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Hypercortisolemic Cushing's patients possess a distinct class of hematopoietic progenitor cells leading to erythrocytosis

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Authors Contribution. LV and FM performed experiments and analyzed the data. MM performed statistical analyses. EG provided patient information, samples and clinical insights on Cushing’s Disease. AF and JB analysed 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.
**Data sharing statement.** Primary data will be disclosed upon request

**ABSTRACT**

Although human cultures stimulated with dexamethasone suggest that the glucocorticoid receptor (GR) activates stress erythropoiesis, the effects of GR activation on erythropoiesis in vivo remains poorly understood. We characterized the phenotype of a large cohort of patients with Cushing’s Disease, a rare condition associated with elevated cortisol levels. Results from hypercortisolemic patients with active Cushing’s were compared with those obtained from eucortisolemic patients after remission and from non-diseased volunteers. Active Cushing’s patients exhibit erythrocytosis associated with normal hemoglobin F levels. In addition, their blood contained elevated numbers of the 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 Cushing’s patients 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 (GR\(_{dim}\) mice)\(^1\) have 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 GR\(_{dim}\) 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 (Dex) plus soluble SCF\(^5\). As a result of this, the picture of the effects of GR on human erythropoiesis is confounded by the fact that in many of these in vitro culture system, 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 glucocorticoids\(^3,6\). Given that glucocorticoids are widely used for treatment of various disorders, including the anemia of patients with Diamond-Blackfan Anemia (DBA), 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’s Disease\(^7\), a rare endocrine disorder (incidence of 1.2-2.4/million/year\(^8\)) characterized by chronic excessive glucocorticoid production due to an adrenocorticotropic hormone-secreting pituitary adenoma\(^9\). Cushing’s Disease is defined not only by excess levels of circulating glucocorticoids, but also by flattened cortisol diurnal rhythms and resistance to suppression by exogenous glucocorticoids. The latter is defined by lack of appropriate serum cortisol suppression after dexamethasone administration, due to autonomous tumoral ACTH production 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 ACTH-secreting tumor, which is the first-line treatment for patients with Cushing’s Disease\textsuperscript{10}. Untreated Cushing’s 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’s patients would greatly increase our knowledge on the effects of long-term treatments with glucocorticoids in humans. To test this hypothesis, we characterize here the blood counts and the response to glucocorticoids in vitro of circulating CD34+ cells in a relatively large cohort of patients with Cushing’s Disease, given the rarity of the disease (13 in the active, A, and 13 in the remission, R, 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\textsuperscript{11}, results were compared with those obtained in parallel from non-diseases controls (matched controls, MC) of similar body mass and age.

**METHODS**

**Human subjects.** Blood from patients with Cushing’s Disease before (A) and after (R) surgical remission (mean time since surgery=14.5 months) and from matched non-diseased controls (MC) was collected at the Mount Sinai Hospital from a prospective cohort study, the clinical registry of which contained 28 A, 11 paired R and 13 MC. Of those patients, this study analyzes blood from 13 A, 13 R (unpaired) and 8 MC (Table 1) provided as de-identified material according to the Mount Sinai Hospital IRB protocol dedicated to studies on Cushing’s Disease (PI = Eliza Geer, at the time Medical Director, Mount Sinai Pituitary Care and Research Center).

**Blood parameters.** Blood values from patients with Cushing’s Disease and MC were determined by the Mount Sinai Hospital clinical laboratory and were retrieved from the patients’ charts.

**Cortisol Levels.** Twenty-four hour Urinary Free Cortisol (UFC) and serum cortisol levels were quantified by gas chromatography-mass spectroscopy as described\textsuperscript{12}. The limit of sensitivity of this assay is 2ng with inter-assay coefficients of variations <10%.
**High performance liquid chromatography (HPLC).** HPLC analysis of globin chains was performed using a Bio-Rad (Hercules, CA) CDM System (CDM 5.1 VII Minion) as described\textsuperscript{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 400gx30min over Ficoll-Hypaque (Amersham-Pharmacia Biotec, Uppsala, Sweden). Human erythroblasts (Erys) were obtained by culturing 10⁶ MNC per mL for 10days in Iscove’s modified Dulbecco’s medium (Mascia Brunelli, Milan, Italy) containing 20% fetal bovine serum (Hyclone, Logan, UT, USA), SCF (10ng/mL) (Amgen, Thousand Oaks, CA), erythropoietin (1u/mL, Amgen), interleukin-3 (IL-3, 1ng/mL, Bouty, Milan, Italy), Dex (10⁻⁶M) and estradiol (10⁻⁶M) (both from Sigma), as described\textsuperscript{5}. Selected experiments were conducted in parallel in cultures supplemented with growth factors plus and minus Dex.

**Colony forming cell (CFC) determinations.** CFC were determined by culturing MNC (3x10⁵ cells/mL) for 12 days in methylcellulose cultures (MethoCult, Stem Cell Technonologies, Inc., Vancouver, BC) stimulated with human SCF (10 ng/mL, Amgen, Thousand Oaks, CA), interleukin-3 (10 ng/mL, Biosource, San Jose, CA), and erythropoietin (Amgen) as described\textsuperscript{14}.

**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) 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 AlexaFluor430 staining. Cultured Erys were profiled with FITC-CD36 and APC-CD235a\textsuperscript{15}. Frequency and phenotype of blood monocytes was assessed by analyzing the percentage of MNC that bound PE-Cy7-CD14, allophycocyanin (APC)-CD169 and PE-CD163 (all from BD Biosciences, San Jose, CA).

**Western Blot analysis.** Whole cell extracts (30μg protein/lane) were separated on SDS-PAGE and transferred to nitrocellulose membranes which were probed with primary antibodies against either GRα (sc-8992H300), GILZ (sc-33780) (all from Santa Cruz Biotechnology, Dallas, TX), pGRS211 (4161, Cell Signaling, Danvers, MA), pGRS203 (Ab195703, Abcam, Cambridge,
MA) and GAPDH (CB1001, Calbiochem, San Diego, CA) and appropriate horseradish peroxidase-coupled secondary antibodies (Calbiochem).

**Statistical Analyses.** Data are presented as box charts (plus min and max value) or as Mean (±SEM), as indicated. Values from A, R and matched MC were compared by Tukey's multiple comparisons test while those obtained with and without Dex within the same group were compared by paired t test using GraphPad 9.2 (SAS Institute, Cary, NC, USA). Differences were considered statistically significant with a p< 0.05.

**RESULTS**

**Active Cushing’s patients exhibit erythrocytosis with nearly normal levels of fetal hemoglobin (HbF).** To clarify the effects of Dex on stress erythropoiesis in vivo, we conducted a thorough characterization of erythropoiesis in a relatively large cohort of hypercortisolemic Cushing’s patients in the active phase (A) and after achieving eucortisolemia due to surgical remission (R) (mean time since surgery=14.5 months), along with non-diseased controls (MC) matched for weight (body mass index=33.3 kg/m² in A and 30.7 kg/m² in MC, range 26.7-34.7, p=0.073) and age (mean age=41 years in both groups).

In the prospective Cushing’s Disease cohort, as expected the mean serum cortisol levels in A were significantly higher than in MC and R (Table 2). The differences in A vs R patients remain statistically significant also in the cohorts of patients included in this study (Tables 1,2).

In the subset of 13 A patients included in this study, hematocrit levels (Hct) were higher than those of MC while hemoglobin and platelet counts were similar among the two groups (Figure 1A,B). Retrospective analyses of the data present in the clinical data base indicate that overall A patients have significantly higher hematocrit levels than R patients and that in the analyses conducted on a subset of 11 paired patients, hemoglobin and platelet levels in A are also significantly greater than in R (Figure 1C). Unexpectedly, hematocrit and cortisol (serum and urine) levels were not correlated neither in A nor in R patients (Figure S1). This lack of correlation may in part be due to the wide intra-patient variability in cortisol values16. Also similar among the two groups and MC were other biophysical properties (red blood cell distribution width, RDW; mean
corpuscular volume, MCV; mean cell hemoglobin, MCH; and mean corpuscular hemoglobin concentrations, MCHC) of the red cells (Figure S2).

White blood cell counts (WBC) were higher in A than in MC and R (Figure 1D). Analysis of WBC subpopulations indicated that the increases are due to greater numbers of neutrophils, while, as expected due to anti-inflammatory effect of GR activation, frequencies of lymphocytes and eosinophils were lower in A than in R and MC (Figure 2A). Although the frequency of monocytes was comparable among groups, the phenotype of CD14+ cells in A 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\(^\text{17,18}\) and CD169 is an interferon-\(\alpha\)-induced inflammatory response probably suppressed by GR\(^\text{19}\), the CD163+CD169- phenotype of the CD14+ cells from A provides an internal control showing that their hypercortisolemia is affecting their hematopoietic cells. In addition, the fact that the CD14+ cells from R patients remained skewed toward the CD163+/CD169- phenotype, suggests that the monocytes maintain a memory of their activation state upon clinical remission. Whether in Cushing’s Disease, the blood monocytes contribute to the erythroid-specific macrophage niche in the bone marrow is not known.

HPLC analyses of red blood cells lysates indicated that A patients expressed levels of \(\alpha\)-and fetal \(\gamma\)-globin chains slightly higher than that of R patients, although overall, these levels were within the range found in MC (Figure 3). These results indicate that active Cushing’s patients experience erythrocytosis with normal HbF levels.

The blood from A Cushing’s patients contains CD34+ cells with a stress-like phenotype. Recent studies of cultures stimulated with BMP4 or Dex have identified a novel class of erythroid progenitor cells defined as stress-progenitor cells that express CD110 (MPL, the thrombopoietin receptor\(^\text{20}\)) and CD36 (the thrombospondin receptor\(^\text{21}\)). In addition, murine Erys generated with Dex express greater levels of CXCR4 (the SDF1/CXCL12 receptor\(^\text{22,23}\)) and in human Erys, GR is chaperoned to the nucleus by the Ca++-protein calreticulin (CARL) in response to Dex\(^\text{15}\). Therefore, to investigate whether in Cushing’s patients constitutive GR activation alters the phenotype of the erythroid progenitor cells, the circulating CD34+ cells from A and R patients, as well as from MC, were profiled with a panel of antibodies that
identify stress-progenitor cells (CD110 and CD36) or proteins regulated by (CXCR4) or that regulate (CALR) the response to glucocorticoids. The panel also included CD117 and CD123, antibodies against receptors for SCF (cKIT) and IL-3, 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 experimental 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/10⁵ MNC in A, R and MC, respectively].

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

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

CD34+ cells from the blood of active Cushing’s patients generate similarly high numbers of erythroid cells when cultured with and without glucocorticoids. MNC from A patients generated similar numbers of Erys in 10 day cultures with or without Dex, a large proportion of which were immature under both conditions. In contrast, and as expected, cells
from MC generated higher numbers of Erys, a good proportion of which were immature, in
culture with Dex than without Dex (Figure 5A,B). Surprisingly, MNC from R patients
generated similarly low numbers of Erys, with a similar maturation profile in cultures with or
without Dex (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 active Cushing’s patients
display an activated GR signal when generated in culture stimulated with and without
glucocorticoids. To provide mechanistic insights into the altered response to Dex of Erys from
A and R patients, biochemical studies of cells generated with and without Dex from MC, A and
R subjects 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. Additionally, we analyzed
levels of GILZ, a downstream target commonly used as a gold standard to measure the
transcriptional activity of GR. As expected given the frequent polymorphism of GRα in the
human population, great variability was observed in the GR content of Erys obtained from
different individuals (see Figures 6A,B, S4). Erys generated with Dex from A, R and MC
expressed equivalent levels of total GRα and GRαS211, but those from A contained lower
levels of GRαS203 than those from R or MC, although the low number (n=2) of A patients
investigated does not allow assessing whether the difference is statistically significant (Figure
6B). The difference in GRαS203 content between A and R was conserved at the stoichiometry
level (i.e. when normalized with respect to the corresponding GR content) (Figure 6C). Finally,
Erys from A contained significantly greater levels of GILZ than those from R or MC (Figure
6B) and Erys generated by A with and without Dex 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’s Disease in the active and remission phases we confirm that chronic exposure to excess glucocorticoids in vivo leads to erythrocytosis and provide 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 Dex. Time-response and ex-vivo amplification studies indicate that the primary human cells that respond to Dex are the BFU-E while CFU-E are insensitive to GR activation. Using cells prospectively isolated from the mouse fetal liver, the Lodish laboratory confirmed these results by identifying steady-state BFU-E as the target of GR activation. By contrast, the sequence of differentiated cells that are progressively generated by human steady-state CD34+ cells ex-vivo in response to Dex are all capable of responding to Dex. Thus, whereas Erys generated in vivo lose the ability to respond to Dex (and SCF) between the BFU-E and the CFU-E stage, those generated in vitro with Dex remain Dex- (and SCF-) 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, has suggested that in cultures stimulated with Dex, 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 been investigated.

By profiling the CD34+ cells from the blood of a large cohort of active (hypercortisolemic) Cushing’s patients, we identify 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 Dex. How representative these stress-specific cells are of the hematopoietic progenitors in the bone marrow of Cushing’s patients remains to be established. In addition to the CD36 and CD110 phenotype, another distinct feature in Cushing’s patients was the increased frequency of CD34+CD133+ cells. Expression of CD133 has been previously noted among CD34+ cells in BM and later documented that its presence was marking very long-term repopulating cells in transplantation
experiments and early hematopoietic progenitors which, if 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’s patients did not show fetal globin increases. 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 GR resistance in Cushing’s and implication 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 (DBA), etc). A general feature of these therapies is that eventually patients became unresponsive to treatment. For its clinical relevance, the mechanisms leading these patients to become unresponsive to glucocorticoids has been the subject of extensive investigation. Human GR (GR/NR3C1) is highly polymorphic, displaying numerous SNPs in the coding region and in regions associated with alternative splicing and mRNA stabilization, resulting in >260 combinations of alternative GR isoforms with a wide range of affinity for glucocorticoids expressed in the human population \(^{29}\). Microenvironmental cues, such as its ligand and/or soluble SCF, may negatively and/or positively contribute 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
Cushing’s patients (including active and in remission) are resistant or insensitive to exogenous glucocorticoid stimulation. In the case of active Cushing’s, the observation that their cells generated with and without Dex 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 are also not responsive to Dex but in this case they generate low numbers of mature Erys in cultures with and without Dex that 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’s is mediated by the polymorphism of the gene and/or its epigenetic regulation. We observe instead an association with the levels of phosphorylation of GRS203. In fact, while Erys from both active and remission patients contain GR phosphorylates at S211, which is required for nuclear translocation, the levels of GR phosphorylated at S203 in patients upon remission is much greater than those observed in 2 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 Cushing’s patients. 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. The hypothesis is consistent with the clinical observation that long-term comorbidities are seen in patients with Cushing’s despite remission, including persistent obesity, increased cardiovascular risk, evidence of abnormal systemic inflammation\textsuperscript{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 glucocorticoid response in patients who became Dex resistant in other disorders\textsuperscript{47,48}. In addition, this hypothesis may be relevant to understand glucocorticoid resistance in patients with DBA, 60% of whom became transfusion dependent upon treatment\textsuperscript{49}. The mechanism that induces loss of glucocorticoid response in DBA is a subject of intensive investigation. Recently, the Blanc laboratory has proposed that DBA patients
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 p57kip2. Based on our results on Cushing’s patients, we speculate that the stress-CFU-E from unresponsive DBA fail to activate p57kip2 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 active Cushing’s patients contains a unique CD34+ cell population in which GR is constitutively active and does not respond to exogenous Dex. Surprisingly, CD34+ cells in the blood from patients in remission are phenotypically normal but retain an abnormal response to exogenous Dex.

REFERENCES


Table 1. Clinical data of the subset of patients and matched controls from the prospective cohort study included in the present investigation. Four patients analyzed both in the active and in the remission phase are indicated in color.

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18
Table 2. Mean cortisol levels in the serum of active (A) and remission (R) Cushing’s patients included in the cohort with respect to the levels observed in non-diseased donors matched for weight, age and sex and in those included in the study (see Table 1 for detail). n.a.=not available

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<th>Mean Cortisol Levels (µg/dL) (prospective cohort study)</th>
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<td>Matched Controls</td>
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<td>Active (A)</td>
<td>22.2 ± 6.3 (n=11) (p=3x10^{-7} vs Controls)</td>
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<td>Remission (R)</td>
<td>8.7 ± 3.9 (n=11) (p=0.006 vs A)</td>
<td>10.2±7.7 (n=14) (p=0.002 vs A)</td>
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LEGENDS TO FIGURES

Figure 1. Cushing’s patients in the active phase (A) express greater hematocrit (Hct), hemoglobin (Hb), platelet (Plt) and white blood cell (WBC) counts than patients in remission (R). A) Photograph of blood tubes from one representative A patient and one matched control. B) Hct, Hb and Plt counts for all the cohort of A and R patients included in the clinical data base (unpaired, left panel, and paired, right panel). C) Hct, Hb and Plt counts of the MC and A patients included in the study. D) White blood cell (WBC) counts for the A and R patients 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 t test and by Tukey's multiple comparisons test in D.

Figure 2. Although the frequency of total monocytes in blood from A and R patients and MC is similar, the frequency of monocytes expressing CD163 which is induced by glucocorticoids in A and R patients is greater than in MC. A) Neutrophil, eosinophil, basophil, monocyte and lymphocyte counts in the blood from A and R patients and MC included in the study. B and C) Percentage of CD14+ cells and of CD14+ cells expressing CD163 or CD169 in the blood from A and R patients and MC. Representative flow cytometry data are presented in B and Mean (±SD) of multiple determinations are presented in C. The number of patients included in each analysis is indicated by n. p values were calculated by Tukey's multiple comparisons test and those statistically significant are indicated in the panel.

Figure 3. Cushing’s patients express overall normal levels of HbF. A) HPLC determination of globin chains levels in red blood cells from representative A and R patients and matched control (each graph a separate subject, as indicated, patients are identified with the same alpha-numerical code indicated in Table 1). B-C) Average α/β-like and γ/β-like ratios in red blood cells from A and R patients and matched controls (MC). P values were calculated by Tukey's multiple comparisons test.

Figure 4. The blood from active Cushing’s patients contains normal numbers of CD34+ cells that express a stress-like phenotype characterized by high cell surface levels of the GR
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 A and R patient and of a matched control (MC) are presented on the left. Cells in the CD34+ gate where then analysed for expression of CD117, CD123, CXCR4, CD110, CD36, CALR and CD133, and the results presented as histograms in grey 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 analysed in parallel. B) Frequency of CD34+ cells in the mononuclear blood cells from multiple A and R patients and matched controls (MC). C) Frequency of CD34+ cells expressing CD117, CD123, CXCR4, CD110, CD36, CALR and CD133 from A and R patients and from matched controls (MC). The number of different individuals included in the various groups is indicated by n. P values were calculated with Tukey's multiple comparisons test and those statistically significant are indicated in the panels.

Figure 5. Erythroid progenitor cells from active Cushing’s patients generate similarly high numbers of Erys in cultures stimulated with and without Dex. A) Total numbers of Erys generated by day 10 in culture of MC, A and R with and without Dex, as indicated. The total number of Erys were calculated by multiplying the total number of cells in each culture per the corresponding percentage of cells with the CD235a/CD36 phenotype determined by FACS. B) Flow cytometry analyses for CD235a/CD36 expression of cells generated at day 10 by CD34+ cells from representative A, R and Cushing’s patients. CD235a/CD36 staining divides erythroid cells in to CD235aneg/CD36pos (Gate1, proerythroblasts, purple), CD235alow/CD36pos (Gate2, basophilic erythroblasts, light green), and CD235amedium/CD36pos (Gate3, polychromatophilic erythroblasts, dark green) and CD235apos/CD36apos (Gate4, orthochromatic erythroblasts, light blue), as reported17. Non-Erythroid cells are CD235anegCD36neg (dark blue) and are mainly lymphocytes. Results are presented as counterplots which allows for a better distinction of the clusters of the individual cell populations. C) Frequency of cells in Gate 1,2,3 and 4 generated by day 10 in culture of MC, A and R with and without Dex (the same colour code than in B). Note that Erys from MC are already mostly mature at day 10 in cultures without Dex. By contrast, at day 10 similar numbers of immature Erys are observed in cultures from A and R cultured with or without Dex. The number of different individuals included in the various groups is indicated by
n. Statistical analyses between minus and plus Dex in the same group were performed by paired t test and values statistically significant are indicated in the panel. Statistical analyses among group was performed by Tukey's multiple comparisons test as most appropriate. Results of the statistical analyses: Panel A: Paired t Test: **MC** minus Dex vs **MC** plus Dex, p=0.001. **A** or **R** minus and plus Dex not statistically different. Tukey's multiple comparisons test: **MC** plus Dex vs. **R** plus Dex: p=0.0003 and vs. **R** minus Dex: p=0.0002; **MC** minus Dex vs. **R** plus Dex: p=0.03 and vs. **R** minus Dex: p=0.02; **A** plus Dex vs. **R** plus Dex: p=0.03 and vs. **R** minus Dex: p= 0.02. **A** minus Dex vs. **R** minus Dex: p= 0.04. All the other comparison are not statistically different. Panel C: Paired t Test: Gate1 and Gate3 for **MC** plus and minus Dex, p=0.02 for both. Gates for **A** and **R** plus and minus Dex are not statistically different. Tukey's multiple comparisons test: no statistically significant differences among Gates in **MC** vs **A** or vs **R** with and without Dex.

**Figure 6.** Erys from active Cushing’s patients contain normal levels of total GR which is poorly phosphorylated in the inactive cytoplasmic-retained S203 form and express high levels of S211 form and of the GR-target gene GILZ in cultures with and without Dex. A) Western blot analyses for total GRα and its nuclear, GRαS211, and cytoplasmic (GRαS203) form, as well of the GR target gene GILZ of Erys generated at day 10 by representative **MC**, **A** and **R** patients (each number a different donor) in culture with and without (A patients only) Dex. 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 Erys generated from **MC** and **R** and **A** patients with Dex. C) Stoichiometry levels of GRαS203 and GRαS211 in **MC**, **A** and **R** patients cultured with Dex. D) Stoichiometry levels of GRαS203 and GRαS211 and levels of GILZ with respect to those of GRαS211 in cultures of **A** patients cultured with and without Dex. In B-D, results are expressed as box chart plus min and max of values observed in multiple *individuals per each group. Statistically significant p values among groups were calculated by Tukey's multiple comparisons test in B and C and by paired t test in D. The number of individuals included in each group is indicated by n.
Figure 5 A-C

A

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B

+ Dex

- Dex

MC

A

R

Day 10

CD36 - FITC

CD235a - APC

C

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P=0.016

P=0.025
Figure S1: Comparison of Hct versus serum cortisol levels in erythroid cells from Active (on the left) and Remission (on the right) patients. Similar data were obtained using 24-hour urine cortisol concentrations (data not shown).
Figure S2: Red blood cell parameters from Active (A), Remission (R) patients and MC (Matched Controls). RDW (Red Blood Cell Distribution Width), MCV (Mean corpuscular Volume), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration). The number of patients included in each analysis is indicated by n. and p values were calculated by Tukey's multiple comparisons test and were all above 0.05.
Figure S3: Mean Fluorescence Intensity (MFI) of CD117, CD123, CXR4 (high and low) CD110, CD36, CALR and CD133 expressed by CD34+ cells from MC, A and R patients, as indicated. The number of patients included in each analysis is indicated by n. and p values were calculated by Tukey's multiple comparisons test. Statistically significant values are indicated within the panel. BD=Below Detection.
Figure S4: Content of total GRα with respect to that of GAPDH in Erys generated from MC and R and A patients with Dex. The GRα content of MC are the same than those in Figure 6B plus values from 4 additional healthy controls previously published in Varricchio et al, Blood (2011) (doi: 10.1182/blood-2010-07-296921). The GRα of A are the same than in Figure 6B plus 3 additional patients not shown. The GRα values for R patients are the same than in Figure 6B. The values among groups are not statistically significant by Tukey's multiple comparisons test.