Early bone marrow alterations in patients with adenosine deaminase 2 deficiency across disease phenotypes and severities

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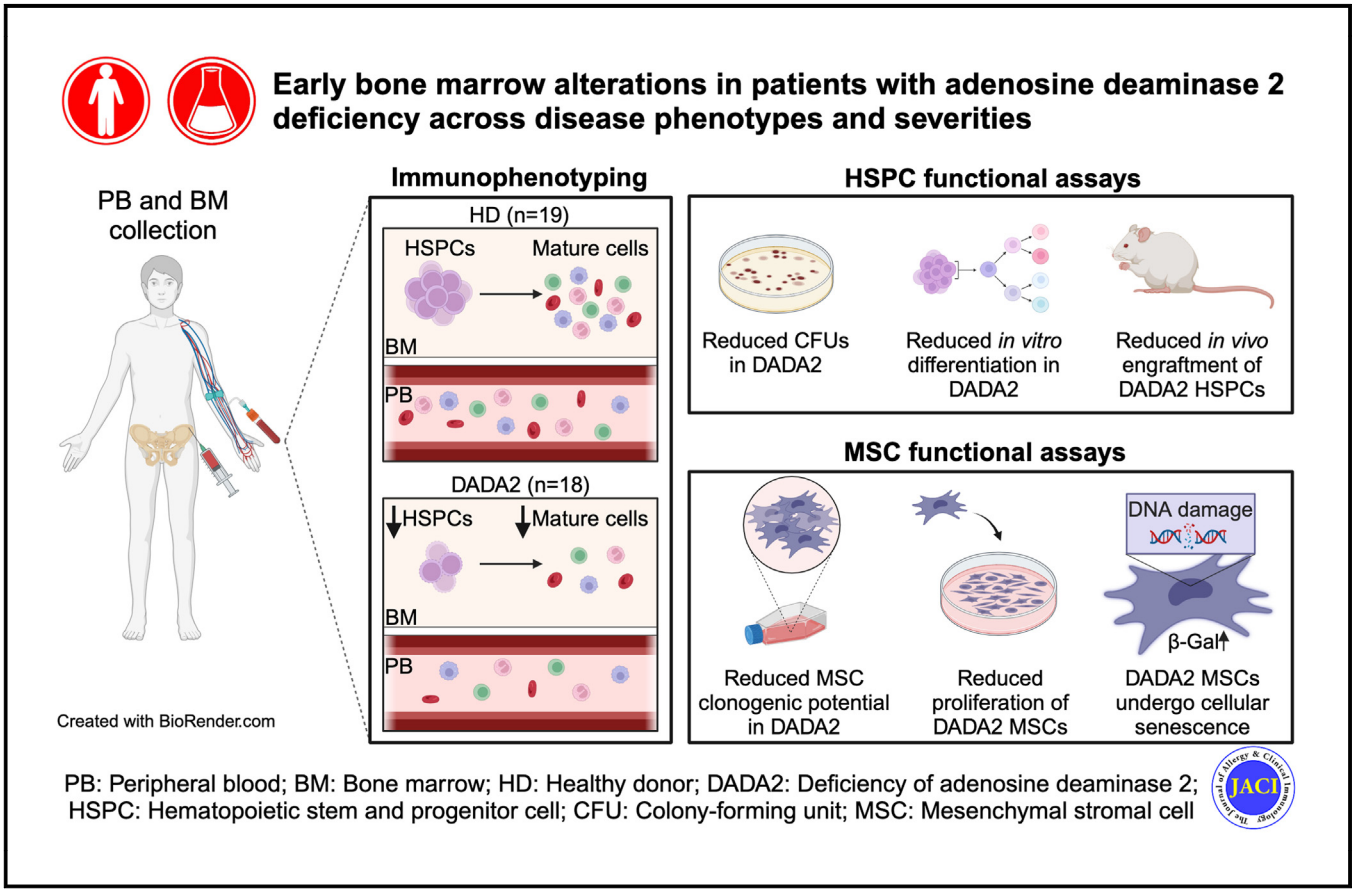
GRAPHICAL ABSTRACT



Capsule summary: Patients with DADA2 show early bone marrow alterations regardless of disease phenotype and severity; this awareness supports regular hematologic monitoring of patients with DADA2, including in cases with subclinical hematologic manifestations.

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Early bone marrow alterations in patients with adenosine deaminase 2 deficiency across disease phenotypes and severities

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Background: Deficiency of adenosine deaminase 2 (DADA2) is a complex monogenic disease caused by recessive mutations in the *ADA2* gene. DADA2 exhibits a broad clinical spectrum encompassing vasculitis, immunodeficiency, and hematologic abnormalities. Yet, the impact of DADA2 on the bone marrow (BM) microenvironment is largely unexplored.

Objective: This study comprehensively examined the BM and peripheral blood of pediatric and adult patients with DADA2 presenting with rheumatologic/immunologic symptoms or severe hematologic manifestations.

Methods: Immunophenotyping of hematopoietic stem cells (HSCs), progenitor cells, and mature cell populations was performed for 18 patients with DADA2. We also conducted a characterization of mesenchymal stromal cells.

Results: Our study revealed a significant decrease in primitive HSCs and progenitor cells, alongside their reduced clonogenic capacity and multilineage differentiation potential. These BM defects were evident in patients with both severe and nonsevere hematologic manifestations, including pediatric patients, demonstrating that BM disruption can emerge silently and early on, even in patients who do not show obvious hematologic symptoms. Beyond stem cells, there was a reduction in mature cell populations in the BM and peripheral blood, affecting myeloid, erythroid, and lymphoid populations. Furthermore, BM mesenchymal stromal cells in patients with DADA2 exhibited reduced clonogenic and proliferation capabilities and were more prone to undergo cellular senescence marked by elevated DNA damage.

Conclusions: Our exploration into the BM landscape of patients with DADA2 sheds light on the critical hematologic dimension of the disease and emphasizes the importance of vigilant monitoring, even in the case of subclinical presentation. (J Allergy Clin Immunol 2025;155:616-27.)

Key words: *Adenosine deaminase 2 deficiency, bone marrow, bone marrow failure, cytopenia, hematopoietic stem cell transplantation, mesenchymal stromal cells, senescence*

Deficiency of adenosine deaminase 2 (DADA2) is a monogenic multisystem disease resulting from autosomal recessive mutations in the *ADA2* gene.[1,2] ADA2, mainly produced by monocytes and macrophages, converts adenosine into inosine. Originally, DADA2 was identified as a form of vasculopathy, exhibiting symptoms resembling polyarteritis nodosa and characterized by early-onset stroke.[1,2] However, the clinical spectrum of DADA2 has expanded, revealing a highly variable presentation. It is now characterized primarily by the combination of 3 main phenotypes: vasculitis and/or inflammation, immunodeficiency, and hematologic disease.[3] These phenotypes are not mutually exclusive, and a specific clinical manifestation can evolve over time, posing challenges in both diagnosis and treatment of the disease. The heterogeneity in the age of onset and disease severity further complicates the clinical landscape.

Current therapeutic approaches for DADA2 focus on controlling inflammation with anti-TNF therapy, which has proved effective in patients exhibiting a predominant inflammatory phenotype.[4] However, it is not beneficial for hematologic manifestations, which are a prevalent aspect of DADA2, affecting up to 56% of patients.[5] Hematologic involvement encompasses a

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Abbreviations used
BFU: Burst-forming unit
BM: Bone marrow
BMF: Bone marrow failure
CFU: Colony-forming unit
DADA2: Deficiency of adenosine deaminase 2
DAPI: 4′-6-Diamidino-2-phenylindole
HD: Healthy donor
HSCT: Hematopoietic stem cell transplantation
HSPC: Hematopoietic stem and progenitor cell
MSC: Mesenchymal stromal cell
PB: Peripheral blood
TEMRA: Effector memory re-expressing CD45RA
T-LGL: T-cell large granular lymphocyte

range of conditions, including anemia (25.6%), lymphopenia (21.4%), neutropenia (20.1%), and thrombocytopenia (8.9%).[5] These manifestations range from mild isolated immune cytopenia affecting a single lineage to severe bone marrow (BM) failure (BMF). Furthermore, some patients may present with lymphoproliferation, lymphoma, macrophage activation syndrome, and hemophagocytic lymphohistiocytosis.[6-10] Severe hematologic features associated with DADA2 are often refractory to standard treatments such as growth factors and immunosuppressants. Therefore, the only curative option for these patients is allogeneic hematopoietic stem cell transplantation (HSCT).[11-14]

While the pathophysiology of vasculitis and inflammation in DADA2 is well documented, the mechanisms underlying hematologic manifestations remain elusive. Reports described abnormalities in erythroid and myeloid precursors and a general reduction in BM function in cases of DADA2-associated BMF.[14-20] However, a comprehensive understanding of the BM abnormalities is yet to be elucidated, and it also remains unknown whether these defects are present in patients who exhibit nonhematologic DADA2 phenotypes.

To unravel the complexities of the BM alterations associated with DADA2, we conducted an in-depth investigation of the hematopoietic stem and progenitor cells (HSPCs) in a cohort of 18 patients, including patients without hematologic complications and those with severe hematologic conditions. We identified significant BM abnormalities in cases considered nonsevere by comparing the BM profiles of patients with solely rheumatologic and immunologic symptoms against the profiles of patients presenting with severe hematologic conditions. These BM defects extend beyond hematopoietic cells, affecting the mesenchymal stromal cell (MSC) compartment within the BM microenvironment.

METHODS

Study approval

Patients with DADA2 were recruited from IRCCS San Raffaele Hospital and IRCCS Ospedale Giannina Gaslini. Diagnosis was based on pathologic or null ADA2 activity[21] and mutation analysis (Table I). Before sample collection, informed consent was obtained from patients and/or their parents. The study protocol RF-2019-12370600 entitled "Expanding the spectrum of adenosine deaminase 2 (ADA2) deficiency: towards a gene therapy approach" (principal investigator: M.G.) received approval from the ethics review boards of the participating institutes, ensuring compliance with the ethical principles outlined in the Declaration of Helsinki. In addition, patients enrolled at the IRCCS San Raffaele Hospital consented to the TIGET09 observational protocol for biological banking approved by the local ethical committee. In patients with DADA2, BM and peripheral blood (PB) samples were collected as part of their diagnostic workup or follow-up. In the case of healthy controls, PB and BM samples were collected after obtaining informed consent according to the TIGET09 research protocol. Healthy donor (HD) BM was obtained from residual aspirates of individuals who had donated BM for allogeneic transplantation at the San Raffaele Hospital.

Flow cytometry of total BM and PB

Cell populations in PB and BM aspirates were identified using a whole-blood dissection cytometry assay previously described.[22] Briefly, after red blood cell lysis, samples were labeled with the fluorescent antibodies targeting specific cell markers reported in Table E1 in this article's Online Repository at www.jacionline.org. Lymphocyte populations underwent more detailed characterization through additional labeling (see Table E2 in the Online Repository at www.jacionline.org). All antibodies were purchased from BioLegend (San Diego, Calif) or BD Biosciences (Franklin Lakes, NJ). Propidium iodide (BioLegend) was used to assess cell viability, and FlowCount beads (BD Biosciences) were added to perform absolute cell quantification. All samples were acquired using BD FACSymphony A5 (BD Biosciences), calibrated with Rainbow beads (Sperotech, Lake Forest, Ill). Raw data were collected using DIVA software and subsequently analyzed with FlowJo software. The combinations of markers used to identify the different hematopoietic subpopulations are listed in Table E3 in the Online Repository at www.jacionline.org.

Isolation of $CD34^+$ cells and colony-forming unit assay

BM $CD34^+$ cells from healthy controls were purchased from Stem Cell Technologies (Vancouver, British Columbia, Canada). Mononuclear cells were isolated from the BM aspirate of patients with DADA2 by density gradient centrifugation using Lymphoprep (Stem Cell Technologies). Patients' $CD34^+$ cells were subsequently purified using a CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD34^+$ cell purity was assessed by flow cytometry. Purified $CD34^+$ cells were then resuspended in complete MethoCult H4434 (Stem Cell Technologies) and plated in triplicate in 35-mm dishes at a density of 1000 cells per dish. After 14 days, erythroid burst-forming units (BFUs), granulocyte-macrophage colony-forming units (CFUs), and granulocyte, erythrocyte, monocyte-macrophage, megakaryocyte CFUs were quantified by light microscopy.

RESULTS

Clinical characteristics of patients with DADA2 and rationale for subgroup analysis

We present a comprehensive cohort of 18 patients with DADA2 from 15 unrelated families. Detailed information on the clinical phenotype, ADA2 genotype, ADA2 enzymatic activity, and ongoing treatments is presented in Table I. The median age at disease onset was 10 years (minimum 2 years; maximum 28 years),

TABLE I. Genotype, ADA2 enzymatic activity, and clinical characteristics of DADA2 patients

Patient	Sex	Age at BM sampling (y)	Age at disease onset (y)	Mutation	ADA2 activity	Clinical Phenotype			Therapy
						Hematologic	Immunologic	Rheumatologic/ inflammatory	
1*	Female	24	2	p.Leu188Pro/ IVS6_IVS7del	Null	Severe neutropenia, thrombocytopenia, T-LGL	Hypogammaglobulinemia, recurrent infections, viral reactivations	Stroke, livedo reticularis	Etanercept, prednisone, G-CSF, immunoglobulin
2*	Female	30	8	p.Leu188Pro/ IVS6_IVS7del	Null	Severe neutropenia, thrombocytopenia, T-LGL	Hypogammaglobulinemia, recurrent infections, viral reactivations, thyroiditis	Stroke, livedo reticularis	Etanercept, G-CSF, immunoglobulin
3*	Female	63	Infancy	p.Arg49Glyfs*4/ p.Trp362Ter	Null	Severe neutropenia, T-LGL	Hypogammaglobulinemia, recurrent infections	—	Etanercept, G-CSF, immunoglobulin
4*	Female	33	28	p.Tyr456Cys/ p.Tyr456Cys	Pathologic	Hodgkin lymphoma, severe neutropenia after CT	—	—	Methylprednisolone
5*	Female	46	18	p.Asn127Ile (het)/n.a.	Pathologic	Severe neutropenia, T-LGL	Hypogammaglobulinemia, necrotizing fasciitis of the leg	Intestinal ischemia, recurrent fevers	Etanercept (after BM), G-CSF, immunoglobulin
6	Female	6	5	p.Arg169Gln/ p.Arg169Gln	Null	—	Recurrent respiratory tract infections, psoriasis, hypo-IgM	Persistent fever, persistent inflammatory indexes, livedo reticularis	Etanercept, immunoglobulin
7	Male	23	11	p.Arg49Glyfs*4/ c.973-2A>G	Null	Lymphopenia, splenomegaly	Mild hypogammag lobulinemia, hypo-IgM	Persistent fever	Etanercept
8	Female	21	Infancy	p.Arg49Glyfs*4/ c.973-2A>G	Pathologic	Lymphopenia	Recurrent respiratory tract infections	Arthralgias	Etanercept
9	Female	10	10	p.Thr360Ala/ p.Thr360Ala	Pathologic	Hypochromic microcytic anemia (poor responder to iron supplementation)	—	Erythema nodosum, cutaneous vasculitis, myalgia, arthralgia	Etanercept (after BM)
10	Male	16	7	p.Thr360Ala/ p.Thr360Ala	Null	—	—	Cerebral and systemic vasculitis, left sensorineural hearing loss with profound hearing loss on the right, recurrent fever, livedo reticularis	Etanercept, steroids
11	Male	16	9	p.Leu188Pro/ p.Gly383Asp	Pathologic	Lymphopenia, mild neutropenia, splenomegaly, lymphoproliferation	Hypogammaglobulinemia, recurrent respiratory tract infections, psoriasis	Aphthous lesions	Etanercept (after BM), immunoglobulin
12	Male	16	13	p.Leu188Pro/ p.Gly383Asp	Pathologic	—	Recurrent respiratory tract infections	—	Etanercept (after BM)
13	Female	14	14	p.Arg34Trp/ p.Pro251Pro	Pathologic	Lymphopenia, thrombocytopenia, hepatosplenomegaly	Hypo-IgM	Recurrent fevers, systemic inflammation, livedo reticularis	Anakinra, etanercept
14	Female	17	11	p.Leu183Pro/ p.Leu183Pro	Pathologic	—	—	Persistent fever with recurrent MAS, vaginal ulcers	Immunoglobulin, steroids, etanercept
15	Male	11	6	p.Gly47Ala/ p.Leu249Pro	Pathologic	—	—	Recurrent/persistent fever, cutaneous vasculitis, arthralgias	Etanercept
16	Female	21	3	p.Thr360Ala/ p.Arg49Glyfs	Pathologic	—	—	Recurrent/persistent fever, livedo reticularis, arthralgia/ arthritis	Etanercept
17	Male	19	16	p.Tyr453Cys/ p.Tyr453Cys	Null	Lymphadenopathy, splenomegaly	Mild hypogammag lobulinemia	Recurrent fever	Etanercept
18	Female	18	6	p.Tyr453Cys/ p.Tyr453Cys	Null	—	—	Recurrent fever, livedo reticularis, persistent inflammatory indexes, arthralgia/ arthritis, myalgia	Etanercept

CT, Chemotherapy; *MAS*, macrophage activation syndrome; *n.a.*, not available.
*Severe patients.

with a median age at analysis of 19.08 years (interquartile range 9.41 years). At the time of analysis, 10 patients (56%) were adults (>18 years old). The observed disease phenotype demonstrated considerable heterogeneity within the cohort. Of patients, 83% presented with rheumatologic/inflammatory manifestations, 61% exhibited immunodeficiency, and 61% displayed hematologic involvement. The most prevalent clinical manifestations included persistent/recurrent fever (50%), livedo (39%), and recurrent infections (39%). The most common laboratory findings were hypogammaglobulinemia (39%) and neutropenia (33%). At the time of analysis, most patients (94%) were receiving anti-TNF treatment, specifically etanercept. Five patients (patients 1-5) presented with severe hematologic symptoms, including cytopenia that either was refractory to treatment or deteriorated over time, sometimes associated with immunodeficiency (Fig E1 in the Online Repository at www.jacionline.org; Table E4 in the Online Repository at www.jacionline.org). Patient 4 experienced prolonged neutropenia following Hodgkin lymphoma treatment, necessitating HSCT. The remaining 4 patients with severe hematologic disease exhibited signs of lymphoproliferation characterized by a noticeable T-cell large granular lymphocyte (T-LGL) infiltrate in the BM. Given the severity of cytopenia and unresponsiveness to treatment, patients 1 and 3 underwent HSCT, while patients 2 and 5 are candidates for HSCT.

The variable degree of disease severity led us to categorize patients into 2 groups based on hematologic criteria: severe, including patients with hematologic indications of HSCT (patients 1-5), and nonsevere, including patients without overt hematologic abnormalities (patients 6-18) except for mild lymphopenia (patients 7, 8, 11, and 12) (Table I; Table E4 in the Online Repository at www.jacionline.org). The decision to analyze these subgroups separately stems from the recognition of their distinct clinical trajectories and treatment responses, emphasizing the importance of conducting a comprehensive examination of their BM composition.

HSPCs in the bone marrow of patients with DADA2 exhibit severe impairment, independent of disease severity

To understand the hematologic impacts of DADA2 and identify potential correlations between specific hematologic manifestations and BM characteristics across patients with severe and nonsevere DADA2 and HD controls, we conducted a detailed cytometric examination of BM cells. A clear reduction in total $CD45^+$ cells was observed in the BM of patients with nonsevere and severe DADA2 compared with HDs, regardless of age (Fig 1, *A*). In contrast, no evident decrease in total $CD45^+$ cells was observed in the PB of any of the patients with severe or nonsevere DADA2, though a tendency for reduced numbers was observed in pediatric patients with nonsevere DADA2 (Fig E2, *A*, in the Online Repository at www.jacionline.org). In line with the decrease in $CD45^+$ cells, $CD34^+$ cells were significantly reduced in the BM of pediatric patients with nonsevere DADA2 and adult patients with severe disease, with the most substantial reduction observed in the latter (Fig 1, *B*). Despite there being no reduction in the proportion of $CD34^+$ cells within the $CD45^+$ population among patients with DADA2, it was noted that the proportion of $CD34^+$ cells was higher in adult patients with nonsevere disease compared with all the other patients and HDs (Fig 1, *C*). In-depth immunophenotyping of HSPC populations in the BM revealed a reduction in the total numbers of both primitive and lineage-committed HSPCs in all patients with DADA2 compared with HDs (Fig 1, *D*). Early T-cell progenitor cells did not show any reduction among all patients. These cells were statistically significantly enriched in the $CD34^+$ cell fraction of pediatric patients with nonsevere DADA2, while an enrichment was also noted in adult patients with both severe and nonsevere DADA2 (Fig 1, *D* and *E*). In PB analysis, no noteworthy differences were found in the absolute numbers or percentages of HSPCs between HDs and patients with DADA2 (Fig E2, *B* and *C*).

Considering the observed depletion of HSPCs in the BM of patients with DADA2, we evaluated their clonogenic and *in vitro* differentiation potential. In patients with both severe and nonsevere DADA2, $CD34^+$ cells displayed a reduced capacity to form CFU granulocyte and macrophage. CFU granulocyte, erythrocyte, monocyte-macrophage, and megakaryocyte were statistically reduced in patients with nonsevere DADA2, while a decrease was also noted in patients with severe disease (Fig 2, *A*). No significant decrease was observed in BFU erythroid formation among all patients with severe and nonsevere DADA2 (Fig 2, *A*). Furthermore, the *in vitro* multilineage differentiation potential analysis revealed a significantly reduced capacity of $CD34^+$ cells from patients with nonsevere DADA2 to differentiate into myeloid, lymphoid, and megakaryocyte/platelet lineages compared with HDs (Fig 2, *B*). While $CD34^+$ cells from patients with severe DADA2 displayed a trend toward reduced differentiation potential into myeloid, lymphoid, and megakaryocyte/platelet lineages, statistical significance was not reached due to the variation and limited sample size. Consistent with the CFU data, no significant reduction in the erythroid differentiation potential of $CD34^+$ cells was observed in any patients with DADA2. However, it should be noted that none of the patients in our cohort were dependent on transfusions. The outcome of these assays could be different in transfusion-dependent patients with severe anemia.

To complement our *in vitro* observations, *in vivo* engraftment experiments with $CD34^+$ cells from 3 patients with nonsevere DADA2 and 3 HDs in NSGW41 mice suggested a tendency toward reduced engraftment capability of DADA2 $CD34^+$ cells at 20 weeks post-transplantation, as indicated by a lower percentage of donor $CD45^+$ cells in the BM, PB, and spleen of NSGW41 mice transplanted with DADA2 $CD34^+$ cells (Fig 2, *C*).

Our findings collectively reveal a profound impact on BM HSPC populations in patients with DADA2, characterized by diminished number and reduced clonogenic, multilineage differentiation, and engraftment capabilities. These alterations are prevalent across all levels of disease severity, highlighting a uniform disruption of hematopoiesis among patients with DADA2, including patients with milder symptoms. These disruptions are present from childhood, indicating an early onset and emphasizing the critical need for prompt recognition and surveillance of affected individuals through periodic hemograms and BM evaluation, if indicated. The goal is to optimize the timing of monitoring and recognize the evolution of the underlying hematologic condition, allowing physicians to anticipate the timing for appropriate definitive therapy when needed.

Mature cell populations are reduced in BM, but not PB, of patients with DADA2

Concurrently with immunophenotyping of the HSPCs, we thoroughly analyzed erythroblasts and mature myeloid, and

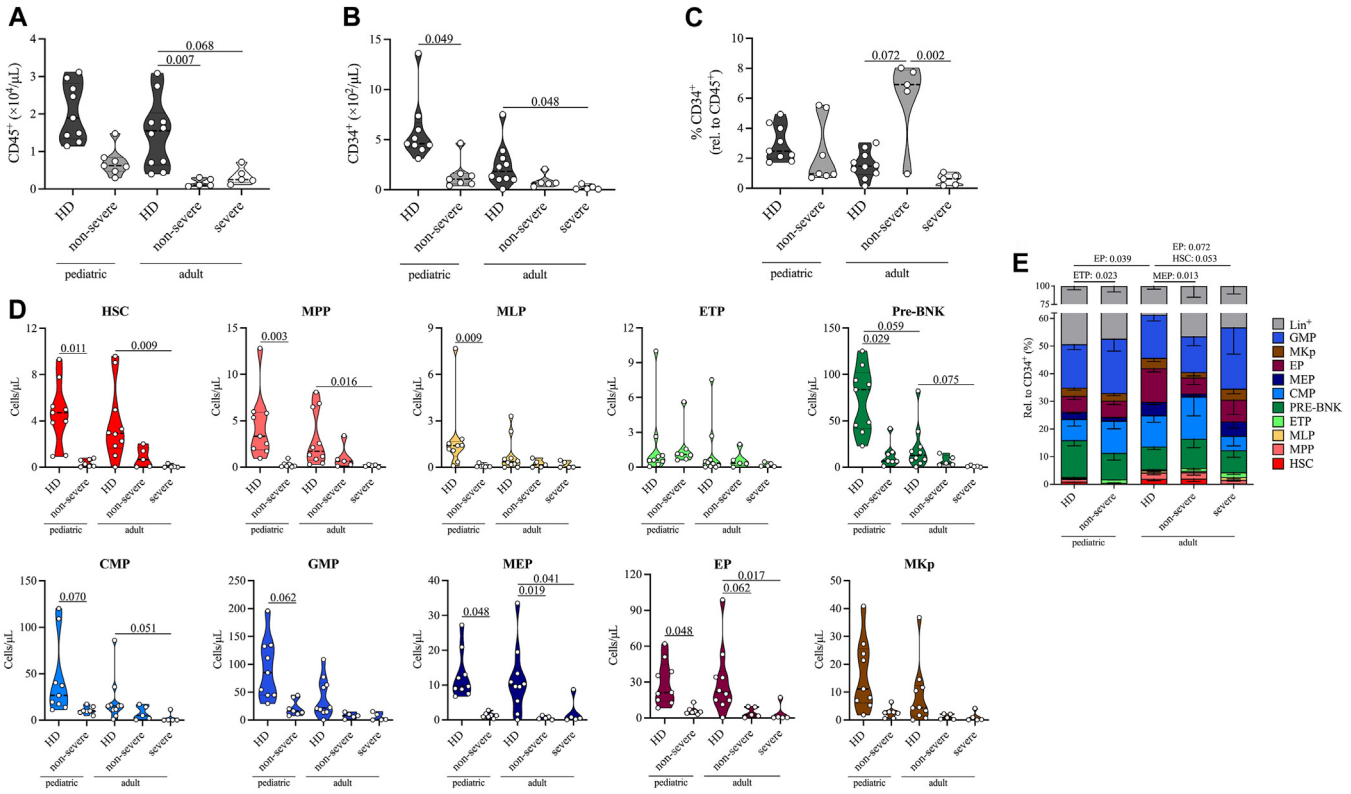

FIG 1. Characterization of CD34$^+$ cells in BM of patients with DADA2. Violin plots depict **(A)** total CD45$^+$ cells per microliter, **(B)** total CD34$^+$ cells per microliter, and **(C)** proportion of CD34$^+$ cells within the CD45$^+$ population in BM of HDs (pediatric n = 9; adult n = 10) and patients with DADA2 with nonsevere (pediatric n = 7; adult n = 5) and severe (n = 5) hematologic conditions. **(D)** Violin plots display the absolute number of cells per microliter of various HSPC populations, including HSC, MPP, MLP, ETP, pre-BNK, CMP, GMP, MEP, EP, and MKp, in BM of HDs and patients with DADA2. **(E)** Stacked bar graph illustrates the composition of CD34$^+$ cells in BM for HDs and patients with DADA2, highlighting the frequency of different HSPC populations among the total CD34$^+$ cells for both groups. Data show mean ± SEM. Statistical significance was determined by nonparametric Kruskal-Wallis test with multiple comparisons. *CMP*, Common myeloid progenitor; *EP*, erythroid progenitor; *ETP*, early T-cell progenitor; *GMP*, granulocyte-monocyte progenitor; *HSC*, hematopoietic stem cell; *MEP*, megakaryocytic-erythroid progenitor; *MKp*, megakaryocyte progenitor; *MLP*, multilymphoid progenitor; *MPP*, multipotent progenitor; *Pre-BNK*, B- and NK-cell precursor.

lymphoid cell populations in BM and PB of patients with DADA2 and HDs. A comparable reduction in the number of erythroblasts was found in BM of patients with nonsevere and severe DADA2 (Fig 3, *A*), with no signs of anemia in the PB (Fig E1; Table E4), pointing to specific alterations within the BM.

In adult patients with severe or nonsevere DADA2, a significant decrease in PMN cell counts was observed in the BM, though their counts in the PB remained within the normal range (Fig 3, *B* and *C*). A similar tendency was observed in pediatric patients. Furthermore, monocytes were reduced in the BM of adult patients with nonsevere DADA2, but not in pediatric patients, with PB counts remaining unaffected. These observations underscore a notable difference in the BM and PB myeloid compartments of patients with nonsevere DADA2.

In the context of lymphoid cells, reductions in several developmental and mature B-cell stages were noted in the BM of patients with nonsevere and severe DADA2 (Fig 3, *D*). A more pronounced decrease in pro-B and pre-B cells was particularly evident among patients with severe DADA2, emphasizing how disease severity distinctly affects these specific lymphoid cell populations (Fig 3, *D*). Conversely, in the PB, no significant differences were found in B cells except for diminished pre-B cell counts in pediatric patients with nonsevere DADA2 (Fig 3, *E*).

Regarding T-cell populations, total BM CD4$^+$ T-cell counts were normal in all patient groups except for adult patients with nonsevere DADA2, which exhibited a minor decline in the BM (Fig 4, *A* and *B*). The proportion of naive CD4$^+$ T cells in the BM was seemingly lower in all patients, with a significant reduction in patients with severe conditions (Fig 4, *A*). However, the PB showed no significant differences in the composition of the CD4$^+$ T cells (Fig 4, *B*). For the CD8$^+$ T-cell compartment, we noted a significant increase in total numbers in the BM of patients with severe DADA2, while the numbers were seemingly reduced in patients with nonsevere DADA2 (Fig 4, *C*). Furthermore, patients with severe disease displayed a reduction in the proportion of naive CD8$^+$ T cells in the BM, alongside an increase in the population of terminally differentiated effector memory re-expressing CD45RA (TEMRA) T cells in the BM and PB (Fig 4, *C* and *D*). In 4 out of 5 patients with severe DADA2, there was evidence of T-LGL infiltrates in the BM, indicative of a lymphoproliferative disorder. This observation suggests a link

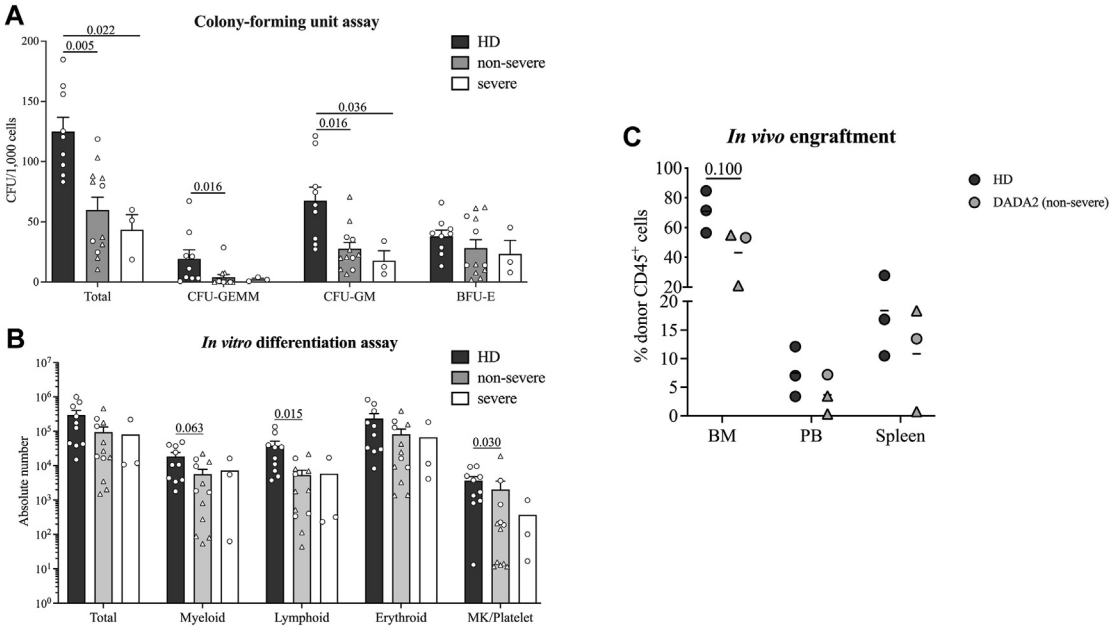

FIG 2. Functional assessment of DADA2 CD34$^+$ cells. **(A)** Bar graph shows the number of total, mixed (CFU-GEMM), myeloid (CFU-GM), and erythroid (BFU-E) colonies derived from 1000 BM CD34$^+$ cells from HDs (n = 9) and patients with DADA2 with nonsevere (n = 11) and severe (n = 3) hematologic conditions. **(B)** Absolute counts of differentiated total, myeloid, lymphoid, erythroid, and MK/platelet cells from 500 BM CD34$^+$ cells from HDs (n = 10) and patients with DADA2 with nonsevere (n = 12) and severe (n = 3) hematologic involvement. **(C)** Engraftment of BM CD34$^+$ cells from HDs (n = 3) and patients with DADA2 (n = 3) in NSGW41 mice, measured 20 weeks post-transplantation, showing the percentage of human donor CD45$^+$ cells relative to total live cells in the BM, PB, and spleen. Adult and pediatric individuals are represented by *circles* and *triangles*, respectively. Data represent mean ± SEM analyzed by nonparametric Kruskal-Wallis test with multiple comparisons. *BFU-E*, BFU erythroid; *CFU-GEMM*, CFU granulocyte, erythrocyte, monocyte-macrophage, megakaryocyte; *CFU-GM*, CFU granulocyte and macrophage; *MK*, megakaryocyte.

between the surge in CD8$^+$ TEMRA T cells and the presence of T-LGL infiltrates in patients with severe DADA2, a correlation that was not found in patients with nonsevere disease. Collectively, these observations highlight the complex immunophenotypic shifts in DADA2, which may be related to the severity of the disease and its associated complications.

DADA2 BM MSCs show impaired clonogenicity and reduced proliferation as a consequence of their premature senescent phenotype

The reduction in both HSPCs and mature cell populations in the BM of patients with DADA2 prompted us to examine their BM niche closely. Recognizing the integral role of MSCs in maintaining the hematopoietic environment, we investigated the functional properties of DADA2 MSCs. In HDs, distinctive clones of fibroblast-like cells (CFU fibroblast) emerged in *ex vivo*–derived HD MSC cultures by day 7, with a substantial increase in clone numbers by day 14 (Fig 5, *A*). Conversely, DADA2 MSCs exhibited a delayed and reduced clonogenic potential, as indicated by a marked reduction in CFU fibroblast numbers. Despite displaying a similar fibroblast-like morphology in culture, DADA2 MSCs appeared flatter and more enlarged than HD MSCs, similar to senescent cells (Fig 5, *B*). Additionally, the proliferative capacity was significantly diminished in DADA2 MSCs, as evidenced by the MTT assay (Fig 5, *C*).

We hypothesized the presence of a senescent phenotype, even at early culture passages. We examined the expression of senescence-associated genes in both HD and DADA2 MSCs. DADA2 MSCs exhibited a notable increase in mRNA levels of cell cycle inhibitors p15 and p16, as well as inflammatory cytokine IL-6 compared with HD MSCs (Fig 5, *D*), suggesting the induction of a senescence-like state in DADA2 MSCs. This finding was further supported by a significant increase in the proportion of DADA2 MSCs exhibiting cytosolic accumulation of SA-β-Gal signal (Fig 5, *E*). Furthermore, confocal microscopy revealed a substantial proportion of DADA2 MSCs with individual or colocalizing 53BP1 and γH2AX nuclear foci (Fig 5, *F*).

Overall, these findings highlight a significant disruption of the MSC compartment within the BM microenvironment of patients with DADA2, characterized by the presence of a senescent phenotype in DADA2 MSCs, which consequently hampers their clonogenic, proliferative, and differentiation potential, possibly impairing the hematopoietic supportive function within the BM niche.

DISCUSSION

Our study revealed profound disruptions in function of the BM in patients with DADA2, evident across various stages of disease activity, clinical complexity, and patient age. Notably, our cohort of patients represents the largest cohort to date in which such a comprehensive analysis of the BM was conducted, highlighting the significance of our findings in elucidating the impact of this

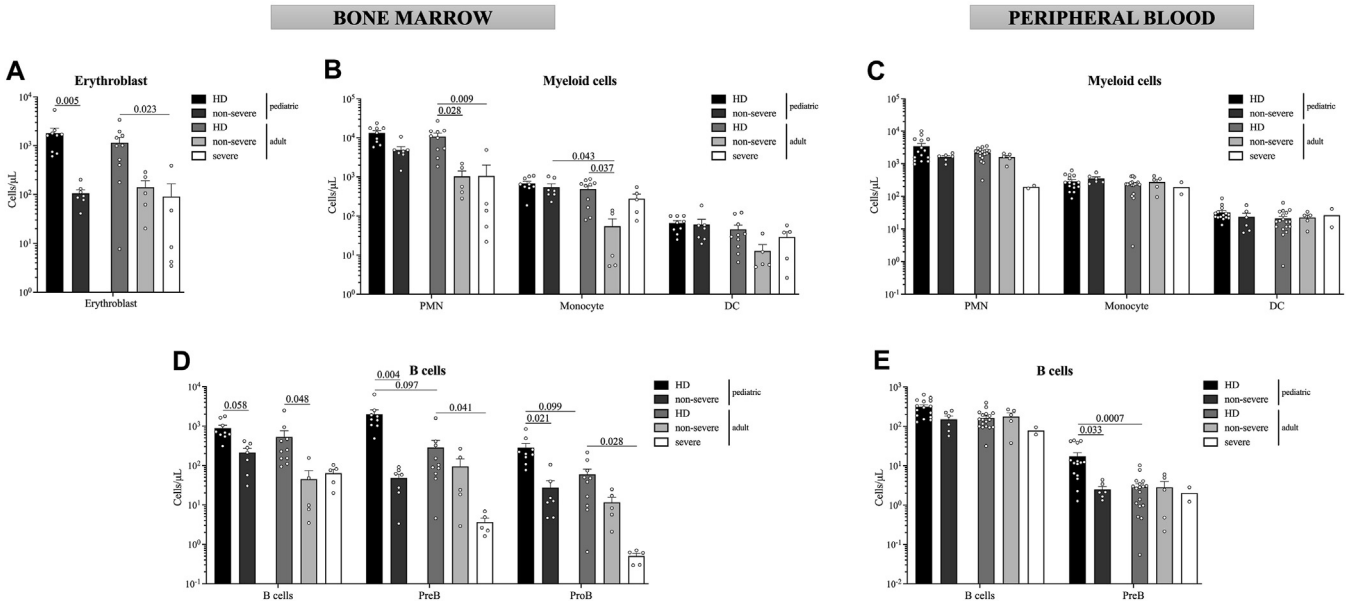

FIG 3. Comparative analysis of mature cell populations in the BM and PB of patients with DADA2. Bar graphs show the absolute number of erythroblast **(A)**, myeloid cells **(B** and **C)**, and B cells **(D** and **E)** per microliter in the BM *(A, B,* and *D)* and PB *(C* and *E)* of HDs (pediatric n = 9 and n = 15 in BM and PB, respectively; adult n = 10 and n = 18 in BM and PB, respectively) and patients with DADA2 with nonsevere (pediatric n = 7 and n = 6 in BM and PB, respectively; adult n = 5 and n = 5 in BM and PB, respectively) and severe (n = 5 and n = 2 for BM and PB, respectively) hematologic involvement. Data show mean ± SEM with the nonparametric Kruskal-Wallis test with multiple comparisons used to determine statistical significance. *DC*, Dendritic cells.

rare disease on the BM. Among the 18 patients, 5 displayed severe hematologic symptoms, with some undergoing HSCT or being assessed for it. These patients showed compromised hematopoiesis, evidenced by the reduction in 1 or more lineages and depletion of both primitive and lineage-committed HSPCs, consistent with prior findings of BM failure in DADA2.[10,16-19,23] This condition leads to progressive cytopenia, significantly affecting multiple hematopoietic lineages.

A compelling aspect of our findings is the marked reduction in BM cellularity in patients presenting with normal blood cell counts and no apparent hematologic disease. Closer examination of HSPC populations revealed a substantial depletion of HSCs, primitive multipotent progenitors, and lineage-committed HSPCs affecting both children and adults with nonsevere DADA2, similar to patients with severe cytopenias. In these patients, we also noted a compromised ability for clonogenic expansion and differentiation into myeloid, lymphoid, and erythroid lineages, indicating a fundamental disturbance in hematopoiesis independently of disease severity. We consistently found decreased levels of various hematopoietic cell populations, including PMN and erythroblast cells, in both nonsevere and severe patient groups. Notably, the decrease in PMN levels in the BM of patients with nonsevere disease was not associated with peripheral neutropenia. This discrepancy might be explained by potential compensatory mechanisms such as extended PMN life span, increased BM release, and mobilization from marginal pools, temporarily preserving normal PMN levels in the circulation.[24]

Additionally, we observed a reduction in BM erythroblasts in all patients, even without severe anemia. This finding is critical as it serves as a diagnostic marker for pure red cell aplasia,[25] identified in some patients with DADA2.[17,23] In some instances, the body may prevent anemia by having the remaining blood cell precursors enhance their proliferation and differentiation, speeding up production to compensate for any decrease and keep red blood cell levels stable.[26,27] This hypothesis is supported by the normal formation of BFU erythroid colonies from patients' HSPCs and the preservation of erythroid differentiation capabilities. These findings indicate a profound hematopoietic disturbance in patients with DADA2, starting far earlier than clinical symptoms manifest. The recurrence of this feature emphasizes the clinical need for detailed and regular monitoring of hematologic parameters in patients with DADA2, independent of their hematologic phenotype or initial symptoms.

Our study further reveals a reduction in pre-BNK progenitors across patients with DADA2, with the most substantial decrease noted in patients with severe DADA2. This consistent decrease in pre-BNK cells correlates with a noticeable decline of several developmental B-cell populations (pre-B, pro-B, and mature B cells) in the BM of patients with DADA2, even though these cells seem normal in the PB. These findings support the results of Yap et al,[28] which identified maturation defects in peripheral B cells in patients with DADA2, likely related to a developmental block at the pre-B cell stage in the BM. Our results further extend this observation as the lack of pre-BNK progenitors hampers B-cell development in DADA2, leading to hypogammaglobulinemia in all severely affected patients. Conversely, most patients with less severe symptoms could maintain adequate immunoglobulin production, suggesting effective maintenance and renewal of plasma cells.

Compared with other types of hematopoietic cells, T cells in patients with DADA2 generally do not exhibit significant abnormalities, although some variability has been observed.[1,28,29]

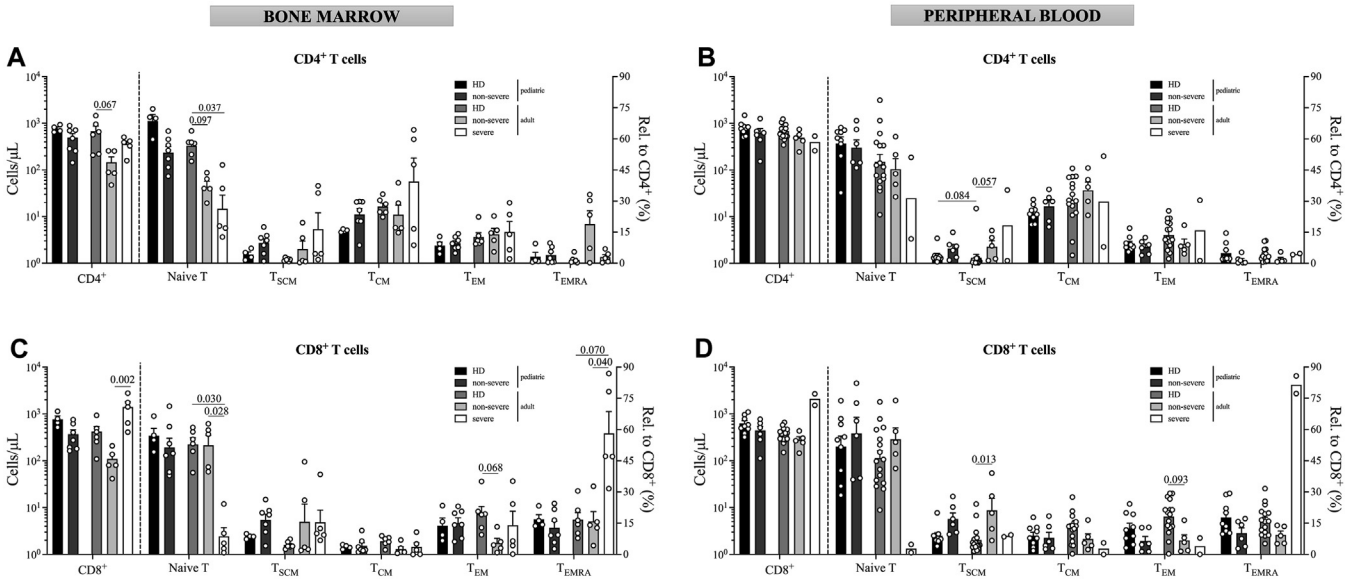

FIG 4. Characterization of T cells in the BM and PB of patients with DADA2. Bar graphs show the absolute number cells of $CD4^+$ **(A** and **B)** and $CD8^+$ **(C** and **D)** T cells in the BM *(A* and *C)* and PB *(B* and *D)* of HDs (pediatric n = 4 and n = 9 in BM and PB, respectively; adult n = 6 and n = 16 in BM and PB, respectively) and patients with DADA2 classified by the severity of their hematologic condition (nonsevere: pediatric n = 7 and n = 6 in BM and PB, respectively; adult n = 5 and n = 5 in BM and PB, respectively, and severe: n = 5 and n = 2 for BM and PB, respectively). The frequency of various $CD4^+$ and $CD8^+$ T-cell populations was also reported: T_{SCM}, T_{CM}, T_{EM}, T_{EMRA}. Data show mean ± SEM, with statistical significance evaluated by nonparametric Kruskal-Wallis test with multiple comparisons. T_{CM}, Central memory T cell; T_{EM}, effector memory T cell; T_{EMRA}, effector memory re-expressing CD45RA T cell; T_{SCM}, stem cell memory T cell.

Our study found minor differences in $CD4^+$ and $CD8^+$ T-cell populations in the BM between HDs and patients with DADA2. However, T cells in the PB were normal. This observation corroborates the finding that early T-cell precursors in the BM of patients with DADA2 are not impacted in DADA2, regardless of symptom severity. These precursors migrate to the thymus and mature into normal T-cell numbers, indicating that their thymic development is possible. Despite no clear abnormalities in circulating T cells, detailed immunophenotyping revealed a significant reduction in naive $CD8^+$ T cells and a relative increase in $CD8^+$ TEMRA cells in both the BM and the PB of patients with DADA2 with severe hematologic symptoms, but not in others. Such terminal differentiation of cytotoxic T cells is associated with lymphoproliferative disease in patients with DADA2.[8,9,14,19,28,30,31] Indeed, 4 out of 5 patients with severe hematologic involvement displayed lymphocytic clusters in BM samples, identified as T-LGL infiltrates by morphologic and cytofluorimetric analysis. While T-LGL leukemia is linked to cytopenias and BMF syndromes,[32,33] its exact role of T-LGL infiltrate in DADA2 is yet to be clarified, potentially contributing to or resulting from the chronic inflammatory state. In cases of T-LGL leukemia associated with DADA2, standard treatment approaches may need adjustments. Typically, T-LGL leukemia is managed through careful observation unless it leads to cytopenia, which requires immunosuppressive therapy such as methotrexate.[34] However, these treatments may increase the infection risk in patients with preexisting immunodeficiency, or they might be ineffective if the BM is compromised independently of T-LGL leukemia. Consequently, genetic diagnosis and disease monitoring may prompt clinicians to consider HSCT as a definitive treatment for cytopenia in patients with T-LGL leukemia.

The hematopoietic dysfunction observed in DADA2 is linked to alterations within the BM niche, specifically affecting MSCs. Our study showed a significant decrease in both the clonogenic and the proliferation capabilities of DADA2 MSCs, a finding that aligns with similar MSC functional impairments seen in other BMF diseases, such as aplastic anemia, Fanconi anemia, and β-thalassemia.[35-39] This notable parallel suggests underlying commonalities in the mechanisms of MSC dysfunction across diverse BMF disorders. Furthermore, DADA2 MSCs demonstrated an early-senescence phenotype, marked by elevated DNA damage, mirroring observations in MSCs from various BMF syndromes and underscoring the recurring theme of MSC senescence within BM disorders.[36,39-41] The impact of the premature senescence on the MSCs in DADA2 demands further investigation.

Delving into the mechanisms behind hematopoietic impairment in DADA2, our recent publication highlighted the critical role of cecr1b, the zebrafish ortholog of ADA2, in regulating both hematopoiesis and inflammation. Disruption of cecr1b led to impaired hematopoiesis and increased inflammation, mirroring what is observed in patients with DADA2.[42] Additionally, studies in patients have shown that BM plasma can hinder the colony-forming ability of HD HSPCs,[14,16] suggesting an extrinsic inhibitory effect potentially linked to inflammatory markers. Chronic inflammation, characterized by elevated levels of TNF-α and type I and II interferons, is a hallmark of DADA2 pathophysiology.[43] Prolonged exposure to these cytokines may lead to stem cell depletion, selective pressures, and BMF.[44-47] For example, TNF-α has been shown to induce apoptosis in myeloid progenitor cells and force quiescent HSCs into an active state, reducing their repopulation capacity.[48,49] In our zebrafish model, administration of human ADA2 or blocking TNF-α increased

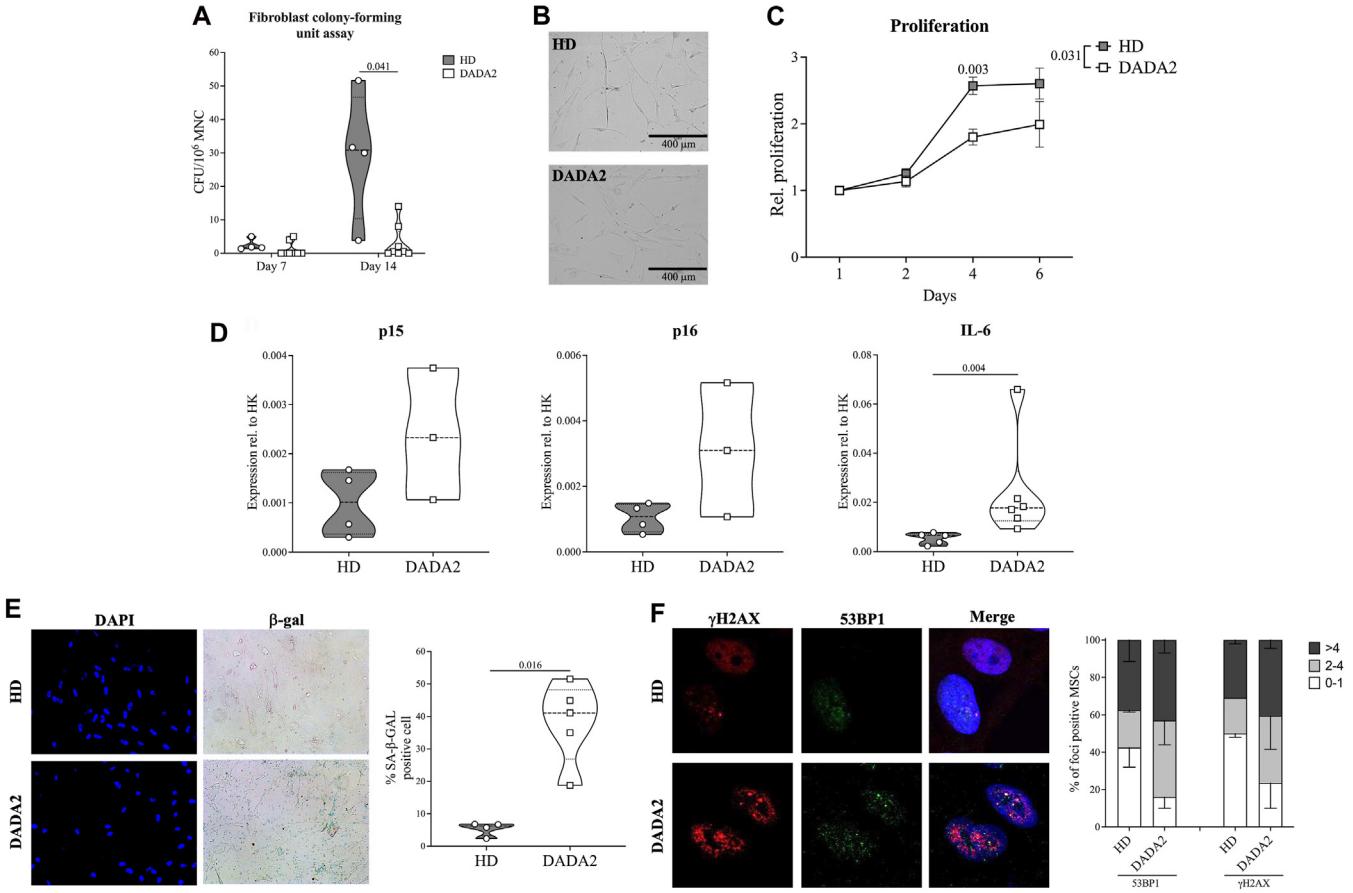

FIG 5. Functional characterization of HD and DADA2 MSCs. **(A)** Violin plot shows fibroblast CFUs in cultures from HDs (n = 4) and patients with DADA2 (n = 8), counted on day 7 and 14 post-seeding, expressed per million MNC plated. **(B)** Representative brightfield microscopic images (40× magnification) of HD and DADA2 MSCs at early passages in culture. **(C)** Time-lapse graph shows the proliferative capacity, measured by fluorometric MTT assay from early passage (P2-P5), comparing HD (n = 5) and DADA2 MSCs (n = 7). Results are mean ± SD of OD relative to day 1 post-seeding. **(D)** Violin plots show the relative expression levels of senescence markers p15 *(left)*, p16 *(middle)*, and IL-6 *(right)* in MSCs from HDs and patients with DADA2. Gene expression data are represented as $2^{-\Delta CT}$ relative to *GAPDH* housekeeping gene. **(E)** The *left panel* shows representative images, while the *right panel* provides quantification of SA-β-Gal staining in early-passage MSCs from HDs and patients with DADA2 using DAPI for nuclear staining. **(F)** Confocal microscopy images *(left panel)* and analysis *(right panel)* depict γH2AX and 53BP1 foci in early passage MSCs from HDs and patients with DADA2 with nuclei highlighted by DAPI. Statistical significance was determined by nonparametric Kruskal-Wallis test with multiple comparisons *(A)* and Mann-Whitney test *(D* and *E)* or repeated measure 2-way ANOVA *(C)*. *HK*, Houskeeping; *MNC*, mononuclear cells.

HSPC counts,[42] suggesting a potential role for inflammatory pathways, including TNF-α, in driving BMF in DADA2. However, the clinical evidence has not consistently supported the efficacy of TNF inhibition in ameliorating BMF in patients with DADA2. Many patients with severe BM involvement, including the patients in our current study, continue to exhibit significant BM abnormalities despite receiving TNF inhibitors. This raises questions about the extent of the contribution of TNF-α–driven inflammation to BMF in DADA2, suggesting that inflammation may not be the sole or primary driver of the disease pathology. Indeed, our data suggest a possible involvement of immune-mediated mechanisms in the progression of DADA2 BMF. Specifically, the marked increase in terminally differentiated cytotoxic T cells in the BM of patients with severe DADA2 hints at their involvement in HSPC destruction, a process previously observed in acquired aplastic anemia.[50] Further research is needed to confirm a similar involvement of cytotoxic T cells in DADA2 BMF.

In conclusion, while our understanding of hematopoietic dysregulation in DADA2 has advanced, many questions remain about how ADA2 influences hematopoiesis. Future research is critical to clarify both the inherent defects in DADA2 HSPCs and the external factors within the BM niche contributing to this dysregulated hematopoiesis, paving the way for more targeted and effective treatments.

DISCLOSURE STATEMENT

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Clinical implications: Patients with DADA2 present with bone marrow alterations long before clinical manifestations. Hematologic monitoring allows for the identification of disease progression and anticipation of appropriate definitive therapy, leading to better outcomes.

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METHODS

In vitro multilineage differentiation assay for CD34$^+$ cells

Non-tissue culture–treated 96-well flat-bottom plates were coated with StemSpan Differentiation Coating Material (Stem Cell Technologies) 2 hours before seeding. Subsequently, BM CD34$^+$ cells from patients or HDs were seeded in SFEM II medium (Stem Cell Technologies) supplemented with hSCF (100 ng/mL), hFLT3 (10 ng/mL), hIL-7 (100 ng/mL), hIL-2 (10 ng/mL) (Novartis, Basel, Switzerland), hTPO (75 ng/mL), hIL-6 (40 ng/mL), hIL-3 (10 ng/mL), hIL-11 (50 ng/mL), hEPO (0.1 U/mL) (Peprotech, Cranbury, NJ), hIL-4 (10 ng/mL) (Miltenyi Biotec), and hLDL (4 μg/mL) (Stem Cell Technologies). Medium was refreshed every 3 to 4 days. After 3 weeks of culture, cells were labeled with the fluorescent antibodies reported in Table E5. All antibodies were purchased from either BioLegend or BD Biosciences. FlowCount beads (BD Biosciences) were added to perform absolute cell quantification. All samples were acquired using BD FACSymphony A5 (BD Bioscience), calibrated with Rainbow beads (Sperotech). Raw data were collected using DIVA software and subsequently analyzed with FlowJo software.

Isolation and culture of BM-derived MSCs

MSCs were isolated and cultured according to previously published protocol.[E1] Briefly, the CD34$^-$ cell fraction of mononuclear cells was plated at a density of 2×10^5 cells/cm^2 in noncoated T75 tissue culture flasks in a basal medium consisting of Dulbecco modified Eagle medium high glucose (Gibco, Life Technologies, Carlsbad, Calif), supplemented with 5% platelet lysate (Stem Cell Technologies), 1% penicillin/streptomycin (EuroClone, Pero, Italy), and 2 mM L-Glutamine (EuroClone). After 1 week, nonadherent cells were removed, and the culture medium was refreshed weekly. When MSCs reached a confluency of greater than 80%, they were harvested using Trypsin-EDTA (EuroClone) and expanded by seeding them at a density of 5×10^3 cells/cm^2.

Fibroblast CFU assay

Fibroblast CFU assay was performed by seeding BM CD34$^-$ mononuclear cells in a basal medium at a density of 1×10^5 cells/cm^2 in 6-well plates. The CFUs were stained with 1% crystal violet (Sigma-Aldrich, St Louis, Mo) and manually counted on day 7 and day 14 after the initial seeding.

MTT assay

Proliferative capacity of MSCs was quantified using the fluorometric MTT assay. MSCs at early passage (P2-P5) were seeded in basal medium at a density of 3000 cells/well in 96-well plates and incubated at 37°C and 5% CO_2. After 1, 2, 4, and 6 days of incubation, the culture medium was removed and replaced with basal medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL; Sigma-Aldrich). After 4 hours, the culture medium containing MTT was removed, and the cells were solubilized with dimethyl sulfoxide, after which the absorbance at 570 nm was immediately measured. The MSC proliferation rate was calculated relative to the signal obtained at day 1 after the initial seeding.

Assessment of senescence and DNA damage in MSCs

Induction of senescence in MSCs was evaluated by SA-β-Gal staining assay (Cell Signaling Technology, Danvers, Mass) and quantitative RT-PCR. For SA-β-Gal staining, MSCs at passages 2 to 5 were seeded on coverslips in basal MSC medium and incubated for 24 hours. Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then incubated overnight with SA-β-Gal staining solution. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), and images were acquired using a Nikon Eclipse inverted microscope. Cells displaying blue signal in the cytosol were considered senescent. Senescence was also evaluated by analyzing the expression of senescence-associated genes (*p15*, *p16* and *IL-6*) in MSCs by quantitative RT-PCR.

To assess DNA damage in senescent MSCs, the cells were grown and fixed on coverslips as described above. Following fixation, cells were permeabilized with 0.1% Triton in PBS for 10 minutes. Immunostaining was performed by incubating the cells for 1 hour at room temperature with either rabbit anti-53BP1 (Bethyl Laboratories, Montgomery, Tex) or Anti-phospho-Histone H2A.X (Ser 139) (Merck, Darmstadt, Germany) antibodies. The cells were then washed 3 times before being incubated for another hour at room temperature with secondary antibodies: donkey anti-mouse IgG, Alexa Fluor 647, or donkey anti-rabbit IgG, Alexa Fluor 568 (Invitrogen/Thermo Fisher Scientific, Waltham, Mass). The cell nuclei were counterstained using DAPI (Sigma-Aldrich). Confocal microscopy images were captured using a Leica TCS SP5 confocal microscope, and the quantification of DNA damage response foci was performed using the ImageJ 64 software (version 1.47).

Measurement of ADA2 enzymatic activity

The activity level of the ADA2 enzyme was quantified in dried plasma spots using a liquid chromatography-tandem mass spectrometry method, as previously described.[E2] An ADA2 activity level less than 0.10 mU/mL was identified as pathologic.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software. For statistical comparison between the different severe and nonsevere patient groups and HD groups, Kruskal-Wallis test was used. In cases where Kruskal-Wallis test indicated a significant difference between groups, a post hoc Dunn test was used for multiple pairwise comparisons. Unpaired, 2-tailed Mann-Whitney *U* test and repeated-measures ANOVA were used for statistical comparison of senescence marker expression and MSC proliferation, respectively. A P value $\leq .05$ was considered statistically significant.

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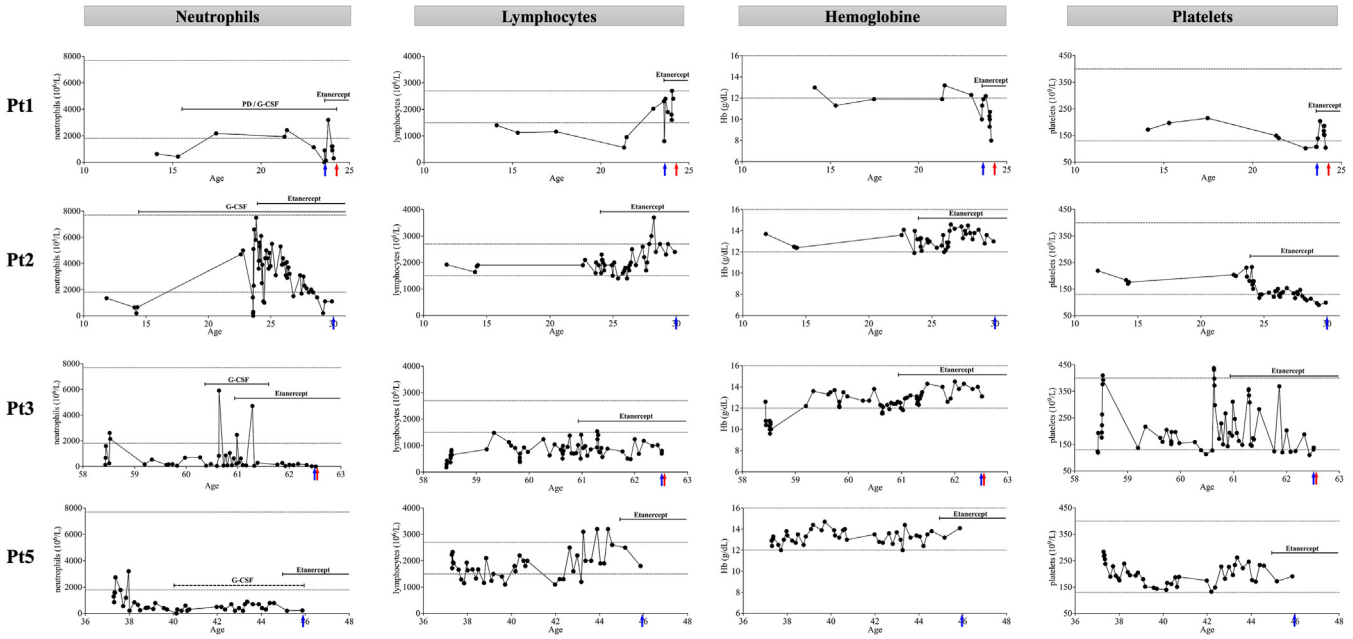

FIG E1. Hemogram data of patients with severe DADA2. Graphs show the longitudinal counts of neutrophils, lymphocytes, Hb, and platelets of 4 patients with severe DADA2 (patients1, 2, 3, and 5). The graphs highlight the treatment history, HSCT interventions *(red arrows)*, and the timing of BM aspirate collection for phenotype analysis *(blue arrows)*. *Dotted lines* represent the normal range. *Hb*, Hemoglobin.

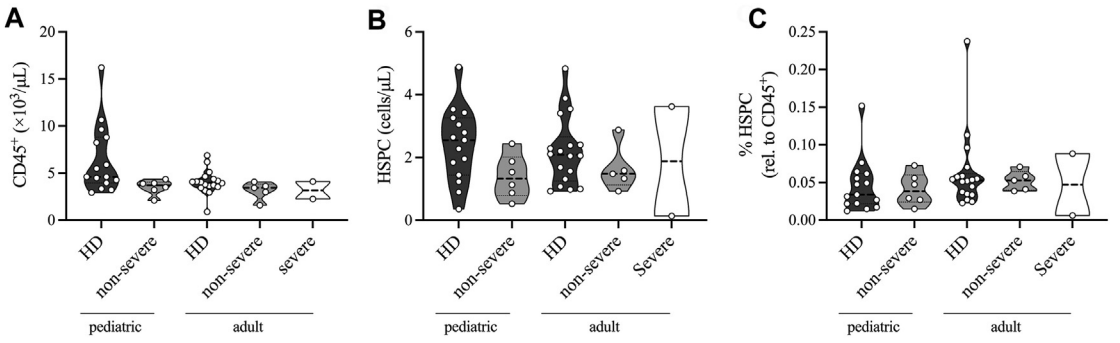

FIG E2. Analysis of $CD34^+$ cells in the PB of patients with DADA2. Violin plots show **(A)** total number of $CD45^+$ cells per microliter, **(B)** absolute number of HSPCs per microliter, and **(C)** proportion of $CD34^+$ cells within the $CD45^+$ cell population, comparing HDs (pediatric n = 15; adult n = 18) with patients with DADA2 having either nonsevere (pediatric n = 6; adult n = 5) or severe (n = 2) hematologic involvement. Statistical significance was calculated by nonparametric Kruskal-Wallis test with multiple comparisons.

TABLE E1. Fluorescent antibodies used in whole-blood dissection assay

Antigen	Clone	Fluorochrome	Vendor	Catalog number
CD3	OKT3	BV605	BioLegend	317322
CD56	5.1H11	PE-Cy5	BioLegend	362516
CD14	M5E2	BV510	BioLegend	301842
CD33	WM53	BB515	BD Biosciences	564588
CD61/41	A2A9/6	PE-Cy7	BioLegend	359812
CD66b	G10F5	BB515	BD Biosciences	564679
CD7	M-T701	BB700	BD Biosciences	566488
CXCR4	12G5	PE	BioLegend	306506
CD38	HB7	BUV737	BD Biosciences	612824
CD45	HI30	BUV395	BD Biosciences	563792
CD90	5E10	APC	BD Biosciences	559869
CD10	HI10a	BV786	BD Biosciences	564960
CD11c	B-ly6	BV650	BD Biosciences	563404
CD34	561	BV421	BioLegend	343610
CD45RA	HI100	APC-H7	BioLegend	304128
CD71	M-A712	BV711	BD Biosciences	563767
CD19	SJ25C1	APC-R700	BD Biosciences	659121

TABLE E2. Fluorescent antibodies used for lymphocyte characterization

Antigen	Clone	Fluorochrome	Vendor	Catalog number
CD3	OKT3	BV605	BioLegend	317322
CD56	5.1H11	PE-Cy5	BioLegend	362516
CD33	WM53	BB515	BD Biosciences	564588
CD66b	G10F5	BB515	BD Biosciences	564679
CD45	HI30	BUV395	BD Biosciences	563792
CD45RA	HI100	APC-H7	BioLegend	304128
CD19	SJ25C1	APC-R700	BD Biosciences	659121
CD4	SK3	BV711	BD Biosciences	563028
CD25	BC96	BV785	BioLegend	302638
CD127	A019D5	APC	BioLegend	351316
CD8	SK1	BUV737	BD Biosciences	612754
CD62L	DREG-56	BV510	BioLegend	304844
CD95	DX2	PE	BioLegend	305608
TCRgd	B1	BV421	BioLegend	331218

TABLE E3. List of markers used to identify different hematopoietic cell populations

Whole-blood dissection	
PMN	$CD45^+$ $CD33^+$ **$CD66b^+$ SSC^{high}**
Monocyte	$CD45^+$ $CD33^+$ **$CD14^+$**
DC	$CD45^+$ $CD33^+$ **$CD14^-$ $CD11c^+$**
B cell	$CD45^+$ $CD33^-$ $CD66b^-$ $CD3^-$ **$CD19^+$ $CD10^-$ $CD34^-$**
Pre-B	$CD45^+$ $CD33^-$ $CD66b^-$ $CD3^-$ **$CD19^+$ $CD10^+$ $CD34^-$**
Pro-B	$CD45^+$ $CD33^-$ $CD66b^-$ $CD3^-$ **$CD19^+$ $CD10^+$ $CD34^+$**
Erythroblast	**$CD45^-$ $CD71^+$**
HSC	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^-$ $CD90^+$ $CD45RA^-$**
MPP	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^-$ $CD90^-$ $CD45RA^-$**
MLP	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^-$ $CD90^-$ $CD45RA^+$**
ETP	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^+$ $CD7^+$**
Pre-BNK	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^+$ $CD7^-$ $CD10^+$ $CD45RA^+$**
CMP	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^+$ $CD7^-$ $CD10^-$ $CD45RA^-$ $CD71^-$ $CD41^-$**
MEP	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^+$ $CD7^-$ $CD10^-$ $CD45RA^-$ $CD71^+$ $CD41^+$**
EP	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^+$ $CD7^-$ $CD10^-$ $CD45RA^-$ $CD71^+$ $CD41^-$**
MKp	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^+$ $CD7^-$ $CD10^-$ $CD45RA^-$ $CD71^-$ $CD41^+$**
GMP	$CD45^+$ $CD14^-$ $CD11c^-$ $CD3^-$ $CD19^-$ $CD56^-$ $CD34^+$ **$CD38^+$ $CD7^-$ $CD10^-$ $CD5RA^-$**
T-cell subsets	
CD4	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ **$CD4^+$ $CD25^-$ $CD127^{mid/+}$**
Naive	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD4^+$ $CD25^-$ $CD127^{mid/+}$ **$CD45RA^+$ $CD62L^+$ $CD95^-$**
SCM	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD4^+$ $CD25^-$ $CD127^{mid/+}$ **$CD45RA^+$ $CD62L^+$ $CD95^+$**
CM	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD4^+$ $CD25^-$ $CD127^{mid/+}$ **$CD45RA^-$ $CD62L^+$ $CD95^+$**
EM	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD4^+$ $CD25^-$ $CD127^{mid/+}$ **$CD45RA^-$ $CD62L^-$ $CD95^+$**
TEMRA	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD4^+$ $CD25^-$ $CD127^{mid/+}$ **$CD45RA^+$ $CD62L^-$ $CD95^+$**
CD8	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ **$CD8^+$**
Naive	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD8^+$ **$CD45RA^+$ $CD62L^+$ $CD95^-$**
SCM	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD8^+$ **$CD45RA^+$ $CD62L^+$ $CD95^+$**
CM	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD8^+$ **$CD45RA^-$ $CD62L^+$ $CD95^+$**
EM	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD8^+$ **$CD45RA^-$ $CD62L^-$ $CD95^+$**
TEMRA	$CD45^+$ $CD66b^-$ $CD33^-$ $CD19^-$ $CD3^+$ $CD56^-$ $TCRgd^-$ $CD8^+$ **$CD45RA^+$ $CD62L^-$ $CD95^+$**

(Continued)

TABLE E3. *(Continued)*

Bold indicates population specific markers.

TABLE E4. Hemogram data of patients with DADA2 at time of BM analysis

Patient	Age at BM sampling	Neutrophils ($\times 10^6$/L)	Lymphocytes ($\times 10^6$/L)	Hemoglobin (g/dL)	Platelets ($\times 10^6$/L)
HD range (children)		1,500-8,000	1,500-7,000	11.5-14.5	130,000-400,000
HD range (adults)		1,800-7,700	1,000-4,800	12-16 (females) 14-18 (males)	130,000-400,000
Pt. 6	6	1,700	2,300	12.7	137,000
Pt. 7	23	4,530	1,030*	16.4	207,000
Pt. 8	21	3,600	1,000*	14.4	200,000
Pt. 9	10	5,000	1,930	12.2	384,000
Pt. 10	16	2,750	1,820	14.8	160,000
Pt. 11	14	1,970	770	15	153,000
Pt. 12	16	2,200	1,390*	16.2	191,000
Pt.13	14	4,500	1,690	12.5	186,000
Pt. 14	17	3,070	1,860	12.2	245,000
Pt. 15	11	1,940	2,600	12.9	240,000
Pt. 16	21	2,860	2,000	12.6	195,000
Pt. 17	19	3,260	2,540	15.3	166,000
Pt. 18	18	4,920	2,530	12.9	204,000

Pt., Patient.
*Below HD range.

TABLE E5. Fluorescent antibodies for analyzing cell populations in the *in vitro* differentiation assay

Antigen	Clone	Fluorochrome	Vendor	Catalog number
CD3	OKT3	BV605	BioLegend	317322
CD56	5.1H11	PE-Cy5	BioLegend	362516
CD235a	GA-R2/HIR2	PE	BD Biosciences	561051
CD33	WM53	BB515	BD Biosciences	564588
CD41	HIP8	PE-Cy7	BioLegend	303718
CD7	M-T701	BB700	BD Biosciences	566488
CD1a	HI149	APC	BD Biosciences	561755
CD5	UCHT2	BUV737	BD Biosciences	612842
CD42b	HIP1	BV786	BD Biosciences	740976
CD15	W6D3	APCfire750	BioLegend	323041
CD10	HI10a	BV510	BioLegend	312219
CD34	561	BV421	BioLegend	343610
CD71	M-A712	BV711	BD Biosciences	563767
CD19	SJ25C1	APC-R700	BD Biosciences	659121