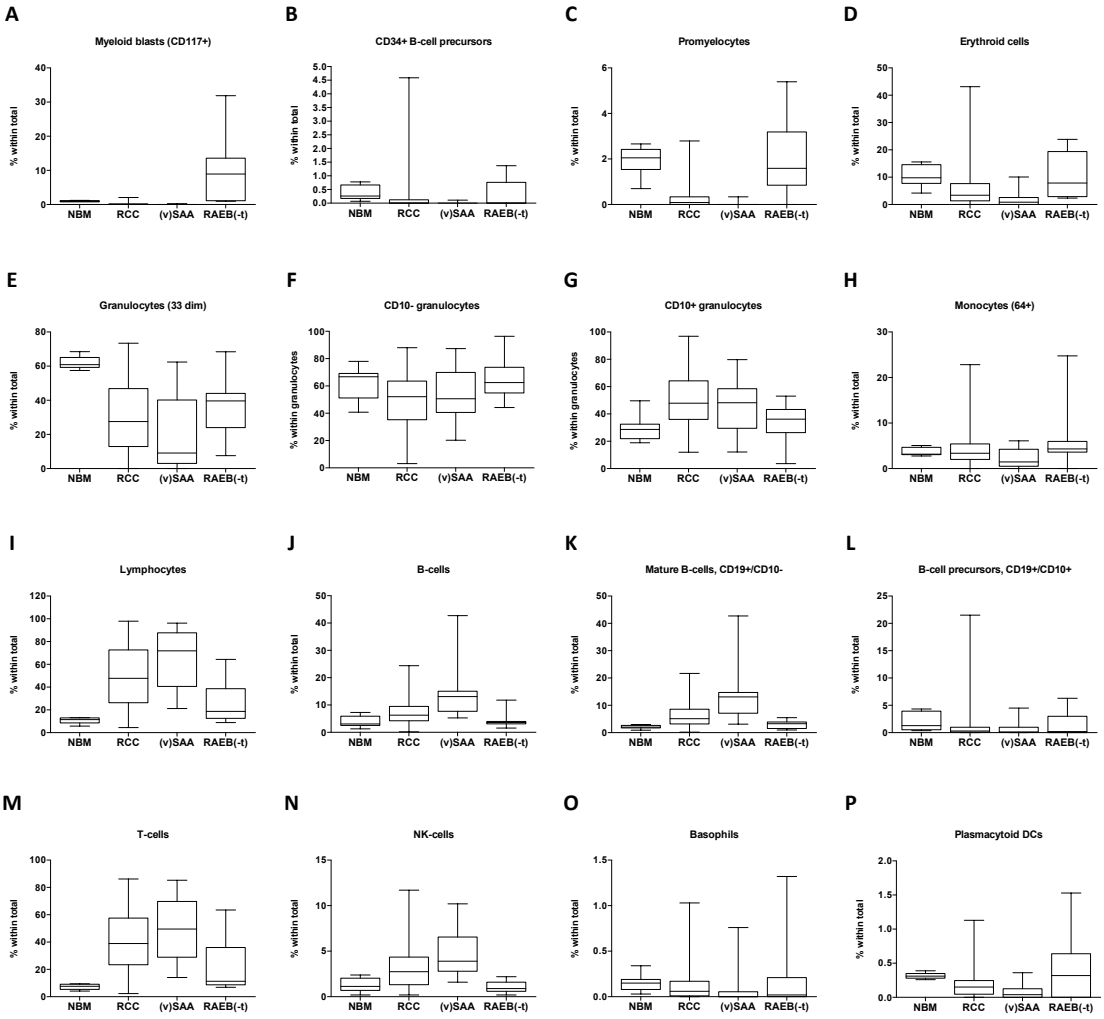
1. Aalbers AM, van der Velden VH, Yoshimi A, Fischer A, Noellke P, Zwaan CM, et al. The clinical relevance of minor paroxysmal nocturnal hemoglobinuria clones in refractory cytopenia of childhood: a prospective study by EWOG-MDS. Leukemia. 2013.

Supplemental Figure

Supplemental Figure 1. Flow cytometric abnormalities in a *GATA2* mutant RAEB patient. (A) Absence of $CD34^+$ B-cell precursors, indicated by an arrow. Granulocytes, in pink, appear to be hypogranular, but the relative granularity is not smaller than 2SD of healthy controls. (B) Within lymphocytes, low percentage of NK cells ($CD3^-CD1656^+$, 0.13% within white blood cells, 0.4% within lymphocytes), high percentage of T cells ($CD3^+$, 93% within lymphocytes, of which large part is $CD3^+/CD1656^+$). (C) Within $CD19^+$ cells, very low number of B-cell precursors ($CD10^+$). (D) Absence of monocytes ($HLA\text{-}DR^+/CD64^+$), also appreciable from (A). (E) Abnormal CD11b-13 pattern of granulocytes. (F) Abnormal CD16-13 pattern of granulocytes.



Supplemental Figure 2. Graphical representation of bone marrow cellular composition in RCC patients and controls. Boxes extend from the 25th to 75th percentile; lines in the boxes indicate medians, whiskers indicate minimum and maximum values. For statistical comparisons between RCC patients and controls, the reader is referred to Table 3 in the manuscript.



Supplemental Tables

Supplemental Table 1. Monoclonal antibody panel

	FITC			PE			PerCP			PE-Cy7/ PC7			APC			APC-Cy7/ APC-H7		
Tube	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**
1	CD3	SK7	BD	CD16.56	B73.1 and C5.9	BD and Zebra/Dako	CD45	2D1	BD	CD10	HI10a	BD	CD19	SJ25C1	BD	CD20	L27	BD
2	CD16	gran/1,5D2	IBL	CD13	L138	BD	CD45	2D1	BD	CD34	8G12	BD	CD117	104D2	Zebra/Dako	CD11b	ICRF44	BD
3	CD36	CLB-IVC7	IBL	CD33	P67.6	BD	CD45	2D1	BD	CD34	8G12	BD	CD11b	D12	BD	CD14	MO-P9	BD
4	CD36	CLB-IVC7	IBL	CD235a	JC159	Zebra/Dako	CD45	2D1	BD	CD117	104D2D1	Beckman Coulter	CD71	LO 1.1	BD	CD34	8G12	BD
5	CD15	MMA	BD	CD64	10.1	Serotech	CD45	2D1	BD	CD34	8G12	BD	HLA-DR	L243	BD	CD11b	ICRF44	BD
6	CD7	3A1/1, 7F3	Sanquin	CD56	C5.9	Zebra/Dako	CD45	2D1	BD	CD19	SJ25C1	BD	CD5	L17F12	BD	CD34	8G12	BD
7	CD34	8G12	BD	CD13.33	L138 and P67.6	BD	CD45	2D1	BD	CD123	6H6	e-Bioscience	CD117	104D2	Zebra/Dako	HLA-DR	L243	BD

Supplemental Table 2. Flow cytometric abnormalities in RCC patients with monosmy 7.

Patient	Myeloid blast cell abnormalities	Erythroid cell abnormalities	Granulocyte abnormalities	Monocyte abnormalities	Total number of abnormalities
1	Expression of CD7, expression of CD56	Heterogeneous expression of CD71, heterogeneous expression of CD36		Expression of CD56, abnormal SSC	6
2		Heterogeneous expression of CD71, heterogeneous expression of CD36			2
3		Heterogeneous expression of CD71, heterogeneous expression of CD36			2
4		Heterogeneous expression of CD71, heterogeneous expression of CD36	Abnormal CD11b-CD13 pattern, abnormal CD16-CD13 pattern		6
5			Abnormal CD16-CD13 pattern	Expression of CD56	2