Patients with hypercortisolemic Cushing disease possess a distinct class of hematopoietic progenitor cells leading to erythrocytosis

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Abstract

Although human cell cultures stimulated with dexamethasone suggest that the glucocorticoid receptor (GR) activates stress erythropoiesis, the effects of GR activation on erythropoiesis *in vivo* remain poorly understood. We characterized the phenotype of a large cohort of patients with Cushing disease, a rare condition associated with elevated cortisol levels. Results from hypercortisolemic patients with active Cushing disease were compared with those obtained from eucortisolemic patients after remission and from volunteers without the disease. Patients with active Cushing disease exhibited erythrocytosis associated with normal hemoglobin F levels. In addition, their blood contained elevated numbers of GR-induced CD163$^+$ monocytes and a unique class of CD34$^+$ cells expressing CD110, CD36, CD133 and the GR-target gene *CXCR4*. When cultured, these CD34$^+$ cells generated similarly large numbers of immature erythroid cells in the presence and absence of dexamethasone, with raised expression of the GR-target gene *GILZ*. Of interest, blood from patients with Cushing disease in remission maintained high numbers of CD163$^+$ monocytes and, although their CD34$^+$ cells had a normal phenotype, these cells were unresponsive to added dexamethasone. Collectively, these results indicate that chronic exposure to excess glucocorticoids *in vivo* leads to erythrocytosis by generating erythroid progenitor cells with a constitutively active GR. Although remission rescues the erythrocytosis and the phenotype of the circulating CD34$^+$ cells, a memory of other prior changes is maintained in remission.

Introduction

Genetic studies have well established that activation of the glucocorticoid receptor (GR),[1] in cooperation with soluble stem cell factor (SCF),[2] is required to recover from anemia induced by exogenous insults in mice. Mice carrying *GR* encoding a protein unable to respond to glucocorticoids (*GRdim* mice)[1] have a normal hematocrit but do not readily recover from the anemia induced by hemolytic agents.[2] In addition, stress inducers such as radiation, prompt the proteolytic cleavage of the membrane-bound form of SCF, increasing the concentration of soluble SCF in the circulation.[2] As in the *GRdim* mice, mice that harbor a *SCF* gene encoding a protein lacking this proteolytic site also exhibit a normal hematocrit and are unable to recover from anemia induced by radiation. Mechanistically, the link between GR activation and soluble SCF is provided by the observation that soluble human SCF is uniquely able to activate the ERK signal that stabilizes the GR protein which in turn enables the cells to respond to glucocorticoids.[3]

By contrast, in humans most of the information available on the functions of GR has been obtained using surrogate *in vitro* models, represented by *ex-vivo* culture of CD34$^+$ cells derived from peripheral blood[4] and stimulated by cocktails of growth factors containing the GR agonist dexamethasone plus soluble SCF.[5] As a result of this, the picture of the ef-

fects of GR on human erythropoiesis is confounded by the fact that in many of these *in vitro* culture systems, human hematopoietic cells are stimulated with soluble murine SCF, which indeed sustains their differentiation, but is unable to activate ERK and to stabilize GR, thus making the cells partially unresponsive to glucorticoids.[3,6] Given that glucocorticoids are widely used for treatment of various disorders, including the anemia of patients with Diamond-Blackfan anemia, knowledge on the *in vivo* effects of chronic GR activation *in vivo* is of great clinical relevance. Unfortunately, the only information available on the effects of chronic GR activation *in vivo* in humans is provided by the erythrocytosis reported for one patient with Cushing disease,[7] a rare endocrine disorder (incidence of 1.2-2.4 cases/million persons/year[8]) characterized by chronic excessive glucocorticoid production due to an adrenocorticotropic hormone-secreting pituitary adenoma.[9] Cushing disease is characterized not only by excess levels of circulating glucocorticoids, but also by flattened cortisol diurnal rhythms and resistance to cortisol suppression by exogenous glucocorticoids. The latter is defined by the lack of appropriate serum cortisol suppression after administration of dexamethasone, due to autonomous production of adrenocorticotropic hormone by the tumor, resulting in increased systemic cortisol levels with lack of normal feedback inhibition. Eucortisolemia, normal circadian rhythms, and normal cortisol feedback inhibition are restored over time after surgical removal of the adrenocorticotropic hormone-secreting tumor, which is the first-line treatment for patients with Cushing disease.[10] Untreated Cushing disease results in increased mortality and multiple morbidities due to chronic hypercortisolemia (visceral obesity, diabetes, hypertension, cardiovascular disease, myopathy, immune suppression, cognitive deficits and mood disorders).

We hypothesized that studies on hypercortisolemic and eucortisolemic Cushing patients would greatly increase our knowledge on the effects of long-term treatments with glucocorticoids in humans. To test this hypothesis, we characterized here the blood counts and the response to glucocorticoids *in vitro* of circulating $CD34^+$ cells in what is a relatively large cohort of patients with Cushing disease, given the rarity of the disease: 13 patients with active disease and 13 in a remission phase). Given the prior observations that glucocorticoids stimulate increases in body mass and that fat cells in the bone marrow exert an important regulatory role on hematopoiesis,[11] results were compared with those obtained in parallel from subjects of similar body mass and age without Cushing disease (matched controls, MC).

Methods

Human subjects

Blood from patients with Cushing disease in an active or remission phase, i.e., before and after surgically induced remission (mean time since surgery, 14.5 months) and from MC was collected at the Mount Sinai Hospital for a prospective cohort study, the clinical registry of which contained 28 patients with active Cushing disease, 11 paired remission cases and 13 MC. Of these patients, this study analyzes blood from 13 with active disease, 13 (unpaired) cases in remission and eight MC (Table 1) provided as de-identified material according to the Mount Sinai Hospital Institutional Review Board-approved protocol dedicated to studies on Cushing disease (Principal Investigator, Eliza Geer, at the time Medical Director, Mount Sinai Pituitary Care and Research Center).

Blood parameters and cortisol levels

Blood values from patients with Cushing disease and from MC were determined by the Mount Sinai Hospital clinical laboratory and were retrieved from the patients' charts.

Twenty-four hour urinary free cortisol and serum cortisol levels were quantified by gas chromatography-mass spectroscopy as described elsewhere.[12] The limit of sensitivity of this assay is 2 ng with interassay coefficients of variation <10%.

High performance liquid chromatography

High performance liquid chromatography analysis of globin chains was performed using a Bio-Rad (Hercules, CA) CDM System (CDM 5.1 VII Minion) as described previously.[13] Individual globin chain levels were quantified on a Shimadzu Prominence instrument with an SPD-10AV diode array detector and an LC-10AT binary pump (Shimadzu, Kyoto, Japan).

Erythroid cultures

Mononuclear blood cells (MNC) were separated by centrifugation at 400 g for 30 min over Ficoll-Hypaque (Amersham-Pharmacia Biotec, Uppsala, Sweden). Human erythroblasts were obtained by culturing 10^6 MNC/mL for 10 days in Iscove modified Dulbecco medium (Mascia Brunelli, Milan, Italy) containing 20% fetal bovine serum (Hyclone, Logan, UT, USA), SCF (10 ng/mL; Amgen, Thousand Oaks, CA, USA), erythropoietin (1 U/mL; Amgen), interleukin-3 (1 ng/mL; Bouty, Milan, Italy), dexamethasone (10^{-6} M) and estradiol (10^{-6} M) (both from Sigma), as described elsewhere.[5] Selected experiments were conducted in parallel in cultures supplemented with growth factors and with or without dexamethasone.

Colony-forming cell assays

Colony-forming cells were assayed by culturing MNC ($3x10^5$ cells/mL) for 12 days in methylcellulose cultures (MethoCult, Stem Cell Techonologies, Inc., Vancouver, British Columbia, Canada) stimulated with human SCF (10 ng/mL; Amgen), interleukin-3 (10 ng/mL, Biosource, San Jose, CA, USA), and erythropoietin (Amgen) as described previously.[14]

Table 1. Clinical data of the subset of patients and matched controls from the prospective cohort study included in the present investigation.

Active phase					Remission phase					Controls			
Patients' number/ID	**Ethnicity**	**Sex**	**Weight Kg**	**Serum cortisol μg/dL**	**Patients' number/ID**	**Ethnicity**	**Sex**	**Weight Kg**	**Serum cortisol μg/dL**	**Patients' number/ID**	**Ethnicity**	**Sex**	**Weight Kg**
P1/02-030	*Caucasian*	*M*	*78.2*	*27.5*	*P25/02-030*	*Caucasian*	*M*	*75.6*	*1*	P16/C18	n.a.	F	na
P6/02-034	*Caucasian*	*F*	*69.3*	*19.2*	*P36/02-034*	*Caucasian*	*F*	na	*19.6*	P23/C23	Caucasian	M	77.9
P8/02-035	*Caucasian*	*M*	*75.3*	*32.2*	*P26/02-035*	*Caucasian*	*M*	*67.7*	*18.9*	P24/C24	Asian	M	107.3
P12/02-037	*Hispanic*	*F*	*71.7*	*15.2*	*P27/02-037*	*Hispanic*	*F*	*67.4*	*3.5*	P29/C26	Asian	M	79.5
P2/02-031	Caucasian	F	86.7	15	P14/02-029	Caucasian	F	101.6	3.1	P33/C27	Caucasian	F	86.9
P10/02-036	African American	F	130.5	24.2	P5/02-023	Caucasian	F	80.4	2.5	P34/C28	Caucasian	F	62
P15/02-038	Caucasian	M	n.a.	5.2	P13/02-017	Caucasian	F	64	7.1	P37/C29	na	F	96
P19/02-040	Caucasian	M	88.1	16.7	P21/02-018	Caucasian	M	103.9	11.1	P38/C30	na	F	na
P20/02-042	Caucasian	F	98	17	P7/02-025	African American	F	88.1	15.9				
P28/02-044	Caucasian	F	68.4	18.9	P32/02-014	Caucasian	F	68.6	24.3				
P30/02-045	Caucasian	F	110.6	24.7	P3/02-032	African American	F	na	13.7				
P31/02-046	Caucasian	F	78.4	32.7	P4/02-033	Caucasian	F	na	2.3				
P35/02-047	Caucasian	M	n.a.	35.2	P17/02-039	Caucasian	F	59.7	9.7				

Four patients analyzed both in the active and in the remission phase are indicated in italics. ID: identity; M: male; F: female; na: information not available.

Flow cytometry

CD34$^+$ cell determinations and phenotyping were conducted by incubating MNC with primary antibodies for 30 min at 4°C and fluorescent signals were measured with a FACS Canto II (Becton and Dickinson, Franklin Lakes, NJ, USA) and analyzed using FlowJo v7.6.4/v10 (Ashland, OR, USA). Cells were exposed to allophycocyanin (APC)-CD34 with either fluorescein isothiocyanate (FITC)-CD36, phycoerythrin-cyanine7 (PE-Cy7)-CD117, Pacific blue-CD123, phycoerythrin (PE)-CXCR4, PE-CD110, PE-CALR or FITC-CD133. Dead cells were excluded by Sytox Blue AlexaFluor 430 staining. Cultured erythroblasts were profiled with FITC-CD36 and APC-CD235a.[15] The frequency and phenotype of blood monocytes were assessed by analyzing the percentage of MNC that bound PE-Cy7-CD14, APC-CD169 and PE-CD163 (all from BD Biosciences, San Jose, CA, USA).

Western blot analysis

Whole cell extracts (30 μg protein/lane) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes which were probed with primary antibodies against either GRα (sc-8992H300), glucocorticoid-induced leucine zipper (GILZ, sc-33780) (all from Santa Cruz Biotechnology, Dallas, TX, USA), pGRS211 (4161, Cell Signaling, Danvers, MA, USA), pGRS203 (Ab195703, Abcam, Cambridge, MA, USA) and GAPDH, (CB1001, Calbiochem, San Diego, CA, USA) and appropriate horseradish peroxidase-coupled secondary antibodies (Calbiochem).

Statistical analyses

Data are presented as box charts (plus minimum and maximum values) or as the mean (± standard error of mean), as indicated. Values from patients with active disease or disease in remission and from MC were compared using the Tukey multiple comparisons test while those obtained with and without dexamethasone within the same groups were compared by a paired *t*-test using GraphPad 9.2 (SAS Institute, Cary, NC, USA). Differences were considered statistically significant with a $P<0.05$.

Results

Patients with active Cushing disease exhibit erythrocytosis with nearly normal levels of fetal hemoglobin

To clarify the effects of dexamethasone on stress erythropoiesis *in vivo*, we conducted a thorough characterization of erythropoiesis in a relatively large cohort of hypercortisolemic Cushing patients in the active phase and after achieving eucortisolemia due to surgically induced remission (mean time since surgery, 14.5 months), along with non-diseased controls matched for weight (body mass index 33.3 kg/m^2 in active-phase patients and 30.7 kg/m^2 in MC; range, 26.7-34.7 kg/m^2, $P=0.073$) and age (mean age 41 years in both groups). In the prospective Cushing disease cohort, as expected the mean serum cortisol levels in the active-phase patients were significantly higher than in MC and remission-phase patients (Table 2). The differences between patients in the active phase and those in remission remained statistically significant also in the cohorts of patients included in this study (Tables 1 and 2).

In the subset of 13 active-phase patients included in this study, hematocrit levels were higher than those of MC while hemoglobin and platelet counts were similar in the two groups (Figure 1A, C). Retrospective analyses of the data present in the clinical database indicated that, overall, active-phase patients had significantly higher hematocrit levels than remission-phase patients and that in the analyses conducted on a subset of 11 patients with paired samples, hemoglobin and platelet levels were significantly higher during the active phase than during remission (Figure 1B). Unexpectedly, hematocrit and cortisol (serum and urine) levels were not correlated either in active-phase or remission-phase patients (*Online Supplementary Figure S1*). This lack of correlation may in part be due to the wide intra-patient variability in cortisol values.[16] Other biophysical properties of red blood cells (red blood cell distribution width, mean corpuscular volume, mean cell hemoglobin, and mean corpuscular hemoglobin concentration) were also similar among the two groups and MC (*Online Supplementary Figure S2*). White blood cell counts were higher in active-phase patients than in MC and remission-phase patients (Figure 1D). Analysis of white blood cell subpopulations indicated that the increases were due to greater numbers of neutrophils, while, as expected due to the anti-inflammatory effect of GR activation, frequencies of lymphocytes and eosinophils were lower in patients with active Cushing disease than in patients

Table 2. Mean cortisol levels in the serum of patients with active Cushing disease and patients in remission included in the whole prospective cohort study with respect to the levels observed in donors without Cushing disease matched for weight, age and sex and in those included in the study (see Table 1 for details).

	Mean cortisol levels, μg/dL (prospective cohort study)	Mean cortisol levels, μg/dL (sub-set of patients in Table I)
Matched controls	8.5±8 (N=13)	na
Active	22.2±6.3 (N=11) ($P=3x10^{-7}$ *vs* MC)	21.3±8.8 (N=13)
Remission	8.7±3.9 (N=11) ($P=0.006$ *vs.* A)	10.2±7.7 (N=14) ($P=0.002$ *vs.* A)

na: not available; MC: matched controls; A: patients with active Cushing disease.

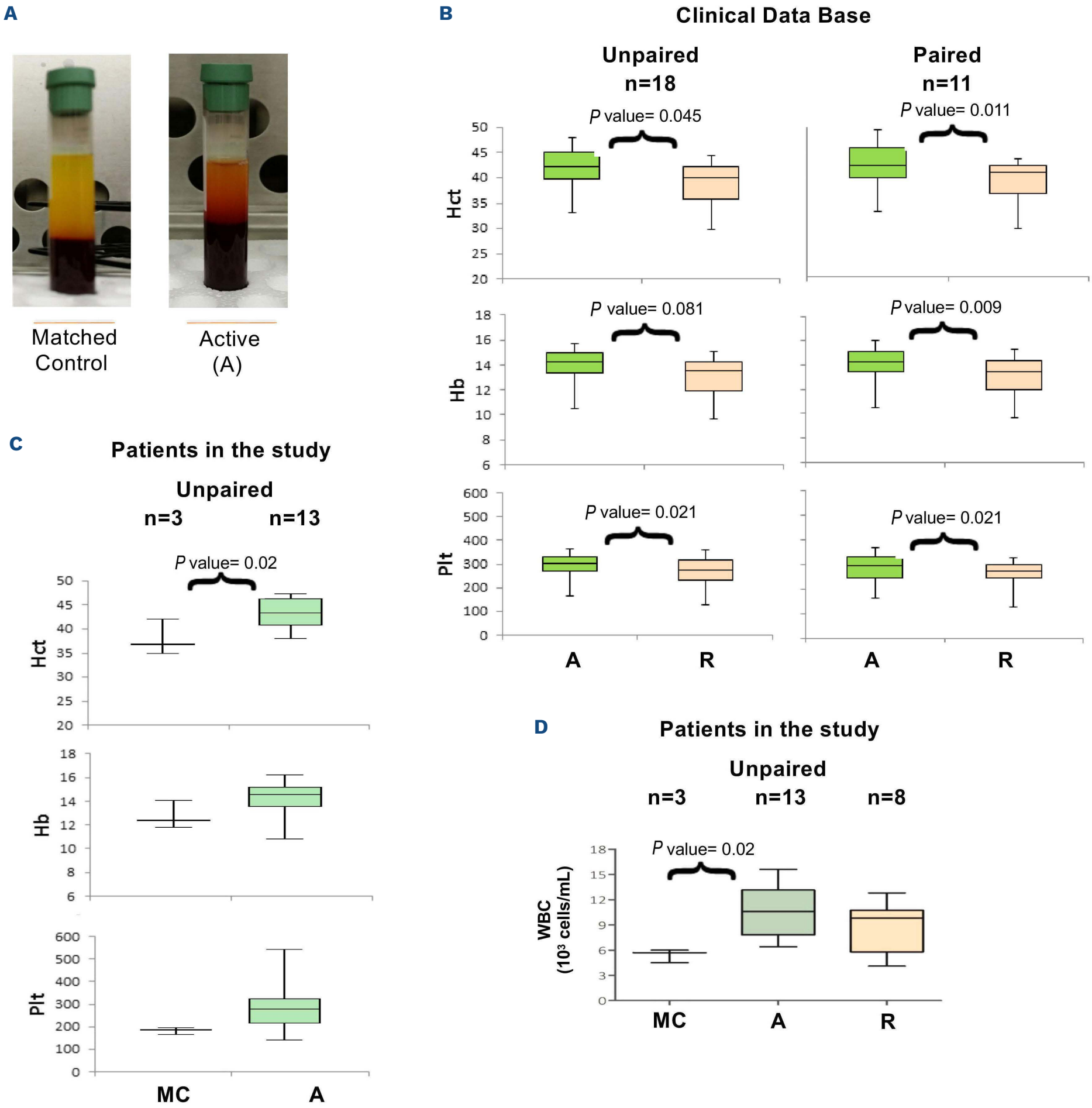


Figure 1. Patients with active Cushing disease have higher hematocrit, hemoglobin level, and platelet and white blood cell counts than patients in remission. (A) Photograph of blood tubes from one representative patient with active Cushing disease and one matched control (MC). (B) Hematocrit, hemoglobin levels and platelet counts for the whole cohort of patients with Cushing disease with active disease or in remission included in the clinical database (unpaired, left panel, and paired, right panel). (C) Hematocrit, hemoglobin levels and platelet counts of the MC and patients with active Cushing disease included in the study. (D) White blood cell counts for patients with active-phase or remission-phase Cushing disease and the MC included in the study. The number of patients included in each analysis is indicated by n. *P* values were calculated in (B) and (C) by a *t* test and in (D) by the Tukey multiple comparisons test. A: patients with active Cushing disease; R: patients with Cushing disease in remission; MC: matched controls (matched for age, weight and sex) without Cushing disease; Hct: hematocrit (%); Hb: hemoglobin (g/dL); Plt: platelet count (x10^9/L); WBC: white blood cell count.

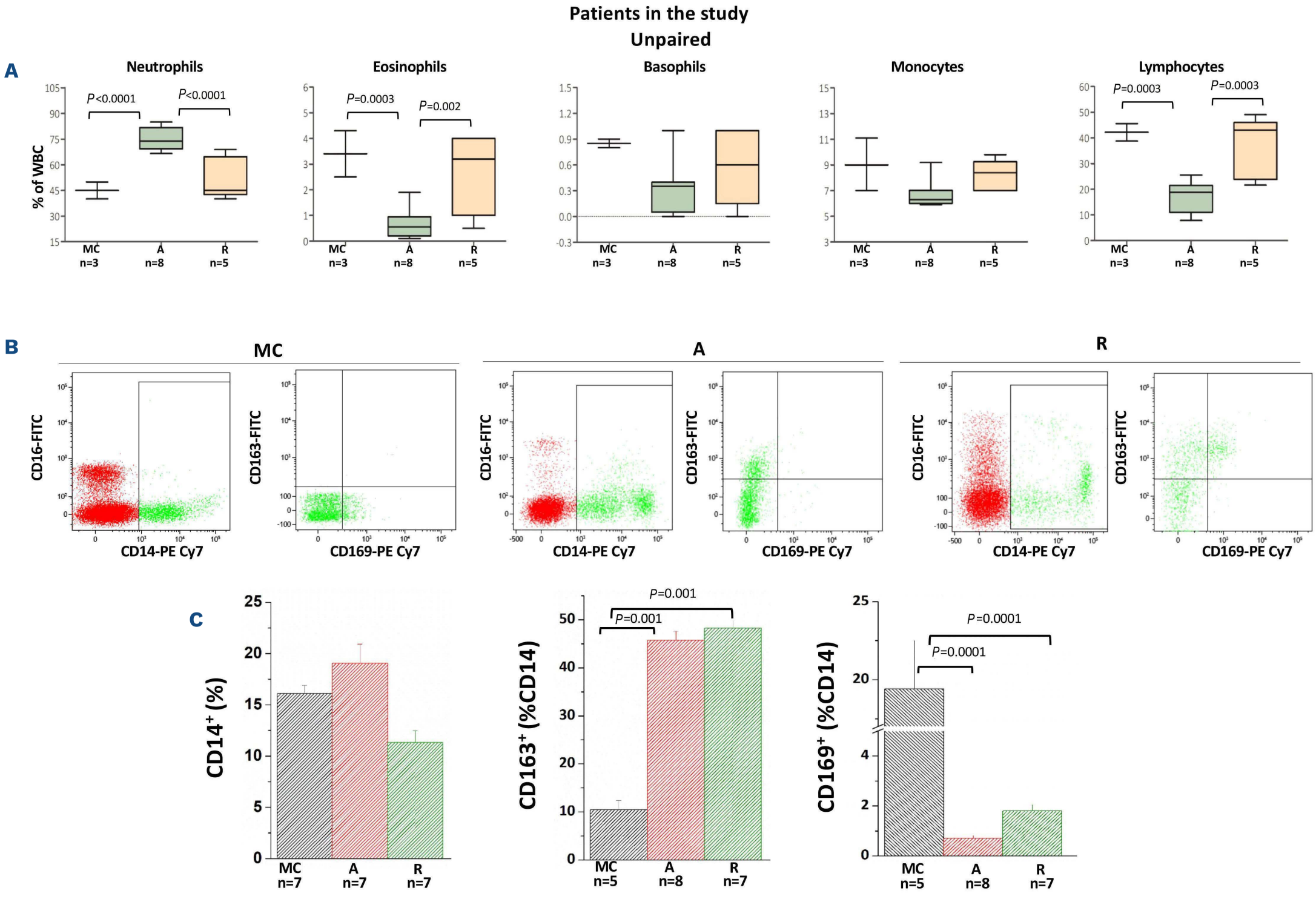


Figure 2. Although the frequency of total monocytes in blood from Cushing patients (with active disease or in remission) and from matched controls is similar, the frequency of monocytes expressing CD163, which is induced by glucocorticoids in patients with Cushing disease, is greater than in matched controls. (A) Neutrophil, eosinophil, basophil, monocyte and lymphocyte counts in the blood from patients with Cushing disease (active and in remission) and matched controls (MC) included in the study. (B, C) Percentages of $CD14^+$ cells and $CD14^+$ cells expressing CD163 or CD169 in the blood from Cushing patients (active and in remission) and MC. Representative flow cytometry data are presented in (B) and the mean (± standard deviation) of multiple determinations are presented in (C). The number of patients included in each analysis is indicated by n. *P* values were calculated by the Tukey multiple comparisons test and those statistically significant are indicated in the panel. WBC: white blood cells; A: patients with active Cushing disease; R: patients with Cushing disease in remission; MC: matched controls (matched for age, weight and sex) without Cushing disease.

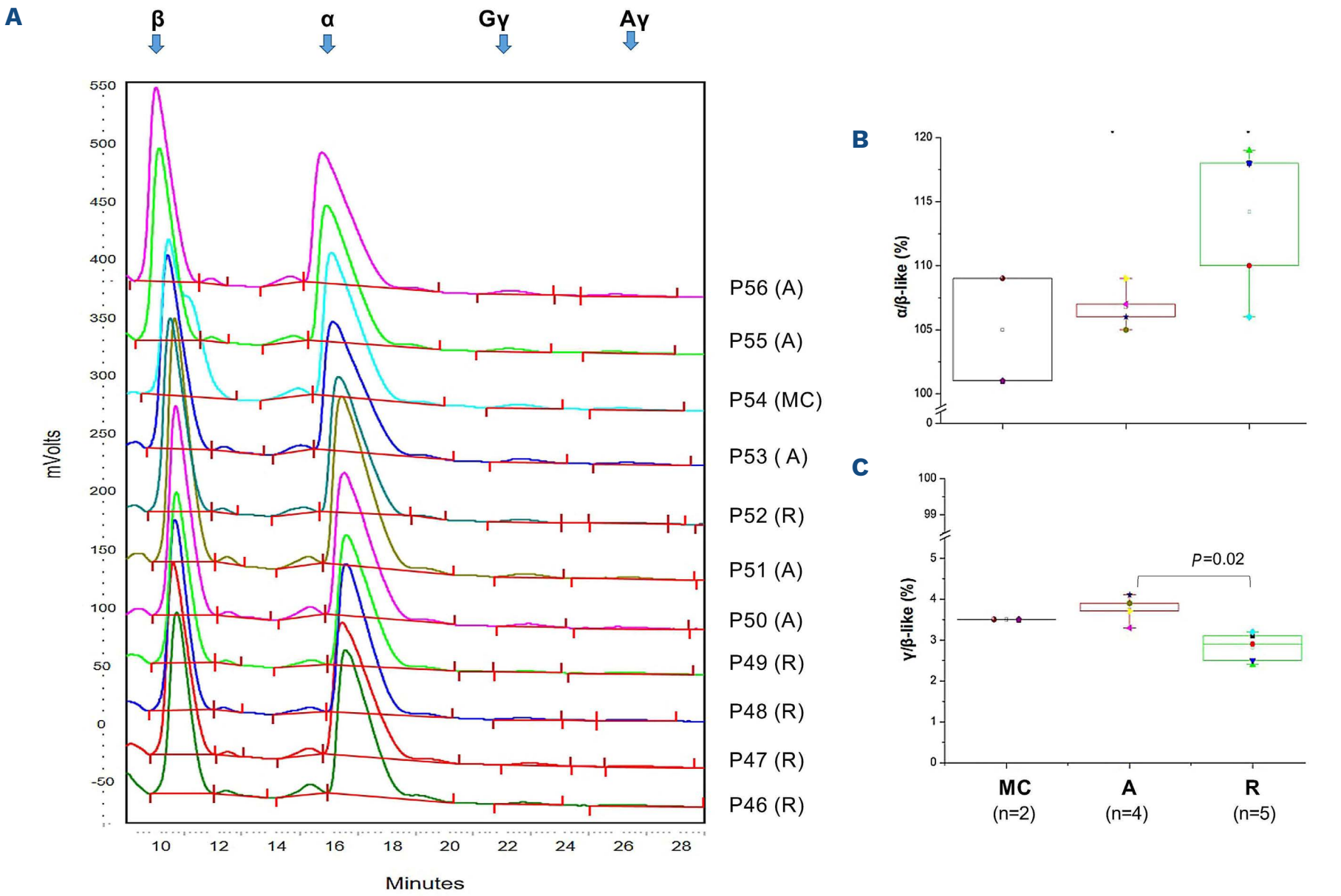


Figure 3. Patients with Cushing disease express overall normal levels of fetal hemoglobin. (A) High performance liquid chromatography determination of globin chain levels in red blood cells from representative patients with active Cushing disease and Cushing disease in remissions and matched controls (each graph represents a separate subject, as indicated; patients are identified with the same alpha-numerical code indicated in Table 1). (B, C) Average ratios of α/β-like and γ/β-like globins in red blood cells from active-phase and remission-phase patients and matched controls. *P* values were calculated by the Tukey multiple comparisons test. A: patients with active Cushing disease; R: patients with Cushing disease in remission; MC: matched controls (matched for age, weight and sex) without Cushing disease.

in remission or in MC (Figure 2A). Although the frequency of monocytes was comparable among groups, the phenotype of CD14$^+$ cells in active-phase patients was skewed toward the CD163$^+$ phenotype while the frequency of CD14$^+$ cells expressing CD169 was reduced in these patients (Figure 2B, C). Since the haptoglobin-hemoglobin scavenger receptor CD163 is induced in monocytes by GR activation[17,18] and CD169 is an interferon-α-induced inflammatory response probably suppressed by GR,[19] the CD163$^+$CD169$^-$ phenotype of the CD14$^+$ cells from patients with active-phase Cushing disease provides an internal control showing that their hypercortisolemia is affecting their hematopoietic cells. In addition, the fact that the CD14$^+$ cells from patients in remission remain skewed toward the CD163$^+$/CD169$^-$ phenotype suggests that the monocytes maintain a memory of their activation state upon clinical remission. Whether the blood monocytes in Cushing disease, contribute to the erythroid-specific macrophage niche in the bone marrow is not known.

High performance liquid chromatography analyses of red blood cell lysates indicated that patients with active disease expressed slightly higher levels of α- and fetal γ-globin chains than those of patients in remission, although overall, these levels were within the range found in MC (Figure 3). These results indicate that patients with active Cushing disease experience erythrocytosis with normal hemoglobin F levels.

The blood from patients with active Cushing disease contains CD34$^+$ cells with a stress-like phenotype

Recent studies of cultures stimulated with BMP4 or dexamethasone have identified a novel class of erythroid progenitor cells defined as stress-progenitor cells, which express CD110 (MPL, the thrombopoietin receptor[20]) and CD36 (the thrombospondin receptor[21]). In addition, murine erythroblasts generated with dexamethasone express higher levels of CXCR4 (the SDF1/CXCL12 receptor)[22,23] and in human erythroblasts, GR is chaperoned to the nucleus

by the Ca^{2+}-protein calreticulin (CARL) in response to dexamethasone.[15] Therefore, to investigate whether constitutive GR activation in Cushing patients alters the phenotype of the erythroid progenitor cells, circulating $CD34^+$ cells from Cushing patients with active disease and disease in remission, as well as from MC, were profiled with a panel of antibodies that identify stress-progenitor cells (CD110 and CD36)[21,24] or proteins regulated by (CXCR4)[23] or that regulate (CALR)[15] the response to glucocorticoids. The panel also included CD117 and CD123, antibodies against receptors for SCF (cKIT[25]) and interleukin-3,[26] as indicators of cell responsiveness to the growth factors used to stimulate the erythroid cultures (Figure 4).

The frequency of $CD34^+$ cells in the blood of the three ex-

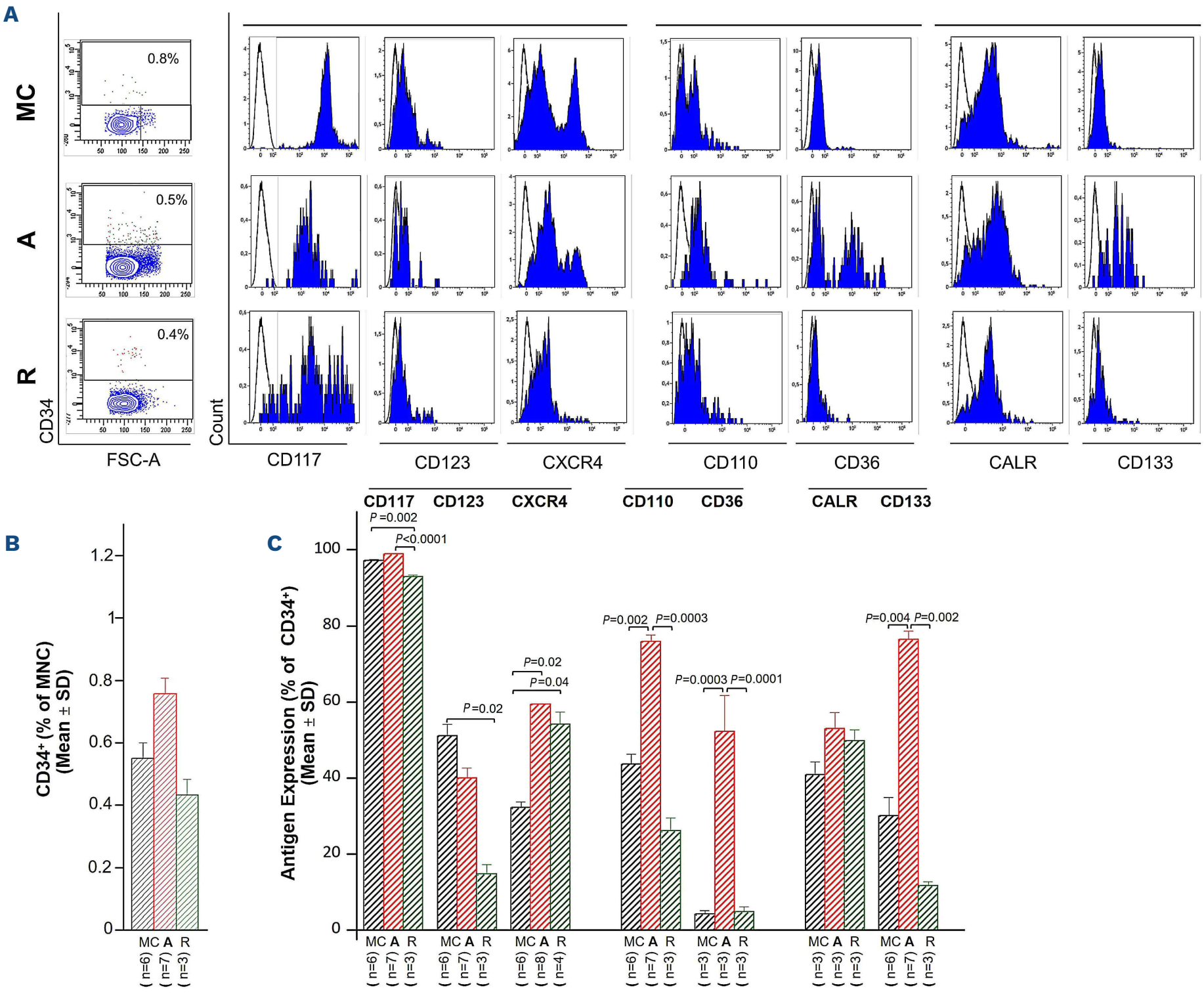


Figure 4. The blood from patients with active Cushing disease contains normal numbers of $CD34^+$ cells that express a stress-like phenotype characterized by high cell surface levels of the glucocorticoid receptor target gene CXCR4, of CD110, CD36 and of the stem cell marker CD133. (A) Representative forward side scatter and CD34 staining of mononuclear cells from the blood of one representative Cushing disease patient with active disease and one in remission as well as the cells from a matched control (MC) are presented on the left. Cells in the $CD34^+$ gate were then analyzed for expression of CD117, CD123, CXCR4, CD110, CD36, CALR and CD133, and the results presented as histograms in gray on the right. The numbers within the plots indicate the frequency of the cell populations within the gate. The blue histograms indicate the signal from irrelevant isotype-matched controls analyzed in parallel. (B) Frequency of $CD34^+$ cells in the mononuclear blood cells from multiple active-phase and remission-phase patients and MC. (C) Frequency of $CD34^+$ cells expressing CD117, CD123, CXCR4, CD110, CD36, CALR and CD133 from active-phase and remission-phase patients and MC. The number of different individuals included in the various groups is indicated by n. *P* values were calculated with the Tukey multiple comparisons test and those statistically significant are indicated in the panels. A: patients with active Cushing disease; R: patients with Cushing disease in remission; MC: matched controls (matched for age, weight and sex) without Cushing disease); FSC: forward scatter; MNC: mononuclear cells; SD: standard deviation.

perimental groups was similar (0.4-0.6% of the MNC) (Figure 4A, B). These frequencies among the three groups remained similar when the cells were functionally evaluated based on their ability to form colonies in semisolid cultures (30.2±27.2, 23.7±13.2 and 16.5±11.5 CFC/10^5 MNC in active-phase patients, remission-phase patients and MC, respectively).

The frequency of CD34$^+$ cells from active phase patients which bind CD117, CD123 and CALR was similar to that from MC (Figure 4A, C). Since response to SCF and interleukin-3 is associated with down-modulation of their receptors,[27] the normal frequency of CD34$^+$ cells expressing these receptors in active-phase patients suggests that they respond readily to SCF and interleukin-3 *in vivo*. In addition, the proportion of CD34$^+$ cells that expressed CXCR4, CD36, CD110 and CD133 was greater in patients with active Cushing disease than in MC. A greater proportion of CD34$^+$ cells from remission-phase patients expressed CXCR4 than did MC cells while a lower proportion of them expressed CD117/CD123, suggesting that CD34$^+$ cells from Cushing patients in remission respond more and less readily than normal to CXCL12 and SCF/IL3, respectively. However, the proportions of CD34$^+$ cells from remission-phase patients expressing all the other antigens were similar to those of the cells from MC (Figure 4A, C). A comparison of the antigen mean fluorescent intensity among groups indicates that, with the exception of CXCR4 which was expressed at levels lower than normal by remission-phase CD34$^+$ cells, and of CD36 and CARL, which were respectively expressed at levels higher and lower than normal by active-phase CD34$^+$ cells, the level of expression per cell of all the other antigens analyzed was comparable among groups (*Online Supplementary Figure S3*).

These results suggest that the circulating progenitor cells from patients with active Cushing disease constitute a unique population, likely generated from hematopoietic stem cells in response to the high cortisol levels found in these patients.

CD34$^+$ cells from the blood of patients with active Cushing disease generate similarly high numbers of erythroid cells when cultured with or without glucocorticoids

MNC from patients with active Cushing disease generated similar numbers of erythroblasts in 10-day cultures with or without dexamethasone, a large proportion of which were immature under both conditions. In contrast, and as expected, cells from MC generated higher numbers of erythroblasts, a good proportion of which were immature, in culture with dexamethasone than without dexamethasone (Figure 5A, B). Surprisingly, MNC from patients in remission generated similarly low numbers of erythroblasts, with a similar maturation profile in cultures with or without dexamethasone (Figure 5C), suggesting that the apparently normal progenitor cells observed in these patients retain some memory of altered response to GR activation.

Erythroid cells generated in culture by CD34$^+$ cells from patients with active Cushing disease display an activated glucocorticoid receptor signal when generated in culture stimulated or not with glucocorticoids

To provide mechanistic insights into the altered response to dexamethasone of erythroblasts from active-phase and remission-phase patients, biochemical studies of cells generated with and without dexamethasone from MC and Cushing patients with active disease or in remission were conducted (Figure 6). These studies compared the content of total GRα, the glucocorticoid binding isoform of the receptor, as well as of GRαS211, which is translocated to the nucleus, and of GRαS203, which is retained in the cytoplasm.[28] Additionally, we analyzed levels of GILZ, a downstream target commonly used as a gold standard to measure the transcriptional activity of GR.[29] As expected given the frequent polymorphism of GRα in the human population,[29] great variability was observed in the GR content of erythroblasts obtained from different individuals (Figure 6A, B, *Online Supplementary Figure S4*). Erythroblasts generated with dexamethasone from active-phase and remission-phase patients as well as MC expressed equivalent levels of total GRα and GRαS211, but those from patients with active disease contained lower levels of GRαS203 than those from remission-phase patients or MC, although the low number (n=2) of active-phase patients investigated does not allow assessment of whether the difference is statistically significant (Figure 6B). The difference in GRαS203 content between the active and remission phases was conserved at the stoichiometry level (i.e., when normalized with respect to the corresponding GR content) (Figure 6C). Finally, erythroblasts from patients with active disease contained significantly greater amounts of GILZ than those from remission-phase patients or MC (Figure 6B) and erythroblasts from active-phase patients generated with or without dexamethasone expressed similar stoichiometric levels of GRαS203 and GRαS211 and similar levels of GILZ when compared to the levels of GRαS211 (Figure 6D).

These results suggest that the lack of response to exogenous glucocorticoids induced by chronic glucocorticoid exposure is mediated by mechanisms that alter the efficiency of the nuclear/cytoplasmic transport of GRα, rather than by those that interfere with the transcription/translation of its mRNA.

Discussion

By studying the erythroid compartments of a relatively large cohort of patients with Cushing disease in active and remission phases we confirm that chronic exposure to excess glucocorticoids *in vivo* leads to erythrocytosis and provide

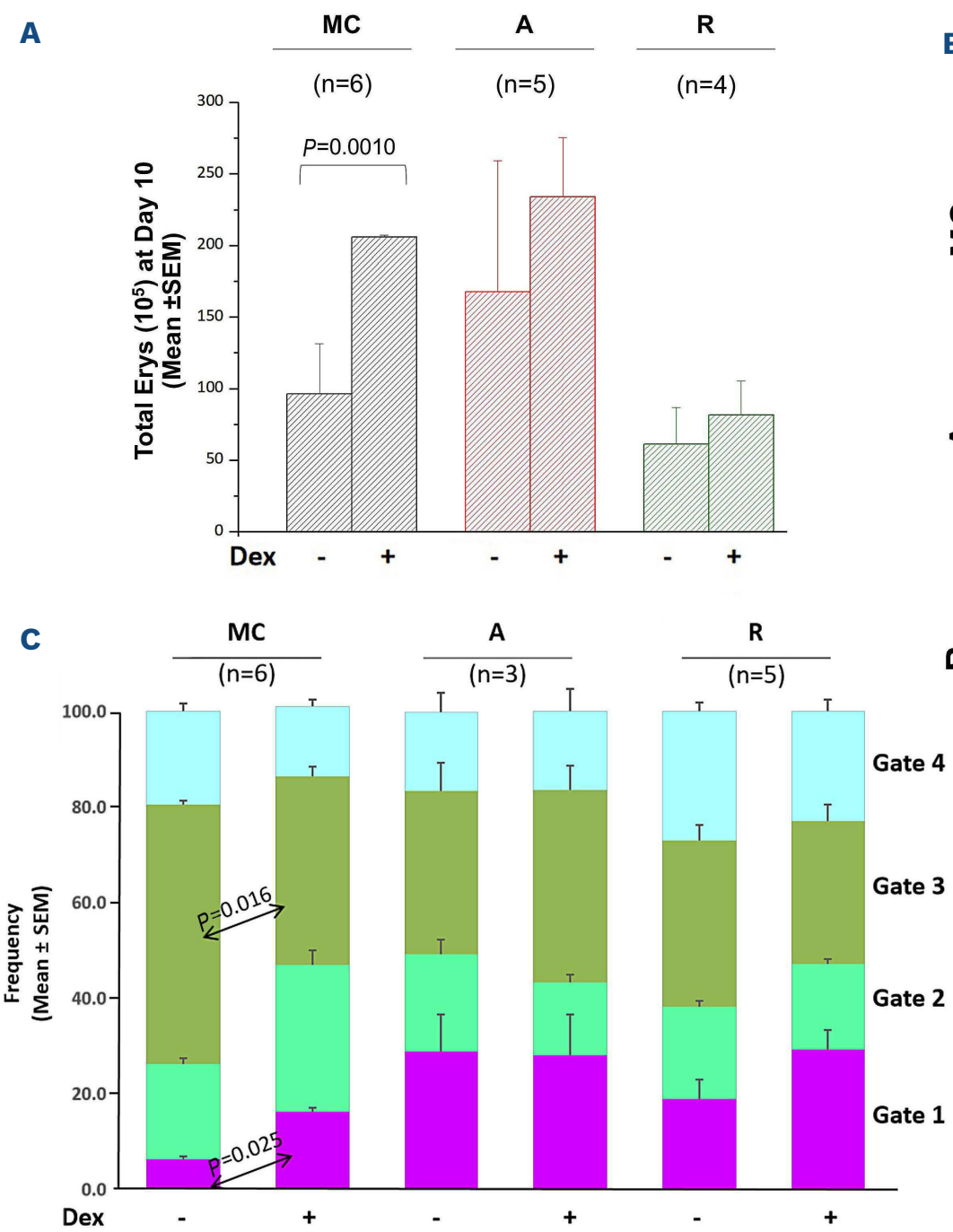
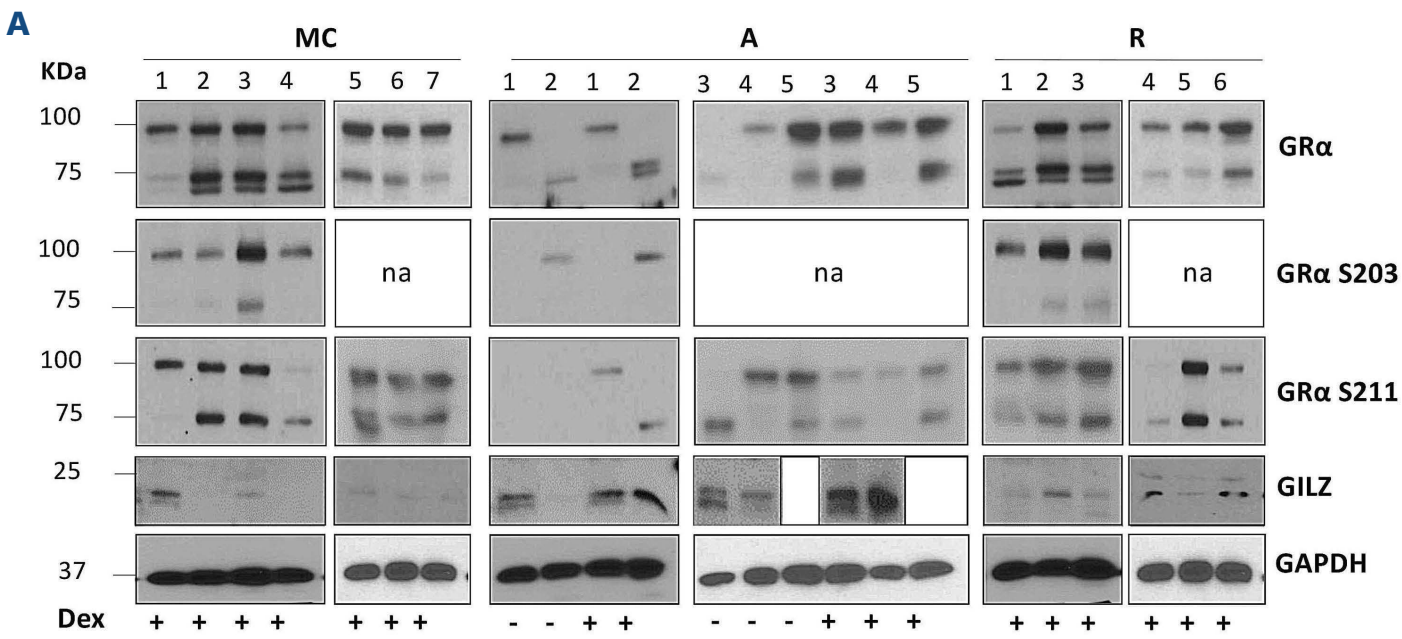


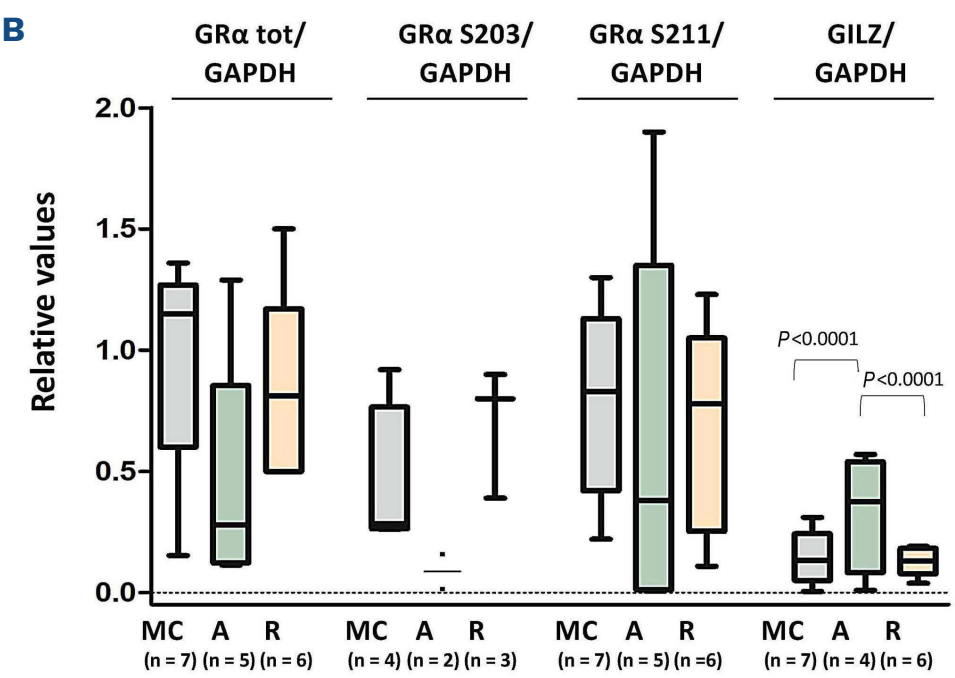
Figure 5. Erythroid progenitor cells from patients with active Cushing disease generate similarly high numbers of erythroblasts in cultures stimulated or not with dexamethasone. (A) Total numbers of erythroblasts generated by day 10 by erythoid progenitor cells from matched controls (MC), patients with active Cushing disease and patients with Cushing disease in remission cultured with or without dexamethasone, as indicated. The total number of erythroblasts was calculated by multiplying the total number of cells in each culture by the corresponding percentage of cells with the CD235a/CD36 phenotype determined by fluorescence activated cell sorting. (B) Flow cytometry analyses for CD235a/CD36 expression of cells generated at day 10 by $CD34^+$ cells from representative active- and remission-phase Cushing patients and MC. CD235a/CD36 staining divides erythroid cells in to $CD235a^{neg}/CD36^{pos}$ (gate 1, proerythroblasts, purple), $CD235a^{low}/CD36^{pos}$ (gate 2, basophilic erythroblasts, light green), and $CD235a^{medium}/CD36^{pos}$ (gate 3, polychromatophilic erythroblasts, dark green) and $CD235a^{pos}/CD36^{pos}$ (gate 4, orthochromatic erythroblasts, light blue), as reported.[17] Non-erythroid cells are $CD235^{neg}CD36^{neg}$ (dark blue) and are mainly lymphocytes. Results are presented as counterplots which allow for a better distinction of the clusters of the individual cell populations. (C) Frequency of cells in gates 1, 2, 3 and 4 generated by day 10 by $CD34^+$ cells from MC and active-phase and remission-phase Cushing patients cultured with or without dexamethasone (the same color code as in panel B). Note that erythroblasts from MC are already mostly mature at day 10 in cultures without dexamethasone. By contrast, at day 10 similar numbers of immature erythroblasts are observed in cultures with or without dexamethasone from active-phase and remission-phase patients. The number of different individuals included in the various groups is indicated by n. Statistical analyses between cultures with or without dexamethasone in the same group were performed by a paired *t* test and statistically significant values are indicated in the panel. Statistical analyses among groups was performed by the Tukey multiple comparisons test as appropriate. Results of the statistical analyses are as follows. (A) Paired *t* test: MC without dexamethasone *vs*. MC with dexamethasone, *P*=0.001; active- or remission-phase Cushing patient with or without dexamethasone, not statistically different. Tukey multiple comparisons test: MC with dexamethasone *vs*. remission-phase patient with dexamethasone, *P*=0.0003 and *vs*. remission-phase patient without dexamethasone, *P*=0.0002; MC without dexamethasone *vs*. remission-phase patient with dexamethasone, P=0.03 and *vs*. remission-phase patient without dexamethasone, *P*=0.02; active-phase patient with dexamethasone *vs*. remission-phase patient with dexamethasone, *P*=0.03 and *vs*. remission-phase patient without dexamethasone, *P*=0.02; active phase patient without dexamethasone *vs*. remission-phase patient without dexamethasone, *P*=0.04. All the other comparisons were not statistically different. (C) Paired *t*-test: gates 1 and 3 for MC with or without dexamethasone, *P*=0.02 for both; gates for active-phase and remission-phase patients with and without dexamethasone are not statistically different. Tukey multiple comparisons test: no statistically significant differences among gates for MC *vs*. active-phase patients or *vs*. remission-phase patients with or without dexamethasone. Erys: erythroblasts; SEM: standard error of mean; Dex: dexamethasone; A: patients with active Cushing disease; R: patients with Cushing disease in remission; MC: matched controls (matched for age, weight and sex) without Cushing disease; FITC; fluorescein isothiocyanate; APC: allophycocyanin.

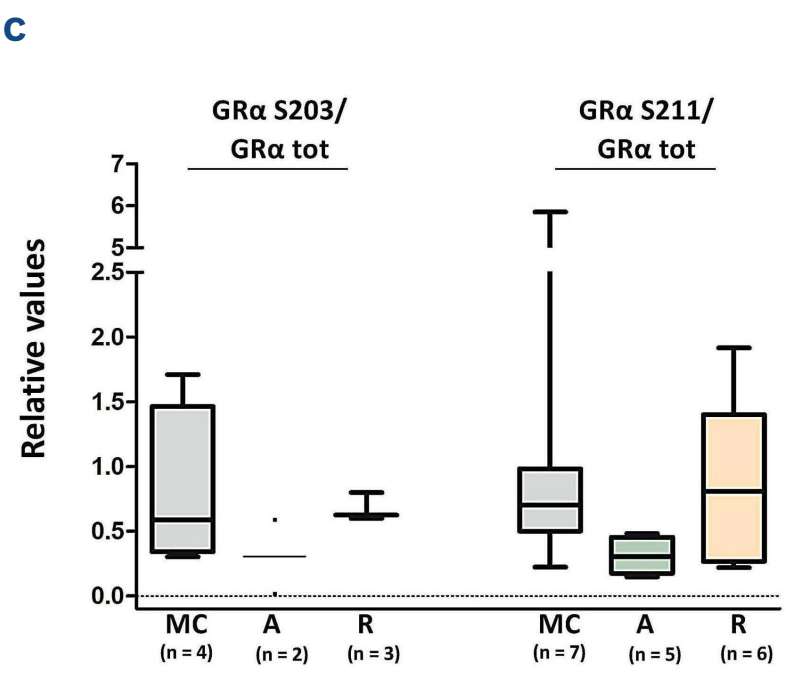
new information on the cells targeted by this receptor *in vivo* and on the biochemical/molecular consequences of its activation.

Cellular targets

There is considerable confusion in the literature on the identity of the erythroid cells targeted by dexamethasone. Time-response and *ex-vivo* amplification studies indicate that the primary human cells that respond to dexamethasone are the burst-forming unit-erythroid (BFU-E) while colony-forming unit-erythroid (CFU-E) cells are insensitive to GR activation.[30] Using cells prospectively isolated from







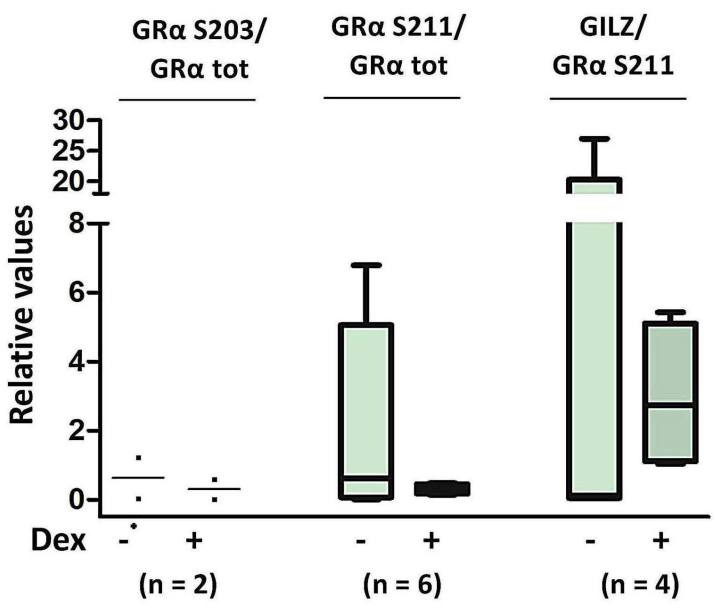


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Figure 6. Erythroblasts from patients with active Cushing disease contain normal levels of total glucocorticoid receptor (GR), which is poorly phosphorylated in the inactive cytoplasmic-retained S203 form, and express high levels of the S211 form and of the GR-target gene GILZ in cultures with and without dexamethasone. (A) Western blot analyses for total GRα and its nuclear (GRαS211) and cytoplasmic (GRαS203) forms, as well of the GR target gene GILZ of erythroblasts generated at day 10 by representative patients with active Cushing disease or disease in remission and matched controls (MC) (each number a different donor) in culture with and without (active-phase patients only) dexamethasone. The position of the molecular weight markers is indicated on the left. (B) Content of total GRα, GRαS203, GRαS211 and GRαS203 with respect to that of GAPDH in erythroblasts generated from cells from active- and remission-phase patients and from MC cultured with dexamethasone. (C) Stoichiometry levels of GRαS203 and GRαS211 in cells from active- and remission-phase patients and from MC cultured with dexamethasone. (D) Stoichiometry levels of GRαS203 and GRαS211 and levels of GILZ with respect to those of GRαS211 in cultures of cells from active-phase patients cultured with or without dexamethasone. In (B-D), results are expressed as box charts with minimum and maximum values observed in multiple individuals in each group. *P* values among groups were calculated by the Tukey multiple comparisons test in (B) and (C) and by a paired *t* test in (D). The number of individuals included in each group is indicated by n. A: patients with active Cushing disease; R: patients with Cushing disease in remission; MC: matched controls (matched for age, weight and sex), without Cushing disease; na: not available; Dex: dexamethasone.

mouse fetal liver, the team headed by Lodish confirmed these results by identifying steady-state BFU-E as the target of GR activation.[31] By contrast, the sequence of differentiated cells that are progressively generated by human steady-state CD34$^+$ cells *ex-vivo* in response to dexamethasone are all capable of responding to dexamethasone.[21,24] Thus, whereas erythroblasts generated *in vivo* lose the ability to respond to dexamethasone (and SCF)[14] between the BFU-E and the CFU-E stage, those generated *in vitro* with dexamethasone remain dexamethasone- (and SCF-)[27] responsive up to the pro-erythroblast stage. The fact that the GR-sensitive BFU-E and CFU-E express, respectively, CD110 and CD105, an antibody that recognizes endoglin,[32,33] has suggested that in cultures stimulated with dexamethasone, multipotent CD34$^+$ cells activate an alternative differentiation pathway that generates stress-specific erythroid progenitors. Whether these stress-specific cell populations are also generated in response to chronic erythroid stress *in vivo* in humans has not yet been investigated.

By profiling the CD34$^+$ cells from the blood of a large cohort of patients with active (hypercortisolemic) Cushing disease, we identified for the first time a population of primary CD34$^+$ cells which express CD36 and CD110, a phenotype that is similar to that of the stress progenitor cells observed in cultures of human CD34$^+$ cells stimulated with dexamethasone.[21,24] How representative these stress-specific cells are of the hematopoietic progenitors in the bone marrow of Cushing patients remains to be established. In addition to the CD36 and CD110 phenotype, another distinct feature in patients with Cushing disease was the increased frequency of CD34$^+$CD133$^+$ cells. Expression of CD133 has been previously noted among CD34$^+$ cells in bone marrow[34] and it was later documented that its presence was marking very long-term repopulating cells in transplantation experiments[35] and early hematopoietic progenitors which, if they co-expressed VEGFR-2,[36] represented endothelial progenitors. Whether the above quantitative changes in distinct phenotypes seen in Cushing patients reflect changes seen *in vivo* in other stress-like conditions is at present unclear and further studies are needed. Of further relevance, previous studies suggested that *in vitro* cultures of CD34$^+$ cells from either peripheral blood or bone marrow from normal subjects gave rise to increased fetal globin levels. Examination of *in vivo* responses during acute erythropoietic demands also documented transient increases in fetal globin.[37] However, such increases were not maintained under chronic stress conditions.[38] Therefore, being under chronic steroid stress, it may not be surprising that Cushing patients did not show increases in fetal globin. Furthermore, it should be added that there is no evidence that addition of steroids *in vitro* specifically contributes to increases in fetal globin. In fact, the opposite is true since addition of steroids to cells at a developmental transition level accelerated the fetal to adult switch.[39]

Molecular mechanisms of glucocorticoid receptor resistance in Cushing disease and implications for chronic glucocorticoid therapy of hematopoietic disorders

The important role played by GR in the stress response inspired the development of GR agonists to treat several stress-related disorders (inflammation, autoimmune diseases, Diamond Blackfan anemia, etc.). A general feature of these therapies is that eventually patients become unresponsive to treatment. Given the clinical relevance of this unresponsiveness, the mechanisms leading patients to lose responsiveness to glucocorticoids have been the subject of extensive investigation. Human *GR* (*GR/NR3C1*) is highly polymorphic, displaying numerous single nucleotide polymorphisms in the coding region and in regions associated with alternative splicing and mRNA stabilization, resulting in more than 260 combinations of alternative GR isoforms with a wide range of affinities for glucocorticoids expressed in humans.[29] Microenvironmental cues, such as its ligand and/or soluble SCF, may contribute negatively and/or positively to the variability of the response to glucocorticoids in the human population.[3,40] In addition, prolonged GR activation may induce epigenetic modifications of the gene promoter, suppressing its expression,[41] and/or post-transcriptional protein modifications, affecting its nuclear trafficking. In this regard, it has been demonstrated that

glucocorticoid stimulation may induce phosphorylation of GR not only at S211, resulting in nuclear translocation and activation of transcriptional activity,[42] but also at S203 which instead retains the receptor in the cytoplasm, inhibiting its transcriptional activity.[43] The data presented here indicate that CD34$^+$ cells from all patients with Cushing disease (including those with active disease and those in remission) are resistant or insensitive to exogenous glucocorticoid stimulation. In the case of active Cushing disease, the observation that cells generated with or without dexamethasone *in vitro* express similarly high levels of GRαS211 and GILZ suggests constitutive activation of GR signaling. By contrast, the CD34$^+$ cells from patients in remission, which are also not responsive to dexamethasone, do generate low numbers of mature erythroblasts in cultures with and without dexamethasone and these express low levels of GILZ, indicating that they are unable to activate GR signaling. Of interest, the large number of patients (and MC) included in the analyses allowed us to exclude, despite the great variability of GR content among cells obtained from different individuals, the hypothesis that variegation in response is due to differences in protein content, making it unlikely that lack of response in Cushing disease is mediated by the polymorphism of the gene and/or its epigenetic regulation. Instead, we observed an association with the levels of phosphorylation of GRS203. In fact, while erythroblasts from both active- and remission-phase patients contained GR phosphorylated at S211, which is required for nuclear translocation, the levels of GR phosphorylated at S203 in patients upon remission was much greater than those observed in two patients in the active phase. These data indicate that nuclear/cytoplasmic trafficking plays a significant role in restraining the cellular response to glucocorticoids also in patients with Cushing disease. They suggest, in fact, that activated GR is constitutively retained in the nucleus of cells from active patients, making them insensitive to *de-novo* stimulation. By contrast, patients in remission are insensitive to *de-novo* stimulation because, due to high levels of S203 phosphorylation, their GR is retained in the cytoplasm, as a memory of the mechanism which attempted to limit their response in the hypercortisolemic state. This hypothesis is consistent with the clinical observation that long-term comorbidities are seen in patients with Cushing disease despite remission, including persistent obesity, increased cardiovascular risk, evidence of abnormal systemic inflammation[44–46] (and high numbers of CD163$^+$ monocytes in blood) (Figure 2), as a memory of their previously hypercortisolemic features. This hypothesis is testable and is consistent with studies that are investigating whether chemical inhibition of S203 may restore response to glucocorticoids in patients with other disorders who became resistant to dexamethasone.[47,48] In addition, this hypothesis may be relevant to understand glucocorticoid resistance in patients with Diamond Blackfan anemia, 60% of whom became transfusion dependent upon treatment.[49] The mechanism that induces loss of glucocorticoid response in Diamond Blackfan anemia is a subject of intensive investigation. Recently, the Blanc laboratory has proposed that patients with Diamond Blackfan anemia become glucocorticoid-unresponsive because their stress-CFU-E fail to self-replicate in response to glucocorticoids since, through a mechanism still to be identified, they are unable to activate p57^{kip2}.[24] Based on our results in Cushing patients, we speculate that the stress-CFU-E from unresponsive Diamond Blackfan anemia patients fail to activate p57^{kip2} because they express high levels of GRαS203 which inhibits the transcriptional activity of the receptor. Thanks to the availability of inhibitors of GRS203, this hypothesis is testable and will be analyzed in separate studies.

In conclusion, blood from patients with active Cushing disease contains a unique CD34$^+$ cell population in which GR is constitutively active and does not respond to exogenous dexamethasone. Surprisingly, CD34$^+$ cells in the blood from patients in remission are phenotypically normal but retain an abnormal response to exogenous dexamethasone.

Disclosures

No conflicts of interest to disclose.

Contributions

LV and FM performed experiments and analyzed the data. MM performed statistical analyses. EG provided patients' information, samples and clinical insights on Cushing disease. AF and JB analyzed the data and wrote the manuscript. TP and ARM designed the study, interpreted the data and wrote the manuscript. All the authors read the manuscript and concur with its content.

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Data-sharing statement

Primary data will be disclosed upon request.

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