Cyclosporine restores hematopoietic function by compensating for decreased Tregs in patients with pure red cell aplasia and acquired aplastic anemia

著者	ダオ チ タイン アン
著者別表示	Dao Thi Thanh An
journal or publication title	博士論文本文Full
学位授与番号	13301甲第4458号
学位名	博士（医学）
学位授与年月日	2016-09-26
URL	http://hdl.handle.net/2297/46468

doi: <https://doi.org/10.1007/s00277-016-2629-7>

Cyclosporine restores hematopoietic function by compensating for decreased Tregs in patients with pure red cell aplasia and acquired aplastic anemia

An T. T. Dao¹ · Hirohito Yamazaki¹ · Hiroyuki Takamatsu¹ · Chiharu Sugimori² · Takamasa Katagiri³ · Hiroyuki Maruyama¹ · Yoshitaka Zaimoku¹ · Kana Maruyama¹ · Trung Q. Ly¹ · Luis Espinoza¹ · Shinji Nakao¹

Received: 30 December 2015 / Accepted: 24 February 2016 / Published online: 11 March 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract Most patients with acquired pure red cell aplasia (PRCA) and some with acquired aplastic anemia (AA) respond well to cyclosporine (CsA), but thereafter often show CsA dependency. The mechanism underlying this dependency remains unknown. We established a reliable method for measuring the regulatory T cell (Treg) count using FoxP3 and Helios expression as markers and determined the balance between Tregs and other helper T cell subsets in 16 PRCA and 29 AA patients. The ratios of interferon- γ -producing CD4⁺ (Th1) T cells to Tregs in untreated patients and CsA-dependent patients were significantly higher (PRCA 5.77 ± 1.47 and 7.38 ± 2.58 ; AA 6.18 ± 2.35 and 8.94 ± 4.06) than in healthy volunteers (HVs; 3.33 ± 0.90) due to the profound decrease in the percentage of Tregs. In contrast, the ratios were comparable to HVs in convalescent CsA-treated AA patients (4.74 ± 2.10) and AA patients in remission after the cessation of CsA treatment (4.24 ± 1.67). Low-dose CsA (100 ng/ml) inhibited the proliferation of conventional T cells (Tconv) to a similar degree to the inhibition by Tregs in a co-culture with a 1:1 Treg/Tconv ratio. The data suggest that CsA may reverse

the hematopoietic suppression in PRCA and AA patients by compensating for the inadequate immune regulatory function that occurs due to a profound decrease in the Treg count.

Keywords Pure red cell aplasia · Aplastic anemia · Regulatory T cells · Cyclosporine A

Introduction

Idiopathic pure red cell aplasia (PRCA) is a refractory erythropoietic failure that is characterized by the absence of erythroblasts in the bone marrow (BM) with normal white blood cell and platelet counts. Although the exact cause is unclear, various effector mechanisms have been proposed, including CD8⁺ T cells, $\gamma\delta$ T cells, large granular lymphocytes, anti-erythropoietin antibodies, and anti-erythropoietin receptor antibodies [1–6]. Despite the various immune mechanisms that underlie erythroid progenitor cell suppression, most PRCA patients respond to cyclosporine (CsA), an immunosuppressant which selectively suppresses T cells. Even more characteristically, PRCA patients show a strong dependency on CsA: they usually require low dose CsA to maintain good erythropoietic function [7]. This is in sharp contrast to the response to CsA in patients with idiopathic aplastic anemia (AA), most of whom can be withdrawn from CsA after achieving remission with antithymocyte globulin (ATG) and CsA therapy [8]. The similar dependency on CsA for hematopoietic function is observed in a subset of AA patients, particularly those who possess DRB1*15:01 [9]. The reason the strong CsA dependency on PRCA patients and this subset of AA patients remains to be elucidated.

CD4⁺CD25⁺ regulatory T cells (Tregs) play an important role in maintaining the homeostasis of the immune

Electronic supplementary material The online version of this article (doi:10.1007/s00277-016-2629-7) contains supplementary material, which is available to authorized users.

✉ Shinji Nakao
snakao8205@staff.kanazawa-u.ac.jp

¹ Cellular Transplantation Biology, Graduate School of Medical Science, Kanazawa University, 13-1 Takaramachi, Kanazawa 920-8641, Japan

² Department of Hematology, Ishikawa Prefectural Central Hospital, 2-1 Kuratsukihigashi, Kanazawa, Ishikawa, Japan

³ Clinical Laboratory Science, Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa, Japan

system by suppressing autoreactive T cell induction and function. Tregs effectively control the development and progression of autoimmune diseases such as type 1 diabetes and psoriasis [10, 11]. Patients with immune-mediated BM failure such as AA have been shown to have significantly reduced numbers of Tregs [12, 13]. However, it is unclear whether the decrease in the Treg count is actively involved in BM suppression through the failure to control autoreactive T cells in these diseases because the BM failure syndromes affect both myelopoiesis and lymphopoiesis; thus, the decrease in Tregs may merely represent T lymphocytopenia which occurs due to a decrease in the number of hematopoietic stem cells. The role of Tregs in the development of immune-mediated BM failure may become clearer by studying patients with PRCA, in whom lymphopoiesis is not affected by causal mechanisms which selectively inhibit erythroid progenitors.

To test this hypothesis, we determined the percentage and absolute count of Tregs as well as helper T cell (Th) subsets, including Th1, Th2, and Th17 cells, in the peripheral blood (PB) of PRCA and AA patients, using an anti-FoxP3 antibody (Ab) and an anti-Helios Ab. This method allows for the functional Tregs to be enumerated more precisely than by conventional methods.

Materials and methods

Patients

Sixteen PRCA patients (idiopathic, $n=12$; thymoma-associated, $n=3$; and large granular lymphocytic leukemia [LGL], $n=1$) and 29 AA patients (non-severe AA, $n=22$; and severe AA, $n=7$) who were referred to our clinic from 2014 to 2015 and responded to CsA or CsA plus rabbit ATG (Thymoglobulin, Sanofi, Japan) therapy were included in this retrospective analysis. Among the 16 PRCA patients, 2 were untreated and 14 were undergoing CsA therapy after showing a good response to CsA ($n=10$) or on CsA dependency ($n=4$) at the time of blood sampling (Table 1). Nine of the AA patients were untreated (severe, $n=3$; non-severe, $n=6$), 5 of the AA patients were in the convalescent phase and undergoing CsA therapy, 6 showed CsA dependency, and 9 were remission after the cessation of CsA therapy. A total of 47 age-matched healthy volunteers (HVs) were used as controls. CsA dependency was defined as two or more relapses of anemia (in the PRCA patients) or pancytopenia (in the AA patients) in association with a dose reduction or the cessation of CsA, which had occurred after more than 1 year of therapy, followed by a good response to CsA. After obtaining informed consent, 10 ml of PB was collected from these patients and the HVs using EDTA-coated tubes. The PB was then

subjected to mononuclear cell (MC) isolation using density gradient medium (Lymphoprep™, Norway). All of the PB samples were analyzed within 8 h after sampling. This study protocol (No. 287) was approved by the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science. Informed consent was obtained from all individual participants included in the study.

Cell sorting

PBMCs from HVs were stained with fluorochrome-labeled antibodies (Abs) specific for CD4 (BD Bioscience), CD45RA (BD Bioscience), CD25 (BD Bioscience), CD127 (eBioscience), CCR7 (R&D), and IL-1RI (R&D). After gating on CD4⁺ lymphocytes, the cells were separated into the indicated populations (CD4⁺CD45RA⁺CD25⁺CD127⁺ (Tconv), CD4⁺CD45RA⁺CD25⁺CD127⁺CCR7⁺IL-1RI⁺ (CCR7⁺IL-1RI⁺ Tregs), CD4⁺CD45RA⁺CD25⁺CD127⁺CCR7⁺IL-1RI⁺ (CCR7⁺IL-1RI⁺ Tregs), and CD4⁺CD45RA⁺CD25⁺CD127⁺CCR7⁺IL-1RI⁺ (CCR7⁺IL-1RI⁺ Tregs)) to a high level of purity (>97 %) by flow cytometry cell sorting with a BD FACS Aria™ Fusion Cell sorter instrument (BD Biosciences).

The phenotypic analysis of Treg subsets and cytokine-producing T cells

CD3⁺CD4⁺ T cells were stained with fluorochrome-labeled Abs specific for FoxP3 (BD Bioscience), and Helios (Biolegend) in combination with or without one of the Abs specific to CD45RA (BD Bioscience), CD31 (eBioscience), CCR4 (BD Bioscience), CTLA4 (BD Bioscience), and Ki67 (eBioscience). All of the intracellular staining procedures were performed using the FoxP3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions. The cells were analyzed by flow cytometry (FCM; FACS Canto II; BD Biosciences).

For the analysis of cytokine production by the Treg subsets, stimulated PBMCs were stained with Abs specific to surface markers, including CD3 and CD4, and then fixed/permeabilized with a FoxP3 Staining Buffer Set (eBioscience), and stained with Abs specific to intracellular proteins, including Helios, FoxP3, IL-2 (BD Bioscience), IL-17, and INF- γ .

For the measurement of the different Th subsets, PBMCs were stimulated by phorbol myristate acetate (PMA; 50 ng/ml, Sigma-Aldrich) and ionomycin (1 μ g/ml, Sigma-Aldrich) in the presence of Golgi Stop (0.75 μ l/ml, BD Bioscience) in a tissue culture incubator for 4 h, and the cells were then washed and stained with fluorescence-labeled anti-CD3 and anti-CD4 Abs and analyzed by FCM to determine the percentage of CD3⁺CD4⁺ T cells that were positive for cytoplasmic

Table 1 Characteristics of PRCA patients

Patient	Age (years)	Sex	Classification	CsA dose (mg/day)	State at blood sampling	WBC ($\times 10^9/l$)	Hb (g/l)	Lym ($\times 10^9/l$)	CD4 ($\times 10^9/l$)
1	69	F	Idiopathic	60	In convalescence	3.850	108	0.8	0.14
2	59	F	Idiopathic	100	CsA dependent	4.280	119	1.4	0.30
3	81	M	Thymoma associated	100	In convalescence	5.600	113	2.1	0.75
4	81	F	LGL leukemia	100	In convalescence	25.000	64	1.4	0.09
5	59	M	Idiopathic	160	In convalescence	3.500	130	2.3	0.51
6	78	M	Idiopathic	160	In convalescence	4.480	100	2.3	0.47
7	57	M	Idiopathic	220	In convalescence	2.780	107	0.8	0.34
8	85	F	Idiopathic	80	CsA dependent	4.600	119	1.2	0.59
9	72	F	Idiopathic	180	In convalescence	3.500	91	1.8	0.50
10	85	F	Idiopathic	80	In convalescence	3.940	81	2.0	0.35
11	45	M	Idiopathic	120	In convalescence	3.300	111	1.7	0.68
12	90	F	Idiopathic	150	In convalescence	6.300	80	1.2	0.61
13	81	M	Thymoma associated	140	CsA dependent	3070	115	2.2	0.43
14	40	F	Idiopathic	120	CsA dependent	2.950	106	1.0	0.20
15	83	F	Thymoma associated	–	Untreated	4.600	62	1.8	0.35
16	66	F	Idiopathic	–	Untreated	3.500	65	2.1	0.70

CsA cyclosporine A, F female, M male, WBC white blood cell, Hb hemoglobin, Lym lymphocyte, LGL large granular lymphocyte

INF- γ^+ (Th1, BD Bioscience), IL-4 $^+$ (Th2, eBioscience), and IL-17 $^+$ (Th17, eBioscience) cells [14].

The assessment of the proliferation and suppressive function of Tregs

Ex vivo-sorted CD4 $^+$ T cell populations were labeled with carboxyfluorescein succinimidyl ester (CFSE; 2.5 μ M; Invitrogen), stimulated with Dynabeads[®] Human T-Activator CD3/CD28 (Life Technology) at a cell/bead ratio of 4:1, as described previously [14]. The cells were cultured for 4 days in the presence of 200 IU of IL-2 (Promega) in OpTmizer[™] CTS[™] T-Cell Expansion medium (Life Technology). To assess the inhibitory function of Tregs, 2×10^4 Tregs were cultured with different ratios of CFSE-labeled conventional T cells (Tconvs). The cells were collected at day 4, and the CFSE dilution was assessed by FCM.

The assessment of Tconv proliferation in the presence of CsA

Tconvs were sorted by FACS Aria Fusion and labeled with CFSE. A total of 1×10^5 cells were cultured in the presence of anti-CD3/CD28 Ab beads with a cell/bead ratio of 4:1 in OpTmizer[™] CTS[™] T-Cell Expansion medium supplemented with 200 IU/ml of IL-2 with different doses of CsA. The cells were collected on day 4, and the CFSE dilution was assessed by FCM.

Statistical analysis

Statistical comparisons of the various lymphocyte subsets were performed using a paired *t* test or the Mann-Whitney *U* test. The GraphPad Prism software program (version 5 for Windows, San Diego, CA, USA) was used for the statistical analyses.

Results

The definition of the Treg subsets by flow cytometry with anti-Helios and anti-FoxP3 antibodies

First, we determined the phenotypes of the Treg subsets of HVs (defined by Helios expression) to validate the use of this antigen as a Treg marker. Although the expression of FoxP3 by CD4 $^+$ T cells decreased in a time-dependent manner after sampling, it was stable within 8 h (data not shown). For this reason, the analyses were performed within 8 h after the sampling of fresh PBMCs. CD4 $^+$ FoxP3 $^+$ Helios $^+$ T cells (Helios $^+$ Tregs) and CD4 $^+$ FoxP3 $^+$ Helios $^-$ T cells (Helios $^-$ Tregs) accounted for 6.5 ± 1.6 and 2.0 ± 0.6 % of the CD4 $^+$ T cells of HVs (Fig. 1a). Helios $^-$ Tregs produced higher amounts of IL-2, IL-17, and IFN- γ than Helios $^+$ Tregs (Fig. 1b). This finding was consistent with previous reports [14–17]. Both subsets highly expressed CTLA4, CCR4 and contained approximately 12 % Ki67 $^+$ cells, indicating that the two T cell subsets were activated in vivo (Fig. S1). The proportion of

Fig. 1 The phenotypic differences between Helios⁺ Tregs and Helios[−] Tregs. The percentages of Helios⁺ and Helios[−] Tregs in the CD4⁺ T cells of 47 healthy volunteers (HVs) with a representative flow cytometry scattergram which was used to determine each of the subset percentages (a). The percentages of IL-2, IL-17 and IFN- γ secreting cells in Helios⁺ and Helios[−] Tregs with a representative scattergram from 6 HVs (b). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

CD31 cells that had recently migrated from the thymus was higher in the Helios⁺ Tregs than in the Helios[−] Tregs (Fig. S1).

To functionally characterize CD4⁺FoxP3⁺Helios⁺ T cells, we sorted CD4⁺CD45RA[−]CD25⁺CD127[−]CCR7[−]IL-1RI[−] (CCR7[−]IL-1RI[−] Treg) and CD4⁺CD45RA[−]CD25⁺CD127[−]CCR7⁺IL-1RI⁺ (CCR7⁺IL-1RI⁺ Treg) fractions that were compatible with Helios⁺ Tregs and Helios[−] Tregs, respectively (Fig. 2a), and tested their effects on the proliferation of T cells stimulated by anti-CD3/CD28 Ab beads. CCR7[−]IL-1RI[−] Tregs showed more divisions than CCR7⁺IL-1RI⁺ Tregs after 4 days of in vitro culture (Fig. 2b). CCR7[−]IL-1RI[−] Tregs were a more potent inhibitor of autologous Tconv proliferation than CCR7⁺IL-1RI⁺ Tregs (Fig. 2c, d).

A decreased percentage and absolute Treg count in PRCA and AA patients

The percentage and absolute counts of Helios⁺ Tregs in PRCA patients were significantly lower than those in HVs including two untreated PRCA patients (3.5 ± 0.2 vs 6.5 ± 1.6 % and $0.0179 \pm 0.0075 \times 10^9$ vs $0.0531 \pm 0.0211 \times 10^9/l$,

respectively), 10 convalescent PRCA patients who were undergoing CsA treatment (3.9 ± 1.6 vs 6.5 ± 1.6 %, $P < 0.0001$ and $0.0161 \pm 0.0070 \times 10^9$ vs $0.0531 \pm 0.0211 \times 10^9/l$, $P < 0.0001$), and four CsA-dependent PRCA patients (4.1 ± 1.9 vs 6.5 ± 1.6 %, $P = 0.0261$ and $0.0158 \pm 0.0102 \times 10^9$ vs $0.0531 \pm 0.0211 \times 10^9/l$, $P = 0.0021$, Fig. 3a, b and Table S1). A similar decrease in the percentage of Tregs was observed in the untreated AA patients and the CsA-dependent AA patients, but not in the convalescent AA patients who were in remission after the cessation of CsA treatment. Of note, the Helios⁺ Treg percentages of the CsA-treated convalescent PRCA patients were significantly lower than those of the CsA-treated convalescent AA patients (3.9 ± 1.6 vs 5.7 ± 0.9 %, $P = 0.0370$) (Fig. 3b). With the exception of the convalescent PRCA patients, who had a significantly lower percentage of Helios[−] Tregs than the HVs, the percentages of Helios[−] Tregs were similar among the PRCA and AA patients and the HVs (Fig. S2). The absolute Helios[−] Treg counts in the convalescent PRCA patients, the CsA-dependent PRCA patients, and the CsA-dependent AA patients were also significantly lower than the counts in the HVs (Fig. S2). The

Fig. 2 The suppression function of Helios⁺ Tregs and Helios⁻ Tregs. The gating strategy and the expression of FoxP3 and Helios in four different T cell populations after sorting (>97 % purity): conventional T cells (Tconv), CCR7⁺IL-1RI⁻ Tregs, CCR7⁺IL-1RI⁺ Tregs, and CCR7⁻IL-1RI⁻ Tregs (a). The proliferation of CFSE-labeled CCR7⁺IL-1RI⁺ Tregs and CCR7⁻IL-1RI⁻ Tregs was analyzed by FCM after 4 days of culture (b). The proliferation of CFSE-labeled Tconvs is shown after co-culture with CCR7⁺IL-1RI⁺ Tregs or CCR7⁻IL-1RI⁻ Tregs (c). The inhibitory function of CCR7⁺IL-1RI⁺ Tregs and CCR7⁻IL-1RI⁻ Tregs was analyzed after 4 days of co-culture. Data were collected from three separate experiments (d)

percentages of Ki67-positive cells in Helios⁺ Tregs were similar among the PRCA patients (untreated, 13.4 ± 7.4 %; convalescent, 13.4 ± 6.3 %; CsA dependent, 11.8 ± 4.3 %), the AA patients who were in remission (13.8 ± 2.9 %), and the HVs (12.5 ± 2.7 %) while the Ki67-positive cell percentages in the untreated AA (7.4 ± 1.5 %, $P = 0.0022$) and in the CsA-dependent AA (8.6 ± 0.6 %, $P = 0.0238$) patients were significantly lower than the percentages in the HVs (Fig. 3c).

When three different CD4⁺FoxP3⁺ T cell subsets (defined by the expression levels of CD45RA and FoxP3) were analyzed, the percentages of CD45RA⁺FoxP3^{low} resting Tregs and CD45RA⁺FoxP3^{high} activated Tregs in all of the three subsets of PRCA patients tended to be lower than those in HVs while the percentages of CD45RA⁻FoxP3^{low} cytokine-producing non-Tregs in the three subsets were higher than those of HVs (Fig. 3e). Representative scattergrams of an

HV, a PRCA patient, and an AA patient are shown in Fig. 3d [18]. Among the AA patients, the activated Treg percentages of the untreated AA patients, the convalescent AA patients, and the CsA-dependent AA patients were significantly lower than those in HVs while the AA patients who were in remission had a similar percentage of activated Tregs ($25.3 \pm 8.6\%$) to HVs ($23.9 \pm 7.4\%$). The percentages of cytokine-producing non-Tregs in untreated AA patients, CsA-dependent AA patients, and AA patients who were in remission were significantly higher than in HVs (Fig. 3e).

The T helper cell subsets in PRCA and AA patients

The ratios of $CD4^+$ T cells to $CD8^+$ T cells were significantly lower in PRCA patients than in HVs (Fig. S3a). In PRCA patients, the $CD4^+$ T cell counts of convalescent and CsA-dependent patients were significantly lower than in HVs (Fig. S3b). In AA patients, the $CD4^+$ T cell counts in CsA-dependent AA patients and AA patients who were in remission were significantly lower than in HVs, while those of untreated AA and convalescent AA patients were comparable to HVs (Fig. S3b).

In contrast to the decreased percentages of Tregs, the percentages of Th1, Th2, and Th17 cells in all of the PRCA subsets were comparable to those in HVs (Fig. S4a, b). In AA patients, the percentages of Th1 and Th2 cells in the four different groups were also comparable to HVs, while the percentages of Th17 cells in untreated AA ($3.1 \pm 2.0\%$, $P = 0.0422$) and CsA-dependent AA ($2.9 \pm 1.6\%$, $P = 0.0392$) patients were significantly higher than in HVs ($1.6 \pm 0.7\%$), (Fig. S4a, b). The absolute counts of Th1, Th2, and Th17 cells were significantly lower than in HVs due to the decreased $CD4^+$ T cell counts in PRCA patients (Fig. S3c). In contrast, the untreated AA patients showed a significant increase in their Th1 and Th17 cell counts in comparison to HVs while the Th1 and Th17 cell counts in other AA patients were comparable or lower than those in HVs due to their decreased $CD4^+$ T cell counts (Fig. S4b).

The ratios of each Th subset to Tregs

As a result of the decrease in the percentage of Tregs, the Th1/Treg and Th2/Treg ratios of the CsA-dependent PRCA patients (7.38 ± 2.58 , $P = 0.0145$ and 1.27 ± 0.37 , $P = 0.0414$) and the Th1/Treg ratio of convalescent CsA-treated PRCA patients (7.49 ± 4.64 , $P = 0.0004$) were significantly higher than those of HVs (3.33 ± 0.90 , 0.83 ± 0.24) (Fig. 3f and Table S1). The Th1/Treg, Th2/Treg, and Th17/Treg ratios of untreated PRCA patients tended to be higher than those of HVs. Similarly, untreated AA patients had significantly increased Th1/Treg (6.18 ± 2.35 , $P = 0.0007$), Th2/Treg (1.36 ± 0.72 , $P = 0.0214$), and Th17/Treg (0.69 ± 0.33 , $P = 0.0008$) ratios and CsA-dependent AA patients had significantly

increased Th1/Treg (8.94 ± 4.06 , $P = 0.0014$) and Th17/Treg (0.81 ± 0.33 , $P = 0.0005$) ratios in comparison to HVs. In contrast to untreated AA and CsA-dependent AA patients, convalescent CsA-treated AA patients and AA patients who were in remission had similar Th1/Treg, Th2/Treg, and Th17/Treg ratios to HVs (Fig. 3f).

The effect of CsA on Tconv proliferation

The normal blood cell counts and lower Treg counts of CsA-treated PRCA patients who were in remission and CsA-dependent AA patients may indicate that, in the blood, CsA complements the inadequate suppression of the Tconv function by Tregs due to their decreased number. We therefore tested the effect of CsA on the proliferation of Tconvs using a CFSE assay. As shown in Fig. 4, CsA inhibited Tconv proliferation in a dose-dependent manner. CsA at concentrations of 50 and 100 ng/ml, which can be achieved in blood by the administration of 2–3 mg/kg of CsA, inhibited the proliferation of Tconvs by 25.9 and 50.4 %, respectively (Fig. 4a, b).

Discussion

Several studies have focused on the role of Tregs in the pathophysiology autoimmune diseases such as AA, type 1

Fig. 3 The imbalance of the Helios⁺ Treg and T helper subsets in the PRCA and AA patients. The expression of FoxP3 and Helios in 47 HVs, 16 PRCA patients, and 29 untreated AA patients (UT untreated, Conval convalescent, CsA-D CsA-dependent, Rem in remission) with representative scattergrams (a). Percentages and the absolute counts of Helios⁺ Tregs in 16 PRCA patients (UT, $n = 2$; Conval, $n = 10$; CsA-D, $n = 4$), 29 AA patients (UT, $n = 9$; Conval, $n = 5$; CsA-D, $n = 6$; and Rem, $n = 9$), and 47 HVs were analyzed (b). The Ki67 expression levels on Helios⁺ Tregs in 12 PRCA patients (UT, $n = 2$; Conval, $n = 6$; CsA-D, $n = 4$), 15 AA patients (UT, $n = 6$; CsA-D, $n = 3$; Rem, $n = 6$), and 6 HVs are shown (c). Representative scattergrams showing the expression of CD45RA and FoxP3 in an HV, a PRCA patient, and an AA patient (rTreg resting Treg, aTreg activated Treg, cTreg cytokine-producing non-Treg) (d). The percentage of rTregs ($CD45RA^+FoxP3^{low}$), aTregs ($CD45RA^-FoxP3^{high}$), and cTregs ($CD45RA^-FoxP3^{low}$) in the total $FoxP3^+CD4^+$ T cells of 30 HVs (25.5 ± 10.0 , 23.9 ± 7.4 , and $50.5 \pm 7.5\%$), 16 PRCA patients (UT, $n = 2$ [4.6 ± 3.3 , 20.5 ± 19.2 , and $74.8 \pm 15.8\%$]; Conval, $n = 10$ [$17.4 \pm 9.9\%$, $P = 0.0091$; $15.1 \pm 9.3\%$, $P = 0.0063$, and $67.5 \pm 7.6\%$, $P < 0.0001$]; and CsA-D, $n = 4$ [$10.6 \pm 7.8\%$, $P = 0.0129$; $13.3 \pm 10.2\%$, $P = 0.0511$, and $76.1 \pm 17.3\%$, $P = 0.0081$]), and 29 AA patients (UT, $n = 9$ [$15.5 \pm 11.2\%$, $P = 0.0224$; $13.5 \pm 7.3\%$, $P = 0.0012$, and $71.0 \pm 12.8\%$, $P < 0.0001$]; Conval, $n = 5$ [$30.4 \pm 11.4\%$, $P = 0.3339$; $15.0 \pm 6.5\%$, $P = 0.0320$, and $54.6 \pm 11.7\%$, $P = 0.2679$]; CsA-D, $n = 6$ [$20.1 \pm 12.6\%$, $P = 0.5666$; $12.5 \pm 3.4\%$, $P = 0.0009$, and $67.4 \pm 10.9\%$, $P = 0.0014$]; and Rem, $n = 9$ [$10.4 \pm 5.9\%$, $P = 0.0002$; $25.3 \pm 8.6\%$, $P = 0.7769$, and $64.3 \pm 9.0\%$, $P = 0.0008$]) were analyzed by FCM. Each of the Treg subsets in PRCA and AA patients was compared to a similar subset of HVs (e). The ratios of Th1/Treg, Th2/Treg, and Th17/Treg in 16 PRCA patients (UT, $n = 2$; Conval, $n = 10$; and CsA-D, $n = 4$) and 26 AA patients (UT, $n = 9$; Conval, $n = 5$; CsA-D, $n = 6$; and Rem, $n = 6$) were compared to those of 32 HVs. The ratios are shown (f). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

diabetes, and psoriasis and have found that CD4⁺CD25⁺FoxP3⁺ Treg numbers were profoundly decreased in patients with active disease [19–23]. Previous studies on bone marrow failure syndromes revealed a decrease in the CD4⁺CD25⁺FoxP3⁺ Treg number, an impaired suppressive function of Tregs on Tconvs, and the reduced trafficking of Tregs to the BM in patients with AA [12, 13]. However, it remains unclear which phenotype best represents functional Tregs in humans. We defined Treg cells as

CD4⁺FoxP3⁺Helios⁺ T cells because Helios, a member of the Ikaros gene family, is expressed by 70–80 % of human FoxP3⁺ T cells and is known to be a good marker for natural Tregs [24, 25]. When we determined the phenotype of the CD4⁺ T cells of HVs and PRCA patients using anti-CD45RA and anti-FoxP3 Abs, we found that CD45RA⁺FoxP3^{low} cells (cytokine-producing non-Tregs) accounted for 50.5±7.5 % of FoxP3⁺ cells in HVs, 67.5–76.1 % in PRCA patients, and 54.6–67.4 % in AA patients;

however, the border between CD45RA⁺FoxP3^{high} cells and CD45RA⁺FoxP3^{low} cells was sometimes unclear in patients with PRCA and AA due to their decreased Treg percentages (Fig. 3d). Moreover, not all cytokine-producing non-Treg cells are reported to be cytokine-producing cells [16, 18]. In contrast, anti-Helios mAbs clearly divided FoxP3⁺ cells into Helios⁺ and Helios⁻ subpopulations (Fig. 1a), and Helios⁺ Treg cells rarely produced many cytokines in comparison to Helios⁻ Treg cells (Fig. 1b). Our co-culture experiments using sorted CCR7⁺IL-1R1⁺ Tregs and CCR7⁺IL-1R1⁻ Tregs, which are compatible with Helios⁻ and Helios⁺ Tregs, respectively, showed that the Helios⁺ Tregs exerted greater inhibitory effects on the proliferation of Tconvs than Helios⁻ Tregs. These results were in agreement with a previous study which found that the Helios⁻ Treg subpopulation harbors a larger population of non-suppressive clones than the Helios⁺ cell subset [17]. These results validated the use of a phenotype Helios⁺ as a marker of functional Tregs.

Our study revealed, for the first time, a significant decrease in the percentage and in the absolute count of Helios⁺ Tregs in PRCA patients regardless of CsA treatment status. Unlike AA patients, lymphopoiesis is not affected by a decrease in the number of hematopoietic stem cells in PRCA patients where only the erythroid precursors are diminished. Our results therefore substantiated that a decrease in the Treg percentage and/or an increase in the Th/Treg ratio contributes to failed hematopoiesis. One may argue that in CsA-treated PRCA patients who are in remission, the marked decrease in Tregs may be caused by the long-term administration of CsA. Several studies have shown the suppressive effects of CsA on Treg numbers [26, 27]. However, in patients with atopic dermatitis, low-dose CsA treatment (2 mg/kg/

day) results in an increase in the number of peripheral Tregs without affecting the CD3⁺, CD4⁺, and CD8⁺ cell populations [28, 29]. Similarly, a lower dose of CsA has been shown to promote Treg development in a rat cardiac allograft model [30]. A recent study also showed that clinically relevant CsA concentrations increased Treg numbers in vitro [31]. Moreover, a decrease in the Helios⁺ Treg percentage was also observed in PRCA and AA patients before CsA therapy, and the normal Treg percentage was maintained in convalescent CsA-treated AA patients. These findings suggest that a decreased in Treg count is related to the disease activity of immune-mediated bone marrow failure.

A striking difference in the response of PRCA and AA patients to CsA was the extent of Treg recovery in association with hematologic recovery. Unlike convalescent CsA-treated AA patients, the Treg percentage and the Th1/Treg ratio remained abnormal in the PRCA patients who responded to CsA. These findings are compatible with the CsA dependency that most PRCA patients show in the clinical setting. A previous study on the helper T cell subsets of PRCA patients demonstrated an increase in the number of Th2 cells in the active phase and the elevation of the Th1/Th2 ratio concomitant with hematological improvement [32]. Our study revealed no significant differences in the percentages of Th1, Th2, and Th17 cells between the PRCA patients and the HVs; however, the absolute counts of Th1, Th2, and Th17 cells were lower in the PRCA patients than in the HVs due to their low CD4 cell count. The apparent decrease in Tregs resulted in a significant increase in the Th1/Treg in convalescent and CsA-dependent PRCA patients. An imbalance between Ths and Tregs has been implicated in the disease activity of several autoimmune diseases, including rheumatoid arthritis, SLE,

Fig. 4 The effect of CsA on conventional T cell proliferation. The expression of CFSE in Tconvs after treatment with different doses of CsA (a). The inhibitory effects of CsA on Tconvs are shown (b); one representative result from three different experiments is shown

and multiple sclerosis [33–36]. Consistent with previous studies on AA using different Treg markers [12, 20], the Th1/Treg, Th2/Treg, and Th17/Treg ratios were markedly higher in our patients with active AA than in HVs. These findings suggest that increased Th1/Treg ratios are correlated with the disease activity of PRCA and AA.

Despite the heterogeneity of its pathogenesis, most PRCA patients respond to monotherapy with CsA [7, 37, 38]. The reason for the uniform response to CsA and the target of CsA remain unknown. The results of the present study suggest that the decreased Treg count is an abnormality that is common to PRCA patients and that CsA may complement the role of Tregs in vivo. This hypothesis was substantiated by our in vitro findings: 50–100 ng/ml of CsA effectively inhibited proliferation of Tconv, to a similar degree to the suppression by Tregs to Tconv at ratios of 1/3 and 1/1 of Tregs (Figs. 2d and 4b). Blood trough levels of CsA ranging 50–100 ng/ml can be achieved by the administration of 2–3 mg/kg of CsA [28]. A recent study by Philippe et al. demonstrated that an optimal CsA trough blood concentration for the treatment of SAA was 100 ng/ml [39].

In the management of PRCA and AA patients who respond to IST, physicians often have difficulties in determining when the CsA dose should be tapered off. The persistently high Th1/Treg ratio of convalescent PRCA patients and the high Th1/Treg ratios detected in patients with CsA-dependent PRCA or AA may indicate that the patients have not acquired tolerance to the autoimmune attack against hematopoietic cells and that CsA treatment, which potentially complements the role of Tregs, should be continued.

In summary, the current study demonstrated a profound decrease in Helios⁺ Tregs and an increased Th1/Treg ratio in PRCA and AA patients who were responsive to CsA. The decrease in the Treg percentage is a potential therapeutic target in PRCA. Clinical trials of cytokines that are known to augment Treg function [40–43] are warranted as a curative treatment in patients with immune-mediated BM failure who are responsive to CsA.

Acknowledgments The authors thank Professor Yasuhiko Yamamoto for his excellent technical assistance in the cell sorting procedures.

Compliance with ethical standards This study protocol (No. 287) was approved by the Ethics Committee for Human Genome/Genome Analysis Research at Kanazawa University Graduate School of Medical Science. Informed consent was obtained from all individual participants included in the study.

Funding This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 24390243 and No. 23659486).

Conflict of interest The authors declare that they have no conflict of interest.

Author contributions A.T.T.D. performed the experiments, analyzed the data, and wrote the manuscript. H.Y., H.T., C.S., T.K., H.M., Y.Z., K.M., T.Q.L., L.E., and S.N. managed the samples and collected the clinical data. S.N. interpreted the data and revised the manuscript. All of the authors approved of the final version of this manuscript.

References

1. Nitta H, Mihara K, Sakai A, Kimura A (2010) Expansion of CD8⁺/perforin⁺ effector memory T cells in the bone marrow of patients with thymoma-associated pure red cell aplasia. *Br J Haematol* 150(6):712–715. doi:10.1111/j.1365-2141.2010.08273.x
2. Nitta H, Harada Y, Hyodo H, Kimura A, Harada H (2012) Expansion of CD8⁺/perforin⁺ T-cells predicts response to ciclosporin A therapy in patients with erythroid hypoplasia/aplasia. *Br J Haematol* 157(5):641–645. doi:10.1111/j.1365-2141.2012.09057.x
3. Michishita Y, Hirokawa M, Fujishima N, Abe Y, Fujishima M, Guo YM, Ubukawa K, Liu J, Yoshioka T, Kameoka Y, Saitoh H, Tagawa H, Takahashi N, Sawada K (2013) CDR3-independent expansion of Vdelta1 T lymphocytes in acquired chronic pure red cell aplasia. *Immunol Lett* 150(1–2):23–29. doi:10.1016/j.imlet.2012.12.006
4. Fujishima N, Hirokawa M, Fujishima M, Wada C, Toyoshima I, Watanabe S, Sawada K (2006) Oligoclonal T cell expansion in blood but not in the thymus from a patient with thymoma-associated pure red cell aplasia. *Haematologica* 91(12 Suppl): ECR47
5. Shinohara K, Mitani N, Miyazaki M, Sakuragi S, Matsuda K, Ogawara S, Saito T, Kaneoka H, Ooji T (2005) Pure red-cell aplasia caused by the antibody to recombinant erythropoietin, epoetin-beta, in a Japanese patient with chronic renal failure. *Am J Hematol* 78(1):15–20. doi:10.1002/ajh.20245
6. Aljama P, Carracedo J, Madueno JA, Ramirez R, Martin-Malo A, de Francisco AL M (2005) Antibody-mediated pure red cell aplasia (PRCA): ensuring future progress by collecting data from a registry. *Nephrol Dial Transplant: Off Publ Eur Dial Transplant Assoc Eur Ren Assoc* 20(Suppl 4):iv27–30. doi:10.1093/ndt/gfh1089
7. Sawada K, Hirokawa M, Fujishima N, Teramura M, Bessho M, Dan K, Tsurumi H, Nakao S, Urabe A, Omine M, Ozawa K, Group PCS (2007) Long-term outcome of patients with acquired primary idiopathic pure red cell aplasia receiving cyclosporine A. A nationwide cohort study in Japan for the PRCA Collaborative Study Group. *Haematologica* 92(8):1021–1028
8. Saracco P, Quarello P, Iori AP, Zecca M, Longoni D, Svahn J, Varotto S, Del Vecchio GC, Dufour C, Ramenghi U, Bacigalupo A, Locasciulli A, Bone Marrow Failure Study Group of the A (2008) Cyclosporin A response and dependence in children with acquired aplastic anaemia: a multicentre retrospective study with long-term observation follow-up. *Br J Haematol* 140(2):197–205. doi:10.1111/j.1365-2141.2007.06903.x
9. Nakao S, Takamatsu H, Chuho T, Ueda M, Shiobara S, Matsuda T, Kaneshige T, Mizoguchi H (1994) Identification of a specific HLA class II haplotype strongly associated with susceptibility to cyclosporine-dependent aplastic anemia. *Blood* 84(12):4257–4261
10. Buckner JH (2010) Mechanisms of impaired regulation by CD4⁺CD25⁺FOXP3⁺ regulatory T cells in human autoimmune diseases. *Nat Rev Immunol* 10(12):849–859. doi:10.1038/nri2889
11. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T (2006) Foxp3⁺ CD25⁺ CD4⁺ natural regulatory T cells in dominant self-tolerance and

- autoimmune disease. *Immunol Rev* 212:8–27. doi:[10.1111/j.0105-2896.2006.00427.x](https://doi.org/10.1111/j.0105-2896.2006.00427.x)
12. Kordasti S, Marsh J, Al-Khan S, Jiang J, Smith A, Mohamedali A, Abellan PP, Veen C, Costantini B, Kulasekararaj AG, Benson-Quarm N, Seidl T, Mian SA, Farzaneh F, Mufti GJ (2012) Functional characterization of CD4⁺ T cells in aplastic anemia. *Blood* 119(9):2033–2043. doi:[10.1182/blood-2011-08-368308](https://doi.org/10.1182/blood-2011-08-368308)
 13. Shi J, Ge M, Lu S, Li X, Shao Y, Huang J, Huang Z, Zhang J, Nie N, Zheng Y (2012) Intrinsic impairment of CD4⁺CD25⁺ regulatory T cells in acquired aplastic anemia. *Blood* 120(8):1624–1632. doi:[10.1182/blood-2011-11-390708](https://doi.org/10.1182/blood-2011-11-390708)
 14. Kim YC, Bhairavabhotla R, Yoon J, Golding A, Thornton AM, Tran DQ, Shevach EM (2012) Oligodeoxynucleotides stabilize Helios-expressing Foxp3⁺ human T regulatory cells during in vitro expansion. *Blood* 119(12):2810–2818. doi:[10.1182/blood-2011-09-377895](https://doi.org/10.1182/blood-2011-09-377895)
 15. Raffin C, Pignon P, Celse C, Debien E, Valmori D, Ayyoub M (2013) Human memory Helios⁺ FOXP3⁺ regulatory T cells (Tregs) encompass induced Tregs that express Aiolos and respond to IL-1β by downregulating their suppressor functions. *J Immunol* 191(9):4619–4627. doi:[10.4049/jimmunol.1301378](https://doi.org/10.4049/jimmunol.1301378)
 16. Golding A, Hasni S, Illei G, Shevach EM (2013) The percentage of FoxP3⁺Helios⁺ Treg cells correlates positively with disease activity in systemic lupus erythematosus. *Arthritis Rheum* 65(11):2898–2906. doi:[10.1002/art.38119](https://doi.org/10.1002/art.38119)
 17. Bin Dhuban K, d’Hennezel E, Nashi E, Bar-Or A, Rieder S, Shevach EM, Nagata S, Piccirillo CA (2015) Coexpression of TIGIT and FCRL3 identifies Helios⁺ human memory regulatory T cells. *J Immunol* 194(8):3687–3696. doi:[10.4049/jimmunol.1401803](https://doi.org/10.4049/jimmunol.1401803)
 18. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochoy G, Sakaguchi S (2009) Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity* 30(6):899–911. doi:[10.1016/j.immuni.2009.03.019](https://doi.org/10.1016/j.immuni.2009.03.019)
 19. Solomou EE, Rezvani K, Mielke S, Malide D, Keyvanfar K, Visconte V, Kajigaya S, Barrett AJ, Young NS (2007) Deficient CD4⁺ CD25⁺ FOXP3⁺ T regulatory cells in acquired aplastic anemia. *Blood* 110(5):1603–1606. doi:[10.1182/blood-2007-01-066258](https://doi.org/10.1182/blood-2007-01-066258)
 20. de Latour RP, Visconte V, Takaku T, Wu C, Erie AJ, Sarcon AK, Desierto MJ, Scheinberg P, Keyvanfar K, Nunez O, Chen J, Young NS (2010) Th17 immune responses contribute to the pathophysiology of aplastic anemia. *Blood* 116(20):4175–4184. doi:[10.1182/blood-2010-01-266098](https://doi.org/10.1182/blood-2010-01-266098)
 21. Ryba-Stanislawowska M, Rybarczyk-Kapturska K, Mysliwiec M, Mysliwska J (2014) Elevated levels of serum IL-12 and IL-18 are associated with lower frequencies of CD4⁺CD25⁺(high)FOXP3⁺ regulatory T cells in young patients with type 1 diabetes. *Inflammation* 37(5):1513–1520. doi:[10.1007/s10753-014-9878-1](https://doi.org/10.1007/s10753-014-9878-1)
 22. Bovenschen HJ, van de Kerkhof PC, van Erp PE, Woestenrenk R, Joosten I, Koenen HJ (2011) Foxp3⁺ regulatory T cells of psoriasis patients easily differentiate into IL-17A-producing cells and are found in lesional skin. *J Invest Dermatol* 131(9):1853–1860. doi:[10.1038/jid.2011.139](https://doi.org/10.1038/jid.2011.139)
 23. Bovenschen HJ, van Vlijmen-Willems IM, van de Kerkhof PC, van Erp PE (2006) Identification of lesional CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells in psoriasis. *Dermatology* 213(2):111–117. doi:[10.1159/000093849](https://doi.org/10.1159/000093849)
 24. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM (2010) Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3⁺ T regulatory cells. *J Immunol* 184(7):3433–3441. doi:[10.4049/jimmunol.0904028](https://doi.org/10.4049/jimmunol.0904028)
 25. Himmel ME, MacDonald KG, Garcia RV, Steiner TS, Levings MK (2013) Helios⁺ and Helios[−] cells coexist within the natural FOXP3⁺ T regulatory cell subset in humans. *J Immunol* 190(5):2001–2008. doi:[10.4049/jimmunol.1201379](https://doi.org/10.4049/jimmunol.1201379)
 26. Coenen JJ, Koenen HJ, van Rijssen E, Hilbrands LB, Joosten I (2006) Rapamycin, and not cyclosporin A, preserves the highly suppressive CD27⁺ subset of human CD4⁺CD25⁺ regulatory T cells. *Blood* 107(3):1018–1023. doi:[10.1182/blood-2005-07-3032](https://doi.org/10.1182/blood-2005-07-3032)
 27. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, Negrin RS (2006) Inhibition of CD4⁺CD25⁺ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 108(1):390–399. doi:[10.1182/blood-2006-01-0329](https://doi.org/10.1182/blood-2006-01-0329)
 28. Baumgrass R, Brandt C, Wegner F, Abdollahnia M, Worm M (2010) Low-dose, but not high-dose, cyclosporin A promotes regulatory T-cell induction, expansion, or both. *J Allergy Clin Immunol* 126(1):183–184. doi:[10.1016/j.jaci.2010.04.032](https://doi.org/10.1016/j.jaci.2010.04.032), **author reply 184**
 29. Brandt C, Pavlovic V, Radbruch A, Worm M, Baumgrass R (2009) Low-dose cyclosporine A therapy increases the regulatory T cell population in patients with atopic dermatitis. *Allergy* 64(11):1588–1596. doi:[10.1111/j.1398-9995.2009.02054.x](https://doi.org/10.1111/j.1398-9995.2009.02054.x)
 30. Kawai M, Kitade H, Mathieu C, Waer M, Pirenne J (2005) Inhibitory and stimulatory effects of cyclosporine A on the development of regulatory T cells in vivo. *Transplantation* 79(9):1073–1077
 31. Fanigliulo D, Lazzerini PE, Capecchi PL, Ulivieri C, Baldari CT, Laghi-Pasini F (2015) Clinically-relevant cyclosporin and rapamycin concentrations enhance regulatory T cell function to a similar extent but with different mechanisms: an in-vitro study in healthy humans. *Int Immunopharmacol* 24(2):276–284. doi:[10.1016/j.intimp.2014.12.021](https://doi.org/10.1016/j.intimp.2014.12.021)
 32. Fujisao S, Tsuda H (1998) Th1/Th2 balance alteration in the clinical course of a patient with pure red cell aplasia and thymoma. *Br J Haematol* 103(2):308–310
 33. Alunno A, Manetti M, Caterbi S, Ibba-Manneschi L, Bistoni O, Bartoloni E, Valentini V, Terenzi R, Gerli R (2015) Altered immunoregulation in rheumatoid arthritis: the role of regulatory T cells and proinflammatory th17 cells and therapeutic implications. *Mediat Inflamm* 2015:751793. doi:[10.1155/2015/751793](https://doi.org/10.1155/2015/751793)
 34. Noack M, Miossec P (2014) Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* 13(6):668–677. doi:[10.1016/j.autrev.2013.12.004](https://doi.org/10.1016/j.autrev.2013.12.004)
 35. Dolff S, Bijl M, Huitema MG, Limburg PC, Kallenberg CG, Abdulahad WH (2011) Disturbed Th1, Th2, Th17 and T(reg) balance in patients with systemic lupus erythematosus. *Clin Immunol* 141(2):197–204. doi:[10.1016/j.clim.2011.08.005](https://doi.org/10.1016/j.clim.2011.08.005)
 36. Buc M (2013) Role of regulatory T cells in pathogenesis and biological therapy of multiple sclerosis. *Mediat Inflamm* 2013:963748. doi:[10.1155/2013/963748](https://doi.org/10.1155/2013/963748)
 37. Sawada K, Fujishima N, Hirokawa M (2008) Acquired pure red cell aplasia: updated review of treatment. *Br J Haematol* 142(4):505–514. doi:[10.1111/j.1365-2141.2008.07216.x](https://doi.org/10.1111/j.1365-2141.2008.07216.x)
 38. Hirokawa M, Sawada K, Fujishima N, Nakao S, Urabe A, Dan K, Fujisawa S, Yonemura Y, Kawano F, Omine M, Ozawa K, Group PCS (2008) Long-term response and outcome following immunosuppressive therapy in thymoma-associated pure red cell aplasia: a nationwide cohort study in Japan by the PRCa collaborative study group. *Haematologica* 93(1):27–33. doi:[10.3324/haematol.11655](https://doi.org/10.3324/haematol.11655)
 39. Philippe M, Henin E, Bertrand Y, Plantaz D, Goutelle S, Bleyzac N (2015) Model-based determination of effective blood concentrations of cyclosporine for neutrophil response in the treatment of severe aplastic anemia in children. *AAPS J* 17(5):1157–1167. doi:[10.1208/s12248-015-9779-8](https://doi.org/10.1208/s12248-015-9779-8)
 40. Samson M, Audia S, Janikashvili N, Ciudad M, Trad M, Fraszczak J, Ornetti P, Maillefert JF, Miossec P, Bonnotte B (2012) Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell

- imbalance in patients with rheumatoid arthritis. *Arthritis Rheum* 64(8):2499–2503. doi:[10.1002/art.34477](https://doi.org/10.1002/art.34477)
41. Bell CJ, Sun Y, Nowak UM, Clark J, Howlett S, Pekalski ML, Yang X, Ast O, Waldhauer I, Freimoser-Grundschober A, Moessner E, Umana P, Klein C, Hosse RJ, Wicker LS, Peterson LB (2015) Sustained in vivo signaling by long-lived IL-2 induces prolonged increases of regulatory T cells. *J Autoimmun* 56:66–80. doi:[10.1016/j.jaut.2014.10.002](https://doi.org/10.1016/j.jaut.2014.10.002)
42. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, Bellucci R, Raderschall E, Canning C, Soiffer RJ, Frank DA, Ritz J (2006) IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* 108(5):1571–1579. doi:[10.1182/blood-2006-02-004747](https://doi.org/10.1182/blood-2006-02-004747)
43. Xia J, Liu W, Hu B, Tian Z, Yang Y (2010) IL-15 promotes regulatory T cell function and protects against diabetes development in NK-depleted NOD mice. *Clin Immunol* 134(2):130–139. doi:[10.1016/j.clim.2009.09.011](https://doi.org/10.1016/j.clim.2009.09.011)