The role of endogenous glucocorticoids in lymphocyte development in melanocortin receptor 2-deficient mice

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ABSTRACT

Glucocorticoids are extensively used in anti-inflammatory therapy and are thought to contribute to the steady-state regulation of hematopoiesis and lymphopoiesis. We have previously established MC2R−/− mice, a model of familial glucocorticoid deficiency, that show several similarities to patients with this disease, including undetectable levels of corticosterone, despite high levels of ACTH and unresponsiveness to ACTH. In this study, we analyzed the possible roles of endogenous glucocorticoids in hematopoiesis and lymphopoiesis in MC2R−/− and CRH−/− mice as models of chronic adrenal insufficiency. Our analysis of total peripheral blood cell counts revealed that the number of lymphocytes was increased and the number of erythrocytes was slightly, but significantly, decreased in MC2R−/− mice. Numbers of immature double negative (CD4 + CD8 + ) thymocytes, transitional type 1 B cells in the spleen, and pre-B cells in the bone marrow, were significantly increased in MC2R−/− mice, suggesting that endogenous glucocorticoids contribute to steady-state regulation of lymphopoiesis. Oral glucocorticoid supplementation reversed peripheral blood cell counts and reduced numbers of T and B cells in the thymus and the spleen. T cells in the thymus and B cells in the spleen were also increased in CRH−/− mice, another animal model of chronic adrenal insufficiency. MC2R−/− mice were sensitive to age-related thymic involution, but they were resistant to fasting-associated thymic involution. Our data support the idea that endogenous glucocorticoids contribute to stress-induced as well as steady-state regulation of hematopoiesis and lymphopoiesis.

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1. Introduction

Glucocorticoids, administered in pharmacological doses, have broad anti-inflammatory and regulatory effects on the host immune response. The powerful anti-inflammatory effects of glucocorticoids form the basis for their utility in the treatment of autoimmune and inflammatory diseases, but their negative side effects substantially limit their clinical efficacy and use [1,2]. Although the endogenous physiological production of glucocorticoids has been suggested to play a role in optimizing the immune repertoire by affecting lymphocyte development and downregulating immune responses [3], the role of endogenous glucocorticoids in the control of lymphocyte development under basal conditions is a matter of debate [4].

A number of different experimental approaches have been used to elucidate the impact of basal levels of glucocorticoids on hemato-lymphopoiesis, especially on T-cell development. Removal of systemic glucocorticoids by adrenalectomy (ADX), an animal model of chronic adrenal insufficiency, results in increased thymus size and cellularity [5], and restoring corticosterone to physiological levels in ADX mice returns the number of thymocytes and ratios of cell subpopulations to normal values [6]. In contrast, glucocorticoid receptor deficient (GR−/−) mice [7,8] and T-cell glucocorticoid receptor-deficient mice [9] failed to show any difference in thymocyte development, selection, or survival relative to their wild-type mice. Thus the role of glucocorticoids in the thymus is controversial, and the role of endogenous glucocorticoid levels on T-cell homeostasis is not well established. Exposure to exogenous glucocorticoids leads to apoptotic loss of not only T cells but also pre-B cells in mice [10,11], and ADX leads to a pronounced increase in spleen weight in mice, suggesting that the endogenous physiological production of glucocorticoids plays a negative role in B-cell development [12,13]. In contrast to lymphoid cells, myeloid cells appear to be resistant to glucocorticoid-induced apoptosis [10].

The secretion of glucocorticoids is stimulated by adrenocorticotrophic hormone (ACTH), which is synthesized and secreted by the
anterior pituitary gland. Corticotropin-releasing hormone (CRH) is synthesized in the hypothalamic paraventricular nucleus (PVN) and secreted into the hypophyseal portal circulation. In response to a variety of stresses, the activity of CRH-secreting neurons in the PVN increases, leading to the secretion of ACTH and glucocorticoids [1]. We previously generated mice with an inactivation mutation of the melanocortin 2 receptor (MC2R) gene. Adult MC2R−/− mice resemble patients with familial glucocorticoid deficiency, a rare form of primary adrenal insufficiency, in several aspects, such as undetectable levels of corticosterone despite high levels of ACTH [14]. We have recently demonstrated that MC2R−/− female is a unique animal model of functional hypothalamic amenorrhea, accompanied with increased expression of CRH in the PVN [15]. In this study, we used MC2R−/− mice to investigate the effects of a permanent lack of glucocorticoids, and demonstrated that glucocorticoid deficiency affects hematopoiesis and immunosuppression under stress conditions such as fasting. Moreover, we used CRH−/− mice, which fail to induce corticosterone, to analyze lymphocyte development in another animal model of adrenal insufficiency.

2. Materials and methods

2.1. Animals

Male MC2R−/− mice on a mixed B6/Balbc background [16] or male CRH−/− mice [17,18] on a B6 background (8–16 weeks old) and their littersmates were used in this study. All the mice were kept under specific-pathogen-free conditions in an environmental controlled clean room in the Laboratory Animal Research Center, NCGM. The experiments were conducted in accordance with the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments. For fasting experiments, mice were given water ad libitum but deprived of food for 36 h. For corticosterone supplementation, corticosterone (Sigma, St. Louis, MO) was administered to 11-week-old mice in the drinking water containing 5 μg/ml corticosterone for 10 days. Because ACTH levels are useful for following patients on glucocorticoid replacement therapy [19], we analyzed ACTH levels in MC2R−/− mice. Corticosterone supplementation (5 μg/ml in drinking water) for 10 days effectively decreased plasma ACTH levels in MC2R−/− mice, although the plasma ACTH level was still higher than in MC2R+/− controls: MC2R−/− mice (nontreated: n = 5) 745 ± 140 pg/ml; MC2R−/− mice (corticosterone-treated: n = 6) 159 ± 70 pg/ml; and MC2R−/− mice (nontreated: n = 5), 82 ± 14 pg/ml. Increasing the dose of corticosterone supplementation to 25 μg/ml in drinking water for 10 days decreased plasma ACTH levels in MC2R−/− mice (n = 5) to 178 ± 32 pg/ml, which was not effective compared to the level achieved by supplementation with 5 μg/ml corticosterone and also significantly decreased thymocyte numbers in MC2R−/− mice (data not shown). The genotypes of all the mice used in the experiment were confirmed by inspecting the morphology of the adrenal glands.

2.2. FACS analysis and antibodies

For analysis of thymocyte developmental stages within the thymus, the following antibodies were used: anti-CD44FITC (IM7; eBioscience, San Diego, CA), anti-CD25PE (PC61.5; eBioscience), anti-CD44biotin (6H11; eBioscience), and anti-CD8APC (53-6.7; eBioscience). Thymocyte developmental stages were distinguished by expression of the cell surface markers CD4 and CD8; double negative (DN), CD4 CD8−; double positive (DP), CD4+CD8+; CD4 single positive (4SP), CD4+CD8−; and CD8 single positive (8SP), CD4−CD8+. Early DN thymocytes were further distinguished by sequential changes in expression of the cell surface markers CD25 and CD44 through the stages DN1 (CD25−CD44+), DN2 (CD25−CD44+), DN3 (CD25+CD44+), and DN4 (CD25+CD44+) [20]. For analysis of T cells and B cells within the spleen, the following antibodies were used: anti-TCRβFITC (H57; eBioscience) and anti-CD19PE (MB19-1; eBioscience). Anti-CD43 biotinylated antibodies were used: anti-B220FITC (RA3-6B2; eBioscience), anti-CD44PE (15/9; eBioscience), and anti-CD3biotin (R2/60; eBioscience). Four B-cell subpopulations were identified: T1 (CD19+CD21+CD24n), T2 (CD19+CD21+CD23+CD24n), MZ (CD19+CD21+CD23+CD24n), and FM (CD19+CD21+CD24+) [21,22]. For analysis of B-cell developmental stages within the bone marrow, the following antibodies were used: anti-B220FITC (RA3-6B2; eBioscience), anti-CD44PE (15/9; eBioscience), and anti-CD3biotin (R2/60; eBioscience). Four B-cell subpopulations were identified: Pro-B (B220+CD43+), pre-B/imature B (B220+CD43−), immature B (B220+IgM+), and mature recirculating B cell (B220+IgM−) [23]. The number of pre-B cells was determined by subtracting the number of immature B cells from the number of pre-B/imature B cells. The absolute cell numbers of each population were calculated from the percentages obtained by using FACS.

2.3. Statistical analysis

All values were calculated as means ± SEM. Comparisons of two groups were performed with Student’s t test. In all analyses, a two-tailed probability of less than 5% (i.e. P < 0.05) was considered statistically significant.

3. Results

3.1. Increased number of lymphocytes and decreased number of erythrocytes in MC2R−/− peripheral blood

Our analysis of total peripheral blood cell counts revealed an increased number of lymphocytes and slightly but significantly decreased number of erythrocytes in MC2R−/− peripheral blood relative to MC2R+/− peripheral blood (Table 1). Hemoglobin levels and hematocrit values were slightly but significantly decreased in MC2R−/− mice compared to MC2R+/− controls (Table 1), whereas mean corpuscular volume (MCV) remained normal, suggesting mild normocytic anemia in MC2R−/− mice. Corticosterone supplementation normalized the peripheral blood numbers of lymphocytes and erythrocytes in MC2R−/− mice, while the same treatment failed to affect peripheral blood cell counts in MC2R+/− controls (Table 1). These results suggest that peripheral blood cells in MC2R−/− mice are more sensitive to exogenous corticosterone than those in MC2R+/− controls.

3.2. Increased number of thymocytes and normal thymic T-cell composition in MC2R−/− mice

Although the total number of thymocytes was significantly higher in MC2R−/− mice than in MC2R+/− mice (Fig. 1A), there were no significant differences in the proportion of CD4- and CD8-defined thymocyte subsets (Fig. 1B) and the proportion of DN cells (DN1–DN4; CD25− and CD44− defined thymocyte subsets) between MC2R−/− and MC2R+/− mice (data not shown), and cell numbers of all the subpopulations were increased in MC2R−/− mice (Fig. 1C). These results suggest that the effect of endogenous corticosterone is not specific for any particular developmental stage of T-cell development in the thymus.
Supplementation of drinking water with corticosterone normalized the number of thymocytes in MC2R+/- mice (Fig. 1A and C) but did not affect the proportion of thymocytes in the corticosterone-treated MC2R +/- mice or in MC2R-/- mice (Fig. 1B). Corticosterone treatment at a higher dose suppressed the number of thymocytes in MC2R-/- mice (data not shown).

3.3. Increased numbers of splenic T and B cells in MC2R-/- mice

We found that proportions of T cells and B cells relative to total thymocytes were not significantly different between MC2R +/- mice and MC2R-/- mice (data not shown), while total cell numbers of both T cells and B cells were increased in MC2R +/- mice (Fig. 1D). Corticosterone supplementation normalized the numbers of splenic T and B cells in MC2R +/- mice (Fig. 1D) but did not affect the proportions in the corticosterone-treated MC2R+/- mice or MC2R-/- mice (data not shown). These results suggest that basal endogenous corticosterone affects both early T-cell development and B-cell development.

3.4. B-cell development in the spleen and bone marrow in MC2R-/- mice

We examined splenic B-cell subpopulations by staining for CD21, CD23, and CD24. All peripheral B-cell fractions in the spleen, MZ B cells, transitional T1 and T2 cells, and follicular B cells were increased in MC2R-/- mice, especially the T1 cells were significantly increased in the mice (Fig. 2A). Then we examined bone marrow B-cell subpopulations by staining for B220, CD43, and IgM [23] and found that the number of pre-B cells was increased in MC2R-/- mice compared to MC2R+/- mice, whereas the number of pro-B cells was not significantly different (Fig. 2B). These results suggest that basal endogenous corticosterone regulates B-cell development in bone marrow.

3.5. Increased numbers of thymocytes and splenic T and B cells in CRH +/- mice

We next analyzed lymphocyte development in another animal model of adrenal insufficiency, CRH +/- mice. The total number of thymocytes was significantly higher in CRH +/- mice than in CRH+/- mice (Fig. 3A), but there were no significant differences in the proportion of CD4- and CD8-defined thymocyte subsets or in the proportion of DN cells (DN1–DN4) between CRH+/- and CRH-/- mice (data not shown). Cell numbers of all the subpopulations were increased in CRH-/- mice (Fig. 3B and data not shown). Proportions of splenic T cells and B cells in CRH-/- mice were not significantly different compared to them in CRH+/- mice (data not shown), but total cell numbers of both T cells and B cells were increased (Fig. 3C). These results are consistent with the notion that endogenous glucocorticoids affect lymphopoiesis, as we observed in MC2R-/- mice.

3.6. Thymocytes in MC2R +/- mice are resistant to apoptosis induced by prolonged fasting and susceptible to thymic involution associated with aging

We previously demonstrated that 36 h of fasting increases serum corticosterone levels in MC2R-/- mice but not in MC2R +/- mice [16]. To clarify the possible involvement of endogenous glucocorticoids in fasting-associated immune suppression, we analyzed thymocyte subpopulations in MC2R-/- mice after 36 h of fasting. Whereas 36 h of fasting significantly decreased the proportion of DP thymocytes among all thymocytes as well as DP thymocyte numbers in MC2R-/- mice, DP thymocytes in MC2R-/- mice were increased.
resistant to fasting-induced changes (Fig. 4A). The thymus is also increased in thymocytes and splenocytes are increased in B-cell subpopulations in spleen and bone marrow in MC2R+/– mice and MC2R+/– mice showed similar involution of their thymus with age (Fig. 4B). Thus, age-related thymic involution is not glucocorticoid dependent.

4. Discussion

Chronic adrenal insufficiency is associated with anemia, whereas increased RBC count, hemoglobin, and hematocrit values are observed in patients with Cushings syndrome [25]. Glucocorticoids enhance formation of murine erythroid colonies and increase proliferation of erythroid cells in the presence of limiting amounts of erythropoietin (Epo). In spite of the fact that the anemia associated with chronic adrenal insufficiency is often masked by a concomitant reduction in plasma volume, our analyses revealed a slight but significantly decreased RBC count, hemoglobin, and hematocrit in MC2R+/– mice (Table 1). Genetic factors may therefore affect the severity of anemia in patients with adrenal insufficiency.

Leukocytes are potentially a direct target for melanocortins [26] and CRH [27] and are also a source of CRH. CRH may modulate the immune and inflammatory responses via two pathways: an anti-inflammatory pathway operated by centrally released CRH, most likely through stimulation of glucocorticoid and catecholamine release, and a proinflammatory pathway through the direct action of peripherally released CRH [27]. We characterized lymphopenia in MC2R+/– mice and CRH+/– mice as models for primary and secondary adrenal insufficiency to clarify the possible roles of melanocortins and CRH in the hematological abnormalities associated with adrenal insufficiency, but we should take into account that adrenal secretion of adrenal androgens is absent or very low in all mammals except primates. Our results that both MC2R+/– mice and CRH+/– mice show increased T cells in thymus and B cells in spleen, consistent with findings in ADX mice [12,13], indicate that glucocorticoids are responsible for the increased lymphocytes observed in adrenal insufficiency. However, we do not rule out the possibility that CRH or ACTH is involved in the regulation of local immune homeostasis under inflammatory conditions [26].

In accordance with the possible role of glucocorticoids in T-cell homeostasis, we found increased numbers of thymocytes in MC2R+/– mice and CRH+/– controls at 3 and 12 months of age. MC2R+/– mice and MC2R+/– mice showed similar involution of their thymus with age (Fig. 4B). Thus, age-related thymic involution is not glucocorticoid dependent.

Fig. 2. B-cell subpopulations in spleen and bone marrow in MC2R+/– mice. (A) Representative FACS profile of splenocytes from MC2R+/– and MC2R+/– mice stained with anti-CD21, -CD23, and -CD24. Bar graph represents the absolute cell numbers of each population: T1 (CD21loCD24hi), T2 (CD21hiCD23hiCD24hi), MZ (CD21hiCD23hiCD24lo), and FM (CD21loCD24hi). (B) Representative FACS profile of bone marrow cells from MC2R+/– and MC2R+/– mice stained with anti-B220, -CD43, and -IgM. Pro-B (B220+/–CD43+), Immature B (IgM–B220+/–), Pre-B (B220+/–IgM), and mature recirculating B cell (Recirc B B220+/–IgM) Bar graph represents the absolute cell numbers of each population. The number of total B cells in bone marrow (B220) was determined by the absolute B220+ cell number, and the number of pre-B cells was determined by subtracting the number of immature B cells from the number of Pre-B/immature B cells (n = 9). Data are shown as mean ± SEM. *, P < 0.05; **, P < 0.01, t test.

Fig. 3. The number of thymocytes and splenocytes are increased in CRH+/– mice. Thymocytes and splenocytes from CRH+/– and CRH+/– mice were analyzed by using FACS for the expression of CD4, CD8, TCR–β and CD19. (A) Bar graph represents total cell number of thymocytes (n = 7). (B) Bar graph represents the absolute cell number of thymocyte subpopulations (n = 7). (C) Bar graph represents the absolute cell numbers of splenic T and B cells (n = 3–6). Data are shown as mean ± SEM. *, P < 0.05; **, P < 0.01, t test.

Fig. 4. Fasting- and age-associated thymic involution in MC2R+/– mice. (A) Thymocytes from MC2R+/– and MC2R+/– mice fasted for 36 h (fasting), and control MC2R+/– mice and CRH+/– mice (non-fasting) were analyzed by using FACS for the expression of CD4 and CD8. Percentage of CD4+CD8– (DN), CD4–CD8+ (DP), CD4+CD8+ (4SP), and CD4–CD8– (8SP) cells in total thymocytes are shown (n = 3). (B) Total numbers of thymocytes in MC2R+/– and MC2R+/– mice at 3 and 12 months of age were analyzed (n = 6–8). Data are shown as mean ± SEM. *, P < 0.05; **, P < 0.01, t test.
membrane glucocorticoid receptor, as in the case of the progesterone receptor and estrogen receptor [30]. It is interesting that GR in the cytosol has been suggested to play a role as part of the TCR complex [31]. In addition, the functional significance of the synthesis of glucocorticoids within the thymus should be analyzed further [32].

Exposure to glucocorticoids leads to apoptotic loss of both T cells and pre-B cells in mice [10,11]. Consistent with the observations that ADX mice lead to a pronounced increase in spleen weight [12,13], we showed that absolute B cell numbers were increased in all peripheral B cell fractions in spleen of ADX and especially pre-B cells are elevated in the bone marrow of ADX mice and are mechanistically distinct from the involution that occurs in fasting or pregnancy.

5. Conclusion

We characterized hematological abnormalities in two animal models of adrenal insufficiency, MC2R−/− and CRH−/− mice, and clarified that endogenous glucocorticoids affect lymphocyte development under basal conditions as well as during fasting. Future studies are required to clarify discrepancies in T-cell development between GR−/− mice and mice with chronic adrenal insufficiencies.

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