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Deficit of circulating CD19^{+}CD24^{hi}CD38^{hi} regulatory B cells in severe aplastic anaemia

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Abstract

Immune aplastic anaemia (AA) is caused by cytotoxic T lymphocytes (CTLs) that destroy haematopoietic stem and progenitor cells. Enhanced type 1 T helper (Th1) responses and reduced regulatory T cells (Tregs) are involved in the immune pathophysiology. CD24^{hi}CD38^{hi} regulatory B cells (Bregs) suppress CTLs and Th1 responses, and induce Tregs via interleukin 10 (IL-10). We investigated circulating B-cell subpopulations, including CD24^{hi}CD38^{hi} Bregs, as well as total B cells, CD4^{+} T cells, CD8^{+} T cells and natural killer cells in 104 untreated patients with severe and very severe AA, aged ≥18 years. All patients were treated with standard immunosuppressive therapy (IST) plus eltrombopag. CD24^{hi}CD38^{hi} Bregs were markedly reduced in patients with AA compared to healthy individuals, especially in very severe AA, but residual Bregs remained functional, capable of producing IL-10; total B-cell counts and the other B-cell subpopulations were similar to those of healthy individuals. CD24^{hi}CD38^{hi} Bregs did not correlate with responses to IST, and they recovered to levels present in healthy individuals after therapy. Mature naïve B-cell counts were unexpectedly associated with IST response. Markedly reduced CD24^{hi}CD38^{hi} Bregs, especially in very severe AA, with recovery after IST suggest Breg deficits may contribute to the pathophysiology of immune AA.

Keywords: aplastic anaemia, flow cytometry, immunosuppressive therapy, lymphocyte subsets, regulatory B cells.

Introduction

Immune aplastic anaemia (AA), caused by T cells that destroy haematopoietic stem and progenitor cells (HSPCs), is successfully treated with immunosuppressive therapy (IST).[1] Cytotoxic T-lymphocytes (CTLs)[6–9] and type 1 T helper (Th1) cytokines,[10–14] such as interferon-γ, are implicated in pathophysiology of immune AA. Frequent human leucocyte antigen (HLA) class I allele loss in HSPCs of patients with AA are evidence of involvement of CTLs.[2–5]

In addition to CTL activation, IFN-γ directly inhibits HSPCs,[7,15–19] and blocks haematopoietic signals of thrombopoietin.[19] Eltrombopag, a thrombopoietin receptor agonist, can stimulate HSPCs in the presence of IFN-γ, which may in part contribute to its efficacy in AA.[19,20]

Regulatory T cells (Tregs) that suppress effector T cells are reduced in AA and their numbers increase in response to IST, suggesting a role of Tregs in the immune pathophysiology.[21,22] A Treg subpopulation correlates with response to IST.[23] Recent studies have revealed immune suppressive functions in regulatory B cells (Bregs). Multiple suppressive B-cell subpopulations with different target cells and functions are collectively referred to as Bregs. Bregs maintain tolerance and immune homeostasis via interleukin 10 (IL-10).[24] Human CD19^{+}CD24^{hi}CD38^{hi} B cells are a well-studied Breg population; they suppress CTLs and Th1 responses, and convert CD4^{+} T cells into Tregs via IL-10, programmed death receptor-ligand 1, CD80, CD86 and CD1d.[25–28] The quantity and function of CD24^{hi}CD38^{hi} Bregs are impaired in various immune disorders, such as systemic lupus erythematosus,[25] rheumatoid arthritis,[27] immune thrombocytopenia[29] and chronic graft-versus-host disease.[30]

B cells and their subpopulations are not well characterised in AA. Peripheral blood CD19^{+} B-cell frequencies are higher in AA compared to myelodysplastic syndrome.[31] IL-10 producing B cells in bone marrow are reduced in AA, suggesting that Breg defects might contribute to the

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pathophysiology.[32] In the present study, we evaluated peripheral blood B-cell subpopulations, including CD24^{hi}CD38^{hi} Bregs, as well as total B cells, CD4^{+} T cells, CD8^{+} T cells and natural killer (NK) cells, at diagnosis and 6 months after institution of IST plus eltrombopag, and further tested their correlations with clinical presentations and outcomes after therapy.

Patients and methods

Patients

A total of 125 previously untreated patients with AA, aged ≥18 years, were included in this retrospective study. All patients were treated with standard IST (horse anti-thymocyte globulin [ATG] and cyclosporine) plus eltrombopag in the National Institutes of Health (NIH) Clinical Center, Bethesda, between July 2012 and October 2018, under protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (clinicaltrials.gov, NCT01623167). Treatment regimens have been described:[20] all patients continued cyclosporine at least until 6 months after institution of IST, eltrombopag was discontinued at 3 months in 25 patients (20%) or continued until 6 months in the remaining patients.

Definitions

Severe AA (SAA) was diagnosed when at least two of the following three criteria were met: an absolute neutrophil count (ANC) of $<0{\cdot}5 \times 10^9$/l, an absolute reticulocyte count (ARC) of $<60 \times 10^9$/l and a platelet count of $<20 \times 10^9$/l. Very severe AA (VSAA) was defined as an ANC of $<0{\cdot}2 \times 10^9$/l in addition to the criteria for SAA. Haematological response to IST was evaluated at 6 months after the institution of ATG, consistent with our previous studies:[20,33] response was defined as blood counts not meeting criteria for SAA, and non-response as blood counts that continued to meet the SAA criteria.

Samples

Blood was sampled within the 4 weeks preceding IST and at 6 months after institution of ATG, after obtaining written informed consent in accordance with the Declaration of Helsinki. Fresh blood anti-coagulated with ethylene diamine tetra-acetic acid was used to quantify absolute numbers of B cells, CD4^{+} T cells, CD8^{+} T cells and NK cells (TBNK subsets). For B-cell subpopulation analysis, peripheral blood mononuclear cells (PBMCs) were separated from heparinised blood using Lymphocyte Separation Medium (MP Biomedicals, Santa Ana, CA, USA) and were cryopreserved in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Gaithersburg, MD, USA) with 20% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 10% dimethyl sulphoxide, according to the standard protocol until use.

Immunostaining of lymphocytes

Absolute numbers of B cells, CD4^{+} T cells, CD8^{+} T cells and NK cells were analysed with BD Multitest™ 6-color TBNK reagent (BD Biosciences, San Diego, CA, USA). The following cell surface markers were used to define B cells, CD45^{+}CD3^{-}CD19^{+}CD16^{-}CD56^{-}; CD4^{+} T cells, CD45^{+}CD3^{+}CD4^{+}CD8^{-}; CD8^{+} T cells, CD45^{+}CD3^{+}CD4^{-}CD8^{+}; and NK cells, CD45^{+}CD3^{-}CD19^{-}(CD16/CD56)$^{+}$ (Figure S1A).

B-cell subpopulations in cryopreserved PBMCs were studied as previously described with modifications.[29,34] Cell counts and viabilities of thawed PBMCs were assessed by automated cell counter with trypan blue, and 1×10^6 live PBMCs were stained for flow cytometry analysis. The CD3^{c}CD19^{+}CD33^{-}glycosylphosphatidylinositol (GPI)-anchor^{+} live B cells were classified based on their expressions of CD24 and CD38: CD24^{hi}CD38^{hi} Bregs, CD24^{low}CD38^{low} mature naïve B cells, CD24^{hi}CD38^{low} memory B cells and CD24^{low}CD38^{hi} plasma cells/plasmablasts (Fig 1A, Figure S1B). GPI-anchor deficient B cells were excluded to avoid underestimation of CD24^{hi}CD38^{hi} Bregs using fluorescent-labelled aerolysin variant (FLAER) because CD24 is a GPI-anchored protein. Absolute numbers of B-cell subpopulations were estimated using their frequencies and mononuclear cell counts in the clinical record. To assess IL-10 production from B cells, 1×10^6 PBMCs were cultured in the presence of 10 μg/ml of CpG (ODN 2006, Thermo Fisher Scientific, Grand Island, NY, USA) and 1 μg/ml of recombinant human CD40 ligand (CD40L; R&D, Minneapolis, MN, USA) in 200 μl of RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin and 1% glutamate for 24 h, as previously published,[34] and a cell activation cocktail, including phorbol-12-myristate 13-acetate, ionomycin and brefeldin A (BioLegend, San Diego, CA, USA), was added during the last 6 h of culture, but brefeldin A was not used for negative controls. Intracellular IL-10^{+} cell frequencies were quantified in CD3^{-}CD19^{+} live B cells and their subpopulations, CD24^{hi}CD38^{hi} Bregs and non-Bregs (Fig 3A, Figure S1C). The following reagents and fluorochrome-conjugated monoclonal antibodies were used to stain B cells: live/dead fixable blue dead cell stain kit for UV excitation (Thermo Fisher Scientific), anti-CD19-BV421 (BioLegend), anti-CD3-V500, anti-CD24-BV605, anti-CD33-PE, anti-CD38-APC (all from BD Biosciences), FLAER-Alexa Fluor 488 (Cedarlane, Burlington, NC, USA), anti-IL-10-PE-Cy7 (eBioscience, Vienna, Austria) and fixation/permeabilisation solution (BD Bioscience).

Statistics

All statistical analyses were performed using the EZR software package (version 1·38), a graphical user interface for R

(version 3·5·2).[35] Data were shown as an *n* (%) or a median (interquartile range, IQR), unless otherwise specified. Fisher's exact test and Mann–Whitney *U*-test were used to compare categorical variables and numerical variables, respectively. Spearman's rank correlation test was used to assess correlations among numerical variables.

Results

Patients

A total of 125 consecutive treatment-naïve patients with AA, aged 18–82 years (median, 40 years), were treated with ATG, cyclosporine and eltrombopag from July 2012 to October 2018. Baseline TBNK lymphocyte subsets were evaluated in 104 of the 125 patients within 4 weeks prior to the therapy, and B-cell subpopulations were analysed in 60 patients whose cryopreserved PBMCs were available. A healthy control dataset of TBNK lymphocyte subsets was obtained from 40 healthy individuals, aged 18–55 years. Cryopreserved PBMCs from 29 healthy individuals, aged 18–72 years, were used as controls of B-cell subpopulations. The patients' characteristics are summarised in Table I and Table SI.

Reduced CD24^{hi}CD38^{hi} Bregs in patients with AA

Peripheral blood CD24^{hi}CD38^{hi} Breg frequencies were markedly reduced in patients with AA compared to healthy individuals, at a median (IQR) of 0·31 (0·14–0·85)% vs. 1·9 (1·3–3·6)% ($P < 0·0001$; Table II, Fig 1), although neither total CD19^{+} B-cell counts nor other B-cell subpopulation frequencies were significantly different between patients with AA and healthy individuals.

NK cells were lower in patients with AA ($P < 0·0001$; Table II) as previously reported.[36] CD4^{+} T cells and CD8^{+} T cells were also reduced in AA ($P = 0·0094$ and $P = 0·022$, respectively), but the difference was due to a negative correlation of CD8^{+} T cell counts with age (correlation efficiency [*r*], −0·42, $P < 0·0001$): age-matched comparisons of 71 patients with AA and 40 healthy individuals, aged 18–55 years, showed that CD8^{+} T cell counts in patients with AA were similar to those in healthy individuals ($P = 0·57$; Table II); CD4^{+} T cell counts tended lower in AA ($P = 0·054$); and total B-cell counts and NK cell counts of patients with AA remained non-significant ($P = 0·29$) and significant ($P < 0·0001$), respectively, compared to healthy individuals.

Reduced CD24^{hi}CD38^{hi} Bregs correlate with severity of AA

An analysis of lymphocyte subsets and baseline clinical parameters, including age, ANC, ARC and platelet counts revealed that only ANC significantly correlated with CD24^{hi}CD38^{hi} Bregs, both in frequency ($r = 0·33$, $P = 0·0098$) and absolute number ($r = 0·31$, $P = 0·016$). CD24^{hi}CD38^{hi} Bregs were significantly lower in 23 patients with VSAA compared to 37 with SAA (median [IQR] 0·18 [0·11–0·34]% vs. 0·50 [0·17–1·4]%, $P = 0·017$; $P = 0·044$ in absolute numbers; Fig 1B, Table SII, SIV). In contrast, CD24^{low}CD38^{low} mature naïve B-cell frequencies negatively correlated with ARC ($r = −0·38$, $P = 0·0024$) and with ANC ($r = −0·33$, $P = 0·0087$), and were significantly higher in VSAA ($P = 0·024$); but negative correlations with blood counts and with severity were not observed in absolute numbers (Table SIV). CD24^{hi}CD38^{hi} Breg and mature naïve

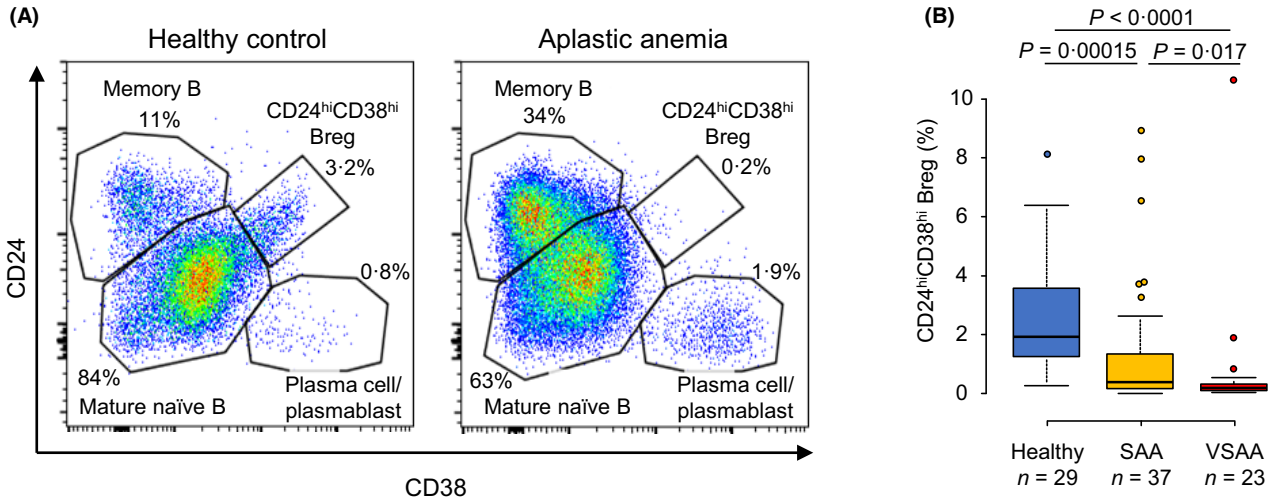

Fig 1. B-cell subpopulations. (A) Representative dot plots in a healthy individual and a patient with AA. CD3^{-}CD19^{+}CD33^{-}GPI-anchor^{+} live B cells were classified based on their expressions of CD24 and CD38: CD24^{hi}CD38^{hi} Bregs, CD24^{low}CD38^{low} mature naïve B cells, CD24^{hi}CD38^{low} memory B cells, and CD24^{low}CD38^{hi} plasma cells/plasmablasts. (B) Comparisons of CD24^{hi}CD38^{hi} Breg frequencies among healthy individuals ($n = 29$), patients with SAA ($n = 37$) and VSAA ($n = 23$). [Colour figure can be viewed at wileyonlinelibrary.com]

Table I. Clinical characteristics of the 104 patients

Characteristics	Value
Number of patients	104
Age, years, median (IQR)	39 (25–56)
Sex, *n* (%)	
Male	52 (50)
Female	52 (50)
Neutrophil, $\times10^9$/l, median (IQR)	0·30 (0·085–0·51)
Reticulocyte, $\times10^9$/l, median (IQR)	20 (9·4–33)
Platelet, $\times10^9$/l, median (IQR)	10 (6·0–15)
Severity, *n* (%)	
Severe	60 (58)
Very severe	44 (42)
GPI-anchor deficient clone, *n* (%)	
<1%	68 (65)
≥1%	36 (35)
Clone size, %, median (IQR)	7·4 (2·5–28)
IST response at 6 months, *n* (%)	
Responder	91 (87)
Non-responder	13 (13)

GPI, glycosylphosphatidylinositol; IST, immunosuppressive therapy; clone size is a summary of 36 patients with ≥1% GPI-anchor deficient clone.

B-cell frequencies did not show a correlation ($P = 0·58$). NK cell and CD8$^+$ T-cell counts were also reduced in VSAA compared to SAA ($P = 0·0084$ and $P = 0·026$, respectively). Age, as described above, significantly correlated with CD8$^+$ T cells ($r = -0·42$, $P < 0·0001$), and mature naïve B-cell frequencies ($r = 0·29$, $P = 0·023$), but did not show a correlation with CD24^{hi}CD38^{hi} Bregs or with other lymphocyte subsets studied.

Responses to IST plus eltrombopag

Initial CD24^{hi}CD38^{hi} Breg displayed no difference between IST-responders and non-responders (Fig 2A, Table III). Among the eight lymphocyte subpopulations studied before therapy, only CD24^{low}CD38^{low} mature naïve B cells correlated with IST responses: 47 responders showed lower initial mature naïve B cells compared to 13 non-responders, both in frequency ($P = 0·014$, Fig 2B, Table III) and absolute number ($P = 0·036$, Table SIV). Responders exhibited a significantly higher ARC compared to non-responders ($P = 0·027$), consistent with a previous report.[33] Clonal evolution was documented in 13 of 104 patients after IST, which did not correlate with Bregs or the other subsets.

Recovery of CD24^{hi}CD38^{hi} Bregs after IST

TBNK lymphocyte subsets and B-cell subpopulations were re-evaluated at 6 months after institution of ATG in 94 and 39 patients, respectively. Total B-cell counts were lower after IST (median [IQR] 74 [46–107]/μl from 137 [73–212]/μl, $P < 0·0001$; Table SIII), but CD24^{hi}CD38^{hi} Bregs increased to levels present in healthy individuals (median [IQR] 2·3 [0·98–4·8]% from 0·31 [0·14–0·85]%, $P < 0·0001$; Fig 2C), in both responders ($n = 34$) and non-responders ($n = 5$). CD24^{low}CD38^{hi} plasma cells/plasmablasts were significantly reduced after IST ($P < 0·0001$). Memory and mature naïve B-cell frequencies did not change after IST, but their absolute numbers were reduced significantly ($P = 0·00084$ and $P = 0·029$, respectively; Table SIV). None of the lymphocyte subsets at 6 months significantly correlated with IST responses, but mature naïve B-cell frequencies showed a higher trend in non-responders at 6 months ($P = 0·068$). Patients who stopped eltrombopag at 3 months and those who continued until 6 months exhibited no difference in their lymphocyte subsets at 6 months (TBNK analysis, $n = 17$ vs. $n = 77$; B-cell subpopulation analysis, $n = 8$ vs. $n = 31$; $P > 0·34$ in all comparisons).

IL-10 production from CD24^{hi}CD38^{hi} Bregs

B cells that produce IL-10 in response to CpG and CD40L stimulation were also significantly reduced in 33 patients with AA compared to 12 healthy individuals (median [IQR] 1·7 [1·1–2·4]% vs. 3·0 [2·4–4·4]%, $P = 0·0017$; Fig 3B), as observed by others,[32] and they showed a positive correlation with CD24^{hi}CD38^{hi} Bregs ($r = 0·37$, $P = 0·012$). IL-10 production from CD24^{hi}CD38^{hi} Bregs was analysable only in 10 of the 33 patients with AA, in which CD24^{hi}CD38^{hi} Bregs were retained but still these were significantly reduced compared to the 12 healthy individuals (median [IQR]

Table II. Age-matched comparisons of lymphocyte subsets between patients with aplastic anaemia (AA) and healthy individuals.

	Healthy individuals		Patients with AA		
Variable, median (IQR)	*n*	Summary measure	*n*	Summary measure	*P*
B cell, /μl	40	163 (106–242)	71	143 (73–225)	0·29
CD24^{hi}CD38^{hi} regulatory B cell, %	29	1·9 (1·3–3·6)	60	0·31 (0·14–0·85)	<0·0001
CD24^{low}CD38^{low} mature naïve B cell, %	29	71 (61–75)	60	65 (52–76)	0·12
CD24^{hi}CD38^{low} memory B cell, %	29	24 (17–31)	60	27 (13–38)	0·56
CD24^{low}CD38^{hi} plasma cell/plasmablast, %	29	1·7 (1·1–3·0)	60	2·1 (1·2–6·1)	0·33
CD4$^+$ T cell, /μl	40	798 (625–1021)	71	698 (452–963)	0·054
CD8$^+$ T cell, /μl	40	400 (296–616)	71	411 (282–537)	0·57
Natural killer cell, /μl	40	322 (202–386)	71	79 (50–112)	<0·0001

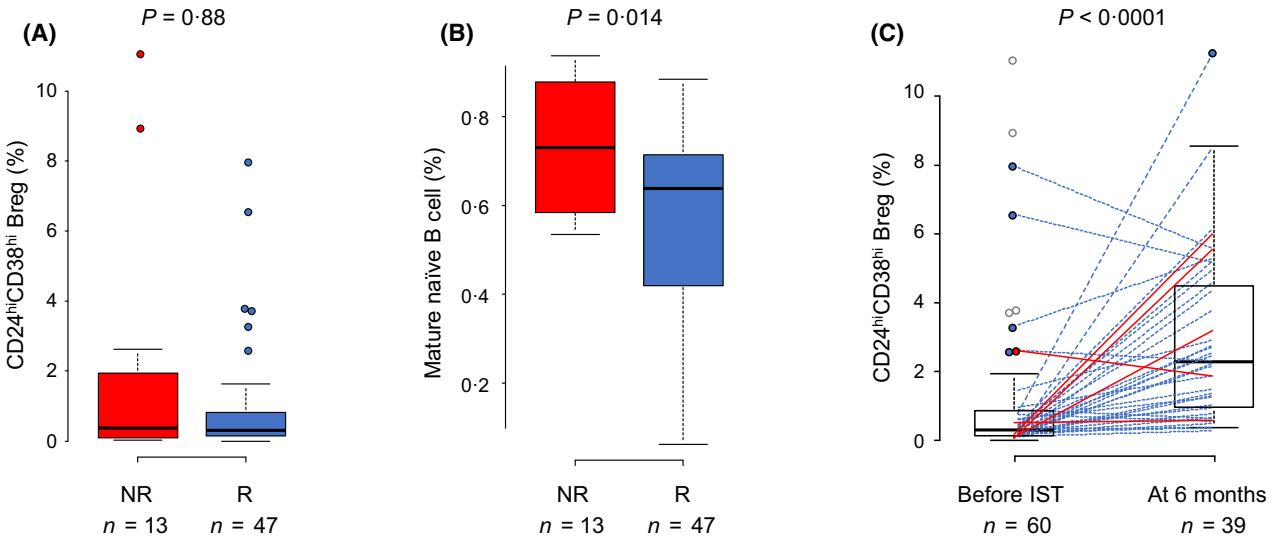

Fig 2. B-cell subpopulation frequencies and response to IST. (A) Initial $CD24^{hi}CD38^{hi}$ Bregs did not correlate with IST responses. (B) Among eight lymphocyte subsets studied, only mature naïve B cells correlated with haematological responses. (C) $CD24^{hi}CD38^{hi}$ Bregs recovered in 6 months after institution of ATG in both responders (blue dot lines, $n = 34$) and non-responders (red solid lines, $n = 5$). R, responders; NR, non-responders. [Colour figure can be viewed at wileyonlinelibrary.com]

Table III. Lymphocyte subsets in IST-responders and -non-responders.

	IST non-responder		IST responder		
Variable,	*n*	Summary measure	*n*	Summary measure	*P*
Age, years, median (IQR)	13	47 (30–56)	91	38 (25–56)	0·25
Male sex, *n* (%)	13	8 (62%)	91	44 (48%)	0·56
Median (IQR):					
B cell (/μl)	13	142 (84–218)	91	136 (73–210)	0·93
$CD24^{hi}CD38^{hi}$ regulatory B cell, %	13	0·37 (0·10–1·9)	47	0·30 (0·15–0·82)	0·88
$CD24^{low}CD38^{low}$ mature naïve B cell, %	13	73 (58–88)	47	64 (42–71)	0·014
$CD24^{hi}CD38^{low}$ memory B cell, %	13	13 (9–33)	47	29 (19–39)	0·076
$CD24^{low}CD38^{hi}$ plasma cell/plasmablast, %	13	2·5 (1·3–3·8)	47	1·9 (1·2–6·3)	0·98
$CD4^{+}$ T cell, /μl	13	535 (280–712)	91	673 (445–917)	0·24
$CD8^{+}$ T cell. /μl	13	197 (107–359)	91	334 (218–502)	0·15
Natural killer cell, /μl	13	64 (45–79)	91	82 (49–113)	0·12

0·76 [0·31–2·2]% vs. 2·8 [2·0–4·1]%, $P = 0·0056$). Intracellular IL-10^{+} cells were significantly enriched in $CD24^{hi}CD38^{hi}$ Bregs both in patients with AA and healthy individuals, and their percentages in $CD24^{hi}CD38^{hi}$ Bregs were comparable between patients with AA and healthy individuals (Fig 3C). These findings indicate that residual $CD24^{hi}CD38^{hi}$ Bregs of patients with AA remain functional.

Bone marrow $CD24^{hi}CD38^{hi}$ Bregs

$CD24^{hi}CD38^{hi}$ Bregs were studied in cryopreserved bone marrow samples of three healthy individuals and four patients with AA at diagnosis and 6 months after institution of IST (Figure SII). Percentages of $CD24^{hi}CD38^{hi}$ Bregs in the three healthy bone marrow samples were 20%, 19% and 13%, respectively, which were much higher than those we have observed in peripheral blood. In contrast, $CD24^{hi}CD38^{hi}$ Bregs in bone marrow of untreated patients with AA were markedly reduced and correlated with those in peripheral blood, and they recovered to even higher levels at 6 months than did cells in peripheral blood.

Discussion

Functions, subsets and repertoires of T cells have been characterised in patients with immune AA, but little has been reported on B cells and their subpopulations. We found that $CD24^{hi}CD38^{hi}$ Bregs were markedly reduced in untreated patients with AA, without significant changes in total B-cell counts and other B-cell subpopulations. $CD24^{hi}CD38^{hi}$ Bregs were especially reduced in VSAA and recovered after IST. Residual $CD24^{hi}CD38^{hi}$ Bregs were functional, capable of producing IL-10. A numerical deficit of $CD24^{hi}CD38^{hi}$ Bregs, that suppress CTLs and Th1 responses and induce Tregs, is

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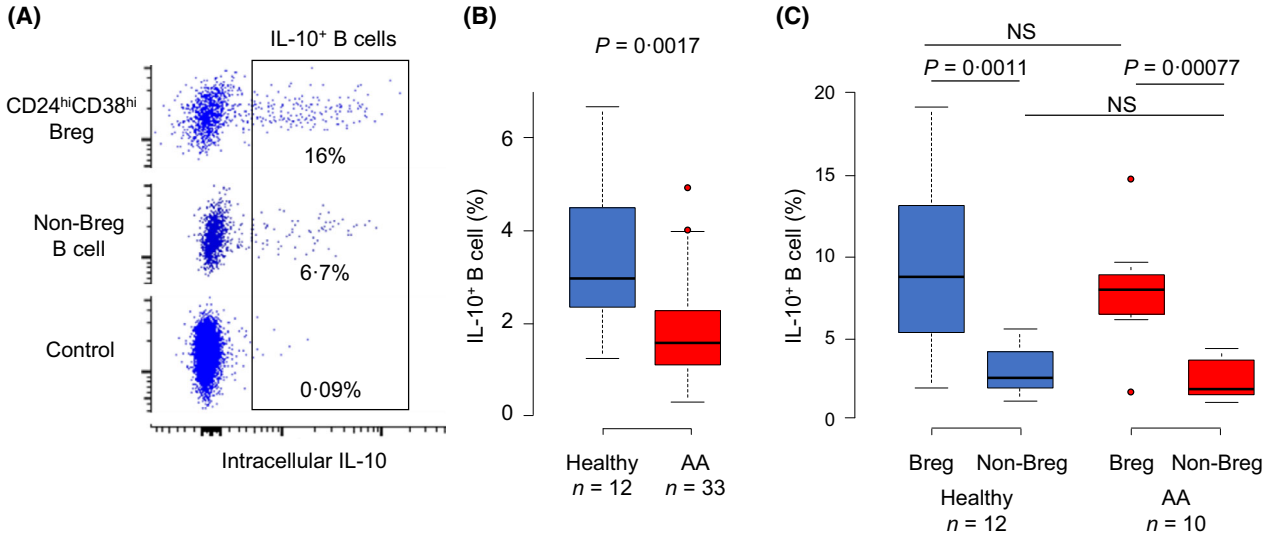

Fig 3. IL-10 production from B cells. (A) Representative dot plots of intracellular IL-10 staining in CD24^{hi}CD38^{hi} Bregs and non-Breg B cells. The control was B cells cultured without brefeldin A. (B) IL-10^{+} B-cell frequencies in total B cells were reduced in patients with AA (n = 33) compared to healthy individuals (n = 12). (C) IL-10^{+} cell frequencies in B-cell subpopulations in healthy individuals (n = 12) and patients with AA (n = 10). Intracellular IL-10^{+} B cells were significantly enriched in CD24^{hi}CD38^{hi} Bregs in both groups. [Colour figure can be viewed at wileyonlinelibrary.com]

consistent with immune pathophysiology of AA, in which autoreactive CD8^{+} CTLs and Th1 responses, in the absence of Treg suppression, are responsible for destruction of HSPCs.[1]

Initial CD24^{hi}CD38^{hi} Breg deficit and recovery of this cell population after IST did not correlate with response to the therapy. Tregs are also reduced in AA and have not correlated with IST response, although a subpopulation of Tregs may be predictive.[23] Other factors, such as residual haematopoiesis[33] and elimination of autoreactive T cells,[37] may be more important. We unexpectedly observed higher CD24^{low}CD38^{low} mature naïve B cells to associate with a lower probability of response to IST. A negative correlation of mature naïve B-cell frequencies with baseline ARC, the strongest predictor of a response to ATG-based IST,[33] may explain this correlation, but it is unclear why mature naïve B cells had a negative correlation with ARC.

Reduced CD24^{hi}CD38^{hi} Bregs in AA, especially in VSAA, may be in part a consequence of marrow failure, as this population represents an immature subset among circulating B cells.[24] But CD24^{hi}CD38^{hi} Bregs recovered in response to IST even in non-responders, suggesting that CD24^{hi}CD38^{hi} Bregs are not just reflecting haematopoiesis.

Reduction of B cells and changes in their subpopulations after IST should be attributable to therapy. Cyclosporine suppresses human B-cell proliferation[38] and reduces Bregs.[39] However, CD24^{hi}CD38^{hi} Bregs in AA remarkably increased after IST on cyclosporine, implying that immune reconstruction by ATG associated with the CD24^{hi}CD38^{hi} Bregs recovery, while eltrombopag appeared not to affect Bregs or the other subpopulations because earlier discontinuation of eltrombopag did make a difference in the lymphocyte subsets studied.

IL-10-producing B cells in bone marrow have been reported to be reduced in patients with AA compared to healthy individuals;[32] we confirmed this finding in peripheral blood, because marrow samples are diluted by peripheral blood, perhaps more so with hypocellular samples. Further the reduction of IL-10^{+} B cells associated with the quantitative deficit of CD24^{hi}CD38^{hi} Bregs, but residual CD24^{hi}CD38^{hi} Bregs in AA were functional, as they produced IL-10 in response to CpG and CD40L.

In addition to B-cell subpopulations, we assessed clinical correlations of conventional lymphocyte subsets, but only NK cells were found to be reduced in AA as previously reported.[36] A distinct lineage origin of NK cells[40] and their short lifespan[41] may associate with a selective defect of NK cells in AA. None of lymphocyte subsets correlated with responses to IST plus eltrombopag.

CD16 and CD24 are GPI-anchored proteins and their cell surface expressions are reduced in GPI-anchor deficient cells.[42] Therefore, we excluded GPI-anchor deficient B cells to avoid underestimation of CD24^{hi}CD38^{hi} Bregs, but NK cell counts were potentially underestimated in the present study because GPI-anchor expressions were not assessed in the TBNK subset analysis. However, the presence of GPI-anchor deficient clones cannot explain the reduced NK cells in AA: CD16^{+}CD56^{-} NK cells that become undetectable by GPI-anchor defects are a minor subpopulation of NK cells,[43] and NK cell counts of patients with AA who did not have GPI-anchor deficient clones remained markedly reduced compared to healthy individuals (P < 0·0001).

While paediatric patients were not enrolled in this retrospective study due to differences in normal lymphocyte counts between children and adults, we did study B-cell subpopulations in three patients aged 4–14 years: CD24^{hi}CD38^{hi}

Bregs were also low in these three children (0·09%, 0·20% and 0·32%), suggesting $CD24^{hi}CD38^{hi}$ Bregs to be reduced in both adult and paediatric AA.

In summary, $CD24^{hi}CD38^{hi}$ Bregs that potentially suppress autoreactive CTLs and Th1 responses were markedly reduced in AA, especially in VSAA, and recovered in response to IST plus eltrombopag. A quantitative deficit in functional $CD24^{hi}CD38^{hi}$ Breg may contribute to pathophysiology in immune AA. We unexpectedly observed higher $CD24^{low}CD38^{low}$ mature naïve B-cell numbers to be associated with a lower probability of responses to IST. B-cell phenotype analysis may be useful for monitoring of AA and predicting outcomes of therapy.

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Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

Yoshitaka Zaimoku, Sachiko Kajigaya and Neal S. Young designed the study and wrote the first draft of the manuscript. Bhavisha A. Patel, Emma M. Groarke and Neal S. Young participated in the patient care. Yoshitaka Zaimoku and Xingmin Feng performed the flow cytometry. Lemlem Alemu and Diego Quinones Raffo managed the patient samples. All authors critically reviewed the draft and approved the final version for publication.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Representative gating strategies. (A) TBNK lymphocyte subset analysis. (B) B-cell subpopulation analysis. (C) Intracellular IL-10 expressions in $CD24^{hi}CD38^{hi}$ Bregs and non-Bregs.

Fig S2. Bone marrow $CD24^{hi}CD38^{hi}$ Bregs. (A) Representative dot plots of a healthy individual and a patient with AA at diagnosis and at 6 months after institution of IST plus eltrombopag. (B) $CD24^{hi}CD38^{hi}$ Breg frequencies in peripheral and bone marrow B cells in patients with AA before IST (black; $n = 4$) and at 6 months after therapy (red; $n = 4$); and those in healthy bone marrow (green; $n = 3$).

Table SI. Clinical characteristics of 60 patients in the B-cell subpopulation analysis.

Table SII. Lymphocyte subsets in SAA and VSAA.

Table SIII. Lymphocyte subsets before and after IST.

Table SIV. B-cell subpopulation comparisons in percentages and absolute numbers.

References

1. Young NS. Aplastic anemia. *N Engl J Med*. 2018;**379**:1643–56.
2. Babushok DV, Duke JL, Xie HM, Stanley N, Atienza J, Perdigones N, et al. Somatic HLA mutations expose the role of class I-mediated autoimmunity in aplastic anemia and its clonal complications. *Blood Adv*. 2017;**1**:1900–10.
3. Katagiri T, Sato-Otsubo A, Kashiwase K, Morishima S, Sato Y, Mori Y, et al. Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia. *Blood*. 2011;**118**:6601–9.
4. Yoshizato T, Dumitriu B, Hosokawa K, Makishima H, Yoshida K, Townsley D, et al. Somatic mutations and clonal hematopoiesis in aplastic anemia. *N Engl J Med*. 2015;**373**:35–47.
5. Zaimoku Y, Takamatsu H, Hosomichi K, Ozawa T, Nakagawa N, Imi T, et al. Identification of an HLA class I allele closely involved in the autoantigen presentation in acquired aplastic anemia. *Blood*. 2017;**129**:2908–16.
6. Hosokawa K, Muranski P, Feng X, Townsley DM, Liu B, Knickelbein J, et al. Memory stem T cells in autoimmune disease: high frequency of Circulating CD8+ memory stem cells in acquired aplastic anemia. *J Immunol*. 2016;**196**:1568–78.
7. Zoumbos NC, Gascon P, Djeu JY, Trost SR, Young NS. Circulating activated suppressor T lymphocytes in aplastic anemia. *N Engl J Med*. 1985;**312**:257–65.
8. Giudice V, Feng X, Lin Z, Hu W, Zhang F, Qiao W, et al. Deep sequencing and flow cytometric characterization of expanded effector memory CD8(+)CD57(+) T cells frequently reveals T-cell receptor Vbeta oligoclonality and CDR3 homology in acquired aplastic anemia. *Haematologica*. 2018;**103**:759–69.
9. Espinoza JL, Elbadry MI, Chonabayashi K, Yoshida Y, Katagiri T, Harada K, et al. Hematopoiesis by iPSC-derived hematopoietic stem cells of aplastic anemia that escape cytotoxic T-cell attack. *Blood Adv*. 2018;**2**:390–400.
10. Lin FC, Karwan M, Saleh B, Hodge DL, Chan T, Boelte KC, et al. IFN-γ causes aplastic anemia by altering hematopoietic stem/progenitor cell composition and disrupting lineage differentiation. *Blood*. 2014;**124**:3699–708.
11. Smith JN, Kanwar VS, MacNamara KC. Hematopoietic stem cell regulation by type I and II interferons in the pathogenesis of acquired aplastic anemia. *Front Immunol*. 2016;**7**:330.
12. Solomou EE, Keyvanfar K, Young NS. T-bet, a Th1 transcription factor, is up-regulated in T cells from patients with aplastic anemia. *Blood*. 2006;**107**:3983–91.
13. Zoumbos NC, Gascon P, Djeu JY, Young NS. Interferon is a mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo. *Proc Natl Acad Sci USA*. 1985;**82**:188–92.
14. Dufour C, Capasso M, Svahn J, Marrone A, Haupt R, Bacigalupo A, et al. Homozygosis for (12) CA repeats in the first intron of the human IFN-gamma gene is significantly associated with the risk of aplastic anaemia in Caucasian population. *Br J Haematol*. 2004;**126**:682–5.
15. Broxmeyer HE, Cooper S, Rubin BY, Taylor MW. The synergistic influence of human interferon-gamma and interferon-alpha on suppression of hematopoietic progenitor cells is additive with the enhanced sensitivity of these cells to inhibition by interferons at low oxygen tension in vitro. *J Immunol*. 1985;**135**:2502–6.
16. Murphy M, Loudon R, Kobayashi M, Trinchieri G. Gamma interferon and lymphotoxin, released by activated T cells, synergize to inhibit granulocyte/monocyte colony formation. *J Exp Med*. 1986;**164**:263–79.
17. Chen J, Feng X, Desierto MJ, Keyvanfar K, Young NS. IFN-gamma-mediated hematopoietic cell destruction in murine models of immune-mediated bone marrow failure. *Blood*. 2015;**126**:2621–31.
18. Omokaro SO, Desierto MJ, Eckhaus MA, Ellison FM, Chen J, Young NS. Lymphocytes with aberrant expression of Fas or Fas ligand attenuate immune bone marrow failure in a mouse model. *J Immunol*. 2009;**182**:3414–22.
19. Alvarado LJ, Huntsman HD, Cheng H, Townsley DM, Winkler T, Feng X, et al. Eltrombopag maintains human hematopoietic stem and progenitor

cells under inflammatory conditions mediated by IFN-gamma. *Blood*. 2019;**133**:2043–55.
20. Townsley DM, Scheinberg P, Winkler T, Desmond R, Dumitriu B, Rios O, et al. Eltrombopag added to standard immunosuppression for aplastic anemia. *N Engl J Med*. 2017;**376**:1540–50.
21. Scheinberg P, Nunez O, Weinstein B, Biancotto A, Wu CO, Young NS. Horse versus rabbit antithymocyte globulin in acquired aplastic anemia. *N Engl J Med*. 2011;**365**:430–8.
22. Solomou EE, Rezvani K, Mielke S, Malide D, Keyvanfar K, Visconte V, et al. Deficient CD4+ CD25+ FOXP3+ T regulatory cells in acquired aplastic anemia. *Blood*. 2007;**110**:1603–6.
23. Kordasti S, Costantini B, Seidl T, Perez Abellan P, Martinez Llordella M, McLornan D, et al. Deep phenotyping of Tregs identifies an immune signature for idiopathic aplastic anemia and predicts response to treatment. *Blood*. 2016;**128**:1193–205.
24. Mauri C, Menon M. Human regulatory B cells in health and disease: therapeutic potential. *J Clin Invest*. 2017;**127**:772–9.
25. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity*. 2010;**32**:129–40.
26. Bosma A, Abdel-Gadir A, Isenberg DA, Jury EC, Mauri C. Lipid-antigen presentation by CD1d(+) B cells is essential for the maintenance of invariant natural killer T cells. *Immunity*. 2012;**36**:477–90.
27. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med*. 2013;**5**:173ra123.
28. Siewe B, Stapleton JT, Martinson J, Keshavarzian A, Kazmi N, Demarais PM, et al. Regulatory B cell frequency correlates with markers of HIV disease progression and attenuates anti-HIV CD8(+) T cell function in vitro. *J Leukoc Biol*. 2013;**93**:811–8.
29. Li X, Zhong H, Bao W, Boulad N, Evangelista J, Haider MA, et al. Defective regulatory B-cell compartment in patients with immune thrombocytopenia. *Blood*. 2012;**120**:3318–25.
30. Khoder A, Sarvaria A, Alsuliman A, Chew C, Sekine T, Cooper N, et al. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood*. 2014;**124**:2034–45.
31. Zhang HF, Huang ZD, Wu XR, Li Q, Yu ZF. Comparison of T lymphocyte subsets in aplastic anemia and hypoplastic myelodysplastic syndromes. *Life Sci*. 2017;**189**:71–5.
32. Gu L, Fu B, Sui X, Xu H. Abnormal expression of b10 cell frequencies: possible relation to pathogenesis and disease severity of aplastic anemia. *Rev Assoc Med Bras (1992)*. 2019;**65**:637–46.
33. Scheinberg P, Wu CO, Nunez O, Young NS. Predicting response to immunosuppressive therapy and survival in severe aplastic anaemia. *Br J Haematol*. 2009;**144**:206–16.
34. Arruda LC, Malmegrim KC, Lima-Junior JR, Clave E, Dias JB, Moraes DA, et al. Immune rebound associates with a favorable clinical response to autologous HSCT in systemic sclerosis patients. *Blood Adv*. 2018;**2**:126–41.
35. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant*. 2013;**48**:452–8.
36. Gascon P, Zoumbos N, Young N. Analysis of natural killer cells in patients with aplastic anemia. *Blood*. 1986;**67**:1349–55.
37. Risitano AM, Maciejewski JP, Green S, Plasilova M, Zeng W, Young NS. In-vivo dominant immune responses in aplastic anaemia: molecular tracking of putatively pathogenetic T-cell clones by TCR beta-CDR3 sequencing. *Lancet*. 2004;**364**:355–64.
38. Hannam-Harris AC, Taylor DS, Nowell PC. Cyclosporin A directly inhibits human B-cell proliferation by more than a single mechanism. *J Leukoc Biol*. 1985;**38**:231–9.
39. Tebbe B, Wilde B, Ye Z, Wang J, Wang X, Jian F, et al. Renal transplant lack recipients treated with calcineurin-inhibitors circulating immature transitional CD19+CD24hiCD38hi regulatory B-lymphocytes. *PLoS ONE*. 2016;**11**:e0153170.
40. Wu C, Li B, Lu R, Koelle SJ, Yang Y, Jares A, et al. Clonal tracking of rhesus macaque hematopoiesis highlights a distinct lineage origin for natural killer cells. *Cell Stem Cell*. 2014;**14**:486–99.
41. Vogel B, Tennert K, Full F, Ensser A. Efficient generation of human natural killer cell lines by viral transformation. *Leukemia*. 2014;**28**: 192–5.
42. Madkaikar M, Gupta M, Jijina F, Ghosh K. Paroxysmal nocturnal haemoglobinuria: diagnostic tests, advantages, & limitations. *Eur J Haematol*. 2009;**83**:503–11.
43. Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology*. 2009;**126**:458–65.

