Early lymphoid progenitors in mouse and man are highly sensitive to glucocorticoids

Hideya Igarashi\textsuperscript{1,2}, Kay L. Medina\textsuperscript{1}, Takafumi Yokota\textsuperscript{1}, Maria Isabel D. Rossi\textsuperscript{1}, Nobuo Sakaguchi\textsuperscript{2}, Philip C. Comp\textsuperscript{3} and Paul W. Kincade\textsuperscript{1}

\textsuperscript{1}Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, 825 Northeast 13th Street, Oklahoma City, OK 73104, USA
\textsuperscript{2}Department of Immunology, Graduate School of Medical Sciences, School of Medicine, Kumamoto University, Kumamoto 860-0811, Japan
\textsuperscript{3}Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

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Abstract

Glucocorticoids are extensively used in anti-inflammatory therapy and may contribute to the normal regulation of lymphopoiesis. This study utilized new information about the early stages of lymphopoiesis in mouse and man to determine precisely which cell types are hormone sensitive. Cycling B lineage precursors were depleted in dexamethasone-treated mice, while mature, non-dividing CD45R\textsuperscript{Hi} CD19\textsuperscript{Hi} lymphocytes, myeloid progenitors and stem cells with the potential for lymphocyte generation on transplantation were spared. Lineage marker-negative (Lin\textsuperscript{-}) IL-7R\textsuperscript{+} Flk-2\textsuperscript{-} pro-lymphocytes also declined, but not as rapidly as the terminal deoxynucleotidyl transferase-positive cells within an early Lin\textsuperscript{-} c-kit\textsuperscript{Hi} Sca-1\textsuperscript{Hi} fraction of bone marrow. Hormone-sensitive cells with additional properties of early lymphoid progenitors (ELP) were identified within the same Lin\textsuperscript{-} c-kit\textsuperscript{Hi} Sca-1\textsuperscript{Hi} subset using human \( b^{+} \) transgenic mice and recombination-activating gene 1 (RAG1)/green fluorescent protein knock-in animals. Furthermore, cells with a recent history of RAG1 expression were more glucocorticoid sensitive than mature lymphocytes in marrow and spleen. Lymphocyte progenitors in mice bearing a human \( b^{+} \) transgene were protected from dexamethasone treatment. However, isolated progenitors from either wild-type or \( b^{+} \) transgenic mice were directly sensitive to the hormone in stromal cell-free cultures, suggesting that additional factors must determine vulnerability to glucocorticoids. B lineage lymphocyte precursors were found to be abnormally elevated in the bone marrow of adrenalectomized or RU486-treated mice. This suggests that glucocorticoids may normally contribute to steady-state regulation of lymphopoiesis. Finally, parallel studies revealed that the earliest events in human lymphopoiesis are susceptible to injury during glucocorticoid therapy.

Introduction

Glucocorticoids are widely used as anti-tumor and anti-inflammatory drugs and beneficial responses likely depend in part on the sensitivity of mature cells of the immune system. However, the same class of hormones may also contribute to normal regulation of T cell development (1), and physiological concentrations can influence such processes as Ig isotype switching and synthesis (2–4). In addition, the immediate precursors of B lymphocytes are preferentially depleted in glucocorticoid-treated animals (5–10). While myeloid cells are spared in this circumstance, it remains unclear precisely when developing progenitors enter hormone-sensitive versus hormone-resistant compartments. There is some sparing of pre-B cells in transgenic mice over-expressing the anti-apoptotic \( b^{+} \) gene, but it is important to know if this is the only determinant of hormone resistance (11). Much remains to be learned about normal cellular processes that are glucocorticoid dependent, as well as cellular targets and consequences of hormone therapy.

The present study was inspired by advances in understanding the very early stages of lymphopoiesis. Very rare lineage marker-negative (Lin\textsuperscript{-}) c-kit\textsuperscript{Hi} Sca-1\textsuperscript{Hi} cells can now be identified as they begin to express lymphoid-determining...
transcription factors and the genes they regulate (12, 13). We designated these early lymphoid progenitors (ELP) if they express terminal deoxynucleotidyl transferase (TdT), recombination-activating gene 1 (RAG1) and/or a human μ transgene, but stress that they are not homogeneous (14). ELP are distinguished from stem cells by their inability to sustain lymphopoiesis for prolonged periods and their down-regulated, but not absent, ability to generate non-lymphoid cells. While the methodology is less well developed for studying human lymphoid cells, new culture and transplantation assays now make it possible to address questions about the importance of hormones for their regulation.

We now report that the most primitive of lymphoid progenitors are immediately and extensively depleted in glucocorticoid-treated mice. We used adrenalectomy and RU486 to learn if glucocorticoids contribute to normal steady-state regulation of lymphopoiesis and conclude that this may indeed be the case. While a Bcl-2 transgene afforded protection from hormonal depletion in vivo, this was not the case under stromal cell-free culture conditions and this anti-apoptosis factor alone could not account for the distinction between lymphopoiesis and progression in myeloid pathways. Some bone marrow specimens taken from hip replacement patients were lymphopenic and particularly for cells thought to represent primitive progenitors of the lymphoid system. Early phases of human B lymphopoiesis are supported by stromal cell coculture systems and can be monitored following engraftment of immunodeficient non-obese diabetic (NOD)/SCID/β2 microglobulin (M)−/− mice with human stem cells. These models afforded an opportunity to explore the responsiveness of defined cell populations to glucocorticoids. The findings implicate this class of hormones in normal steady-state regulation of lymphopoiesis and precisely define progenitors that are vulnerable to anti-inflammatory therapy.

Methods

Animals

C57BL/6J and NOD/SCID/β2M−/− mice were purchased from The Jackson Laboratory and maintained in our laboratory animal facility. Adrenalectomized or sham-operated mice were also obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained with physiological saline as drinking water. RAG1/green fluorescence protein (GFP) knock-in mice were described previously (15, 16). Breeding pairs of RAG1−/− human μ transgenic mice were a gift from R. Hardy (Fox Chase Cancer Center, Philadelphia, PA, USA). The human bcl-2 transgenic mice were kindly provided by Linda F. Thompson (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA).

Murine cell sorting and flow cytometry

Bone marrow cells were harvested and enriched for lineage-negative cells by incubation with antibodies to lineage markers, anti-Gr-1 (Ly-6G; RB6-8C5) and anti-CD11b/Mac-1 (M1/70) for myeloid cells, anti-CD19 (1D3) and anti-CD45R/B220 (RA3/6B2) for B lineage cells and TER119 for erythroid cells, followed by negative selection with the MACS cell separation system (Miltenyi Biotec, Auburn, CA, USA). These partially lineage-depleted cells were further stained with FITC-rat anti-mouse lineage markers (Gr-1, Mac-1, CD2, CD45R, CD19, CD3 and CD8), PE-anti-mouse Sca-1 and aliphophycocyanin anti-mouse c-kit. Lineage-positive cells were electronically gated out and lineage-negative fractions were sorted as c-kit+ Sca-1+ or c-kit− Sca-1− cells on a MoFlo (Cytomation, Fort Collins, CO, USA). In experiments using RAG1/GFP knock-in mice, PE-conjugated rat anti-mouse Lin antibodies were used instead of FITC-labeled antibodies. All mAbs were purchased from PharMingen (San Jose, CA, USA). To measure the RNA contents of single cells, Pyronin Y staining was conducted according to published reports (17, 18). Briefly, 5.0 × 10^6 cells were resuspended in 1.5 ml of Hoechst buffer (HBSS supplemented with 20 nmol of HEPES pH 7.2, 1 mg ml−1 of glucose and 10% FCS). Pyronin Y (Sigma Chemical Co., St Louis, MO, USA) was added to the cell suspensions at a final concentration of 100 ng ml−1 and incubated for 45 min at 37°C. Cells were washed once with Hoechst buffer and then stained for cell surface antigens. For intracellular staining, first, surface staining of cells was conducted. The cells were then fixed with 1% PFA in PBS and permeabilized with 70% ethanol at −20°C for >30 min and washed twice with the staining buffer. To establish the proliferative fraction, cells were incubated with the antibody anti-Ki-67 (mib-1, PharMingen) for 1 h on ice, as described by Zupo et al. (19). TdT was detected using the FITC kit from Supertechs (Bethesda, MD, USA). Flow cytometry was performed on a FACS Calibur (Becton Dickinson, San Diego, CA, USA) and the data were analyzed with Flojo software (Treestar, San Carlos, CA, USA).

Murine cell culture

Details of stromal cell-free, serum-free culture of early murine lymphocyte precursors are described elsewhere (20). Briefly, sorted cells were cultured with X-VIVO15 medium (BioWhittaker, Walkersville, MD, USA) containing 1% detoxified BSA (Stem Cell Technologies, Vancouver, British Columbia, Canada), 2 mM L-glutamine, 5 × 10−5 M 2-mercaptoethanol, 100 units ml−1 penicillin and 100 mg ml−1 streptomycin. Recombinant mouse stem cell factor (20 ng ml−1), Flk-2/Flt3 ligand (100 ng ml−1) (R&D Systems, Minneapolis, MN, USA) and recombinant mouse IL-7 (1 ng ml−1) (Endogen, Cambridge, MA, USA) were included to drive B lymphoid lineage differentiation.

RU486 treatment

Mifepristone (RU486) (BIOMOL Research Labs., Inc., Butler Pike Plymouth Meeting, PA, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg ml−1. This DMSO stock solution was diluted 1/10 with 1-M acetic acid just prior to use, then diluted again into PBS containing 1 mg ml−1 BSA to a final concentration of 250 μg ml−1. Mice were treated with 2 ml (500 μg) of this working solution via three intraperitoneal injections 24 h apart, and their bone marrow was analyzed 24 h later (21).

Human bone marrow collection

Cord blood samples were obtained from placentas of healthy newborns collected at the Oklahoma University Hospital (Oklahoma City, OK, USA). Adult bone marrow samples
were collected from patients between 24 to 88 years of age that were undergoing hip replacement surgery at the Bone and Joint Hospital (Oklahoma City, OK, USA). Patient records with no identifying information were used to group samples according to recent history of treatment with anti-inflammatory drugs such as prednisone. An Investigational Review Board approved all procedures involving human specimens.

Human marrow cell isolation and staining for FACS analysis

Four-color immunofluorescence analysis was used for the identification of B cell precursor populations in total nucleated bone marrow cell suspensions from adult marrow. Briefly, single-cell suspensions were obtained by flushing or gently vortexing the bone marrow. After staining, erythrocytes were lysed using the Becton Dickinson lysis buffer according to the manufacturer's instructions and CD45 antibody was used to assess the percentages of leukocytes in each sample. Intracellular staining of TdT was done by permeabilizing cells after surface staining described above.

Isolation of CD34+ cells and cell sorting

Mononuclear cord blood cells were collected by Ficoll/Hypaque (Lymphocyte Separation Medium, Cellgro-Mediatech, Hensdon, VA, USA) centrifugation within 24 h of collection. Depletion of erythroid, myeloid, NK, T and B cells was done before the CD34 selection. Cells were incubated on ice for 30 min with the following mouse anti-human antibodies: CD10, CD19, CD3, CD13, CD14, CD16, CD33, CD56, and glycoporphrin A, washed with Hanks and negatively selected using the BioMag goat anti-mouse IgG-coated beads (PerSeptive Biosystems Inc., Framingham, MA, USA) following manufacturer's instructions. Enrichment for CD34+ cells from cord blood mononuclear cells (CBMNC), total bone marrow and marrow depleted of erythroid, myeloid, NK and T (Lin-) cells was performed following manufacturer's instructions using the Dynal CD34 Progenitor Cell Selection System (Dynal A.S., Oslo, Norway). CD34+ enriched cells were stained with CD34–FITC and CD38–PE before sorting using a MoFlo (Cytomation). CD34+CD38- cells were determined using gating previously described (22). Post-sort analysis revealed the purity of the sort populations to be greater than 95%.

Co-cultures of human cells

The murine bone marrow stromal cell line, MS-5 was generously provided by J. Mori. Non-irradiated MS-5 stromal cells were prepared at a concentration of 1 × 10^5 cells per well in 96-well tissue plates (Corning, Corning, NY, USA) or 5 × 10^4 cells per well in 24-well tissue plates (Corning) 1–2 days prior to the seeding of hematopoietic progenitor cells at a concentration of 1–2 × 10^3 cells ml^{-1}. Cells were cultured in α-MEM (Cellgro-Mediatech) supplemented with 10% FCS (Hyclone, Logan, UT, USA) and a combination of rhSCF (100 ng ml^{-1}) and recombinant human granulocyte colony stimulating factor (rH-G-CSF) (10 ng ml^{-1}) as described by Nishihara et al. (23). The cultures were maintained for 5–8 weeks and fed every week by removing half of the medium and replacing it with fresh medium. Cytokines were added with each feeding. The generation of myeloid and B lineage cells was evaluated after 4 weeks of culture.

Transplantation of human cells into NOD/SCID/β2M−/− mice

Magnetically enriched CD34+ cells from cord blood were depleted in PBS with 0.1% BSA (fraction V, Sigma) and 2 × 10^5 injected into the tail vein of sub-lethally irradiated (100 cGy from a ^{137}Cs source) 6-week-old male or female NOD/SCID/β2M−/− mice. A total of 5 × 10^6 CD34+ CBMNC were co-injected as a source of accessory cells (24). The efficiency of engraftment was confirmed by the analysis of peripheral blood.

Corticosteroid treatment

The influence of glucocorticoids on human lymphopoiesis was assessed by adding 10^{-7} M hydrocortisone (Sigma) to the co-cultures described above. The cultures were fed weekly and maintained for a total of 4 weeks. The cells were harvested, counted, stained and analyzed by FACS. Hormone sensitivity was also investigated with NOD/SCID/β2M−/− chimeras. The animals were injected with human CD34+ cord blood cells as described above. After 7 weeks, 21 days time-release pellets containing dexamethasone (0.24 mg released per day; Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously under anesthesia. Treated and mock-injected mice were sacrificed 48 h later and bone marrow was harvested for FACS analysis. The same treatment protocol was used to investigate the sensitivity of murine lymphocytes to dexamethasone. For murine bone marrow culture studies, dexamethasone (Sigma; D-4902) was used at a final concentration of 100 nM.

Results

Rapid depletion of lymphocyte progenitors in bone marrow of dexamethasone-treated mice

A wealth of information is available about stages of B lymphopoiesis in murine bone marrow and new approaches allow identification of the most primitive of lymphoid progenitors (13, 14). Previous studies demonstrated that pre-B cells are glucocorticoid sensitive (5–11). This was confirmed in our experiments where CD45R^a^CD43^a^− pre-B cells as well as CD45R^a^CD43^b^− pro-B cells were depleted within 24 h of hormone treatment (data not shown and Fig. 4 below). Almost unaffected were a small, but distinct, subset of hormone-resistant lymphocytes that were likely to be mature recirculating B cells. This follows from the fact that resistant lymphocytes had relatively high densities of CD45R and CD19, lacked the Ki-67 nuclear proliferation antigen and had little RNA identified with the Pyronin Y stain (Fig. 1). In contrast to this depletion, CD45R^a^TER119+ erythroid cells and GR-1^a^ myeloid cells were retained in normal to elevated proportions (data not shown).

Our analysis then focused on earlier categories of lymphoid progenitors that can be found within the Lin– fraction of bone marrow. There is inconsistent nomenclature between laboratories, but functional pro-lymphocytes (also known as common...
lymphoid progenitors) display a distinctly low density of c-kit and express TdT (25). The α chain of the IL-7R and Flk-2 additionally identify pro-lymphocytes that can rapidly give rise to B, NK and, in some circumstances, T lineage cells (20, 26, 27). We found virtually no cells with any of these characteristics in mice treated for 3 days with dexamethasone (Fig. 2A).

High expression of c-kit and Sca-1 antigens designates hematopoietic stem cells and their immediate progeny. Within that primitive category, cells initiating the lymphoid differentiation program, i.e. ELP can be identified according to their synthesis of TdT and/or RAG1 (12, 13). A time course experiment revealed that Lin− c-kithi Sca-1th cells were very rapidly depleted (Fig. 2B). Human μ chain transgenic mice provide an independent means of detecting ELP in the Lin− c-kithi Sca-1th fraction and display of CD27 is an additional distinguishing characteristic (12). Cells with these properties were almost completely eliminated by hormone treatment (Fig. 2C). We conclude that the most primitive of lymphoid progenitors rapidly disappear from the marrow of glucocorticoid-treated mice. More-differentiated cells in the lymphoid series are also depleted, while myeloid/bone marrow and spleen were spared (Fig. 3B and C). Furthermore, many GFP− sIgM+ B cells in the spleen were unaffected by 72 h of treatment with dexamethasone (Fig. 3C). This category was not completely hormone resistant however, inasmuch as numbers of total nucleated cells in the spleen declined 40-fold. These results demonstrate that initiation of the lymphocyte differentiation program coincides with or slightly precedes the acquisition of glucocorticoid sensitivity.

A bcl-2 transgene affords partial protection from the suppressive effects of dexamethasone

It has been extensively documented that glucocorticoids elicit an apoptotic response in immature lymphocytes and that this response can be offset by over-expression of Bcl-2 (11, 28–30). We wondered if the same was true for lymphocyte progenitors that might be depleted via different mechanisms. The Eμ/Ig enhancer/promoter used to prepare these mice does not direct human Bcl-2 expression in myeloid lineage cells, and we determined that transgene expression is detectable at least from the Lin− c-kitlo Sca-1lo stage (data not shown). Examination of bcl-2 transgenic animals then revealed that all of the major categories of B lineage lymphocytes in bone marrow resisted dexamethasone treatment (Fig. 4A). Lin− c-kitlo cells were then placed in defined stromal cell- and serum-free cultures before being exposed to the same hormone. This revealed that functional pro-lymphocytes able to generate CD19+ cells from either normal or bcl-2 transgenic animals were direct hormone targets (Fig. 4B). It is noteworthy that the sorted Lin− c-kitlo fractions also contained some progenitors capable of generating CD11b/Mac-1+ myeloid cells. As was the case for cultures of human progenitors (see Fig. 7) these non-lymphoid cells were unaffected by the hormone. Thus, ectopic expression of Bcl-2 provides hormone resistance to lymphoid progenitors in a natural environment. However, stromal cell-free culture experiments indicate that Bcl-2 levels alone cannot account for the difference between lymphoid and myeloid cells.

Naturally produced glucocorticoids may negatively influence lymphopoiesis

The above findings define lymphopoietic populations that might suffer as a result of anti-inflammatory therapy, but do not implicate endogenous glucocorticoids in normal steady-state regulation. However, it has been reported that neonatal adrenalectomy causes increases in thymus size (31) and we now show that numbers of B lineage precursors in bone marrow were expanded in mice treated with the glucocorticoid...
antagonist drug RU486 (Fig. 5A). A similar response was
documented in adrenalectomized animals (Fig. 5B). This was
particularly the case for pre-B cells, which are the most
numerous marrow progenitors, and ones that would be the
most affected by any alterations in lymphopoiesis. Their
numbers in adrenalectomized mice averaged 4-fold more
than those in sham-operated and control animals. These
findings indicate that adrenal hormones normally contribute to
some degree in limiting lymphopoiesis within bone marrow.

Therapeutic use of glucocorticoids may selectively ablate
lymphopoiesis in humans

Lymphopoiesis normally continues in bone marrow throughout
life (32–34). There are changes in the phenotypes and
functional properties of lymphoid progenitors during the
fetal/neonatal period, but their proliferation and the ratio of
one subset to another are remarkably constant thereafter (34).
In contrast to this normal pattern, we have found exceptional
patient samples where terminal deoxynucleotidyl transferase-
positive (TdT+) and B cell progenitors (CD34+ CD10+ CD19−/−)
were virtually absent (Fig. 6). Mature B and T cells, as well as
myeloid cells identified by light scatter properties or CD33
expression were always present, suggesting a high degree of
selectivity. The samples were obtained from patients un-
dergoing hip replacement surgery and where treatment with
prednisone was common. However, these individuals used
various combinations of other medications and numbers were
insufficient to assign lymphocyte deficiency to any particular
therapy. Therefore, two experimental models were used to
determine if and how treatment with glucocorticoids influences
lymphopoiesis in humans.

The addition of 10−7 M concentrations of hydrocortisone
completely and selectively blocked the generation of CD19+
cells in cultures initiated with CD34+ Lin− cord blood cells (Fig.
7A–C). In contrast, the production of myeloid cells was hor-
mone resistant. Immunodeficient NOD/SCID/β2M−/− mice were
then engrafted with human cord blood CD34+ cells before
treatment with time-release dexamethasone pellets (Fig. 7D–
G). The marrow of mock-injected animals
contain cells representing all stages of human B lymphopoi-
esis (35). The analysis shown in Fig. 2(D–G) was gated on
human CD34+ cells, and highly selective depletion of CD19+
and TdT+ lymphocyte precursors was found in hormone-
treated mice. By contrast, absolute numbers of undifferentiated
CD34+ CD10− TdT− cells were unaffected (Fig. 7 and additional
data not shown). These observations demonstrate that the very
early stages of lymphopoiesis in humans are preferentially
sensitive to anti-inflammatory therapy.

Discussion

Many studies indicate that the glucocorticoids used in anti-
flammatory therapy could disrupt replenishment of the
immune system by newly formed lymphocytes. Here we used several animal models to study discrete populations of bone marrow cells and conclude that hormone sensitivity coincides with the earliest lymphoid fate specification steps in very rare progenitors. All of the progeny of those primitive cells are depleted in hormone-treated mice, but mature non-dividing lymphocytes and non-lymphoid cells are very selectively spared. While lymphopoiesis was protected in Bcl-2 transgenic mice, progenitors were still sensitive in culture and levels of this anti-apoptotic protein are not sufficient to account for the resistance of myeloid lineage cells. Additional experiments suggested that this class of hormones may participate in

**Fig. 3.** Glucocorticoid sensitivity is acquired at the most primitive stage and lost as a very late event in B lymphopoiesis. Heterozygous RAG1/GFP knock-in mice were treated with placebo (control) or dexamethasone-containing pellets (DxRx) for 72 h. (A) Bone marrow cells were harvested and rigorously lineage-negative cells were prepared by cell sorting. The boxed region contains ELP marked by GFP expression in a Lin- Sca-1+ c-kit+ CD27+ fraction. (B) GFP expression persists in pro-B (CD45RLo CD43Hi), pre-B (CD45RLo CD43Lo) and B cells (CD45RHi CD43- ) that are hormone sensitive (shaded area). (C) Splenocytes were also harvested and stained to illustrate GFP expression among the CD45R+ slgM+ B cell fraction.

**Fig. 4.** A bcl-2 transgene provides partial glucocorticoid protection for lymphocyte progenitors but does not explain their normal hormone sensitivity. (A) Transgenic mice expressing a human bcl-2 gene controlled by an Ig enhancer/promoter were treated for 72 h with dexamethasone-containing pellets (DxRx). Marrow cells were then harvested, stained for major lymphocyte populations and analyzed by flow cytometry. Percentages are given for CD45R+ CD43- B cells, CD45RLo CD43Lo pre-B cells, as well as CD45R- CD43Hi pro-B cells in the boxed regions. (B) Lin- c-kit+ Sca-1+ lymphoid progenitor cells were sorted from littermate or transgenic mice and exposed to the indicated concentrations of hormone in serum- and stromal cell-free cultures. Seven days later, cells were recovered and analyzed for the presence of CD19+ B lineage or CD11b/Mac-1+ myeloid cells. Total number of cell yield/input, CD19+ yield/input and CD11b/Mac-1+ yield/input were determined by simply dividing numbers of recovered total cells, CD19+ cells and CD11b/Mac-1+ cells by the numbers of progenitors used to initiate the cultures, respectively.
normal steady-state regulation of lymphopoiesis. Finally, human lymphopoietic cells were preferentially inhibited by exposure to glucocorticoids in culture and in chimeric animals.

A number of previous studies documented the selective suppression of lymphopoiesis in the marrow of glucocorticoid-treated mice (5–11). However, this did not include evaluation of rare, primitive progenitors that have only recently been defined (12, 13). We have now found that proliferating CD45Rlo cells with abundant RNA identified by Ki-67 and Pyronin Y staining were almost completely eliminated. This reduction of cycling cells is in agreement with our observation in human marrow, where cycling B cells from treated patients were almost absent (data not shown). Garvy et al. showed that chronic elevation of glucocorticoids in murine plasma reduced the number of cycling B cells in bone marrow and induced apoptosis. They implanted cortisone tablets subcutaneously and measured plasma concentrations of corticoids during the interval of 6 h to 15 days. Cell cycle analysis demonstrated a reduction in B220+ cells in S phase (8). This would include relatively abundant pro-B and pre-B populations in bone marrow. Less-differentiated lymphoid progenitors can be found in the small Lin- fraction that lacks any blood cell lineage-associated markers and identified according to expression of the enzyme TdT (20, 25, 27). We found that these cells declined much more rapidly following hormone treatment than the CD43+ CD45Rlo pro/pre-B cells (Fig. 4). Thus, the kinetics of depletion are consistent with a precursor–product relationship between these two categories of progenitors. Most of the Lin- TdT+ pre-lymphocytes have a distinctive low density of c-kit and a majority expresses the IL-7Rα chain (20, 26). It is now clear that they are exquisitely hormone sensitive, regardless of whether they stain for the Ki-67 proliferation antigen.

Even earlier Lin- cells that take longer to generate lymphocytes express high densities of c-kit and Sca-1 (12, 13). They can be distinguished from stem cells by the display of CD27 and Flk-2, while they differ from myeloid progenitors in expressing TdT, a human transgene, and RAG1 (12, 13). These ELP give rise to B, T and NK lineage cells and we previously found that ELP in adult mice were selectively sensitive to estrogen (12). The present study revealed them to be depleted in glucocorticoid-treated animals (Fig. 5).

Multiple mechanisms have been proposed for glucocorticoid-induced cell death (36–38). For example, the hormone can block trans-activation and gene expression by direct interaction with nuclear factor-κB (NF-κB), or compete with NF-κB for binding to CBP/p300. In addition, glucocorticoids up-regulate levels of IκB, effectively lowering amounts of NF-κB (39–41). The cell survival-related c-myc protein is depressed in some cell types following hormone exposure (42–45). On the other hand, CDK2, CDK4 and CDK6 proteins required for progression in the cell cycle are down-regulated. Reciprocally, the p27Kip1 and p21Cip1 inhibitors of cell division are increased (42, 46–52). Additionally, glucocorticoids can elicit DNA fragmentation and apoptosis via caspase-independent pathways (53–55). A key question here is why cells committed to lymphoid differentiation fates are steroid hormone sensitive, while myeloid progenitors are not. Substantial protection was afforded by a human bcl-2 transgene, suggesting that the hormone may initiate an apoptotic program. The same was previously found with respect to estrogen sensitivity (56). However, responsiveness was highly dependent on the environment and purified progenitors from bcl-2 transgenic mice were glucocorticoid sensitive in stromal cell-free, serum-free cultures. It is noteworthy that human Bcl-2 was expressed at detectable levels from at least the Lin- c-kitlo Sca-1lo stage. Contact with stromal cells is known to decrease hormone sensitivity and spontaneous apoptosis of lymphoid progenitors in vitro (57–59). Therefore, the life/death decision must hinge on a balance between pro-apoptotic and anti-apoptotic signals. In

**Fig. 5.** Physiological regulation of B lymphopoiesis by glucocorticoids. Mice were treated with the glucocorticoid receptor antagonist RU486 or adrenalectomized to suppress glucocorticoid-related functions. (A) Four days after intraperitoneal administration of RU486 or vehicle only (control), bone marrow cells were harvested and stained with FITC-conjugated anti-CD45R and PE-conjugated anti-CD43 before being resolved by flow cytometry. Regions containing CD45R+ CD43- B cells, CD45Rlo CD43-lo pre-B cells and CD45Rhi CD43hi pro-B cells are illustrated with boxes. The percentages of cells in these regions are also shown. (B) Absolute numbers of B lineage cells, defined by total cell counting and flow cytometry as CD45R+ total B lineage cells, CD45Rlo CD43- B cells, CD45Rhi CD43-lo pre-B cells and CD45Rhi CD43hi pro-B cells from the adrenalectomized or RU486-treated mice bone marrow are shown in bar graphs together with total nuclear (NC) cells. The means and standard deviations were calculated from three individuals in each group. Sham-operated and vehicle administration mice represented controls for adrenalectomy and RU486 treatments, respectively.
of hormones could determine thresholds for negative selection and elimination of autoreactive B cells.

It has been previously established that sex steroids participate in steady-state regulation of lymphopoiesis and the observations reported here suggest this is also the case for glucocorticoids (66). However, there are notable differences between these classes of hormones. While estrogen receptors are only expressed by primitive lymphoid progenitors in adult bone marrow, glucocorticoid receptors are more widely distributed and can also be detected on progenitors in fetuses (67). Furthermore, there is a much wider spectrum of cellular targets for this class of hormones and even small non-dividing lymphocytes have some sensitivity to glucocorticoids.

It is unclear if glucocorticoids influence lineage choice decisions at the level of multipotent hematopoietic progenitors or alternatively, if the hormones suppress development of cells that have already committed to lymphoid fates. We have no clonal data to directly address this important issue but have found no net increase in numbers of myeloid cells in hormone-treated cultures. On the other hand, glucocorticoids are associated with an increase in granulocytes in vivo [data not shown and (68)]. Interestingly, myelopoiesis and erythropoiesis are rescued in patients receiving allogeneic bone marrow transplants and cortisone. However, there was a delay in B cell recovery in these patients (69). We found that cells from one patient treated with cortisone grew with normal clonogenic frequency and yield of CD19+ cells from CD34+ CD38− stem/progenitors (M.I.D. Rossi and P.W. Kincade, unpublished data). Additionally, marrow from glucocorticoid-treated mice generated lymphocytes normally when transplanted into irradiated recipient mice (data not shown). Thus, the effect of cortisone is reversible or immediate lymphoid progenitors are more sensitive targets of glucocorticoids than stem cells.

ELP have not been precisely defined in humans, and there is evidence that they undergo developmental age-related changes (34, 70). Patterns of selective cellular loss in patients might be informative in this regard. For example, some marrow specimens had very few TdT+ CD10− cells and were also depleted with respect to TdT+ CD10+ and CD10+ CD19− lymphocytes (Fig. 6). In contrast, mature IgM+ B cells and CD33− myeloid cells were retained. These findings are consistent with proposed models for human B lineage differentiation but do not reveal the precise reason for the deficiency or the nature of the target cells. Culture models and chimeric mice permitted more detailed analyses and exposure to defined concentrations of hormone. These findings confirm and extend a previous report that physiological concentrations (10−7 M) of cortisol (natural steroid) and dexamethasone (synthetic steroid) induced apoptosis of human B cell progenitors in culture (71). This response was blocked by the addition of RU486. Some species differences have been found between lymphoid progenitors in humans and mice. For example, IL-7 is essential for B lymphopoiesis in adult mice but has no clear role in humans (72). Additionally, human cells express a unique subset of glucocorticoid receptors not found in rodents (73). Regardless, we found that lymphoid progenitors in both species were preferentially sensitive to natural and synthetic glucocorticoids.

Sex steroids have proven to be powerful experimental tools for establishing precursor–product relationships between
Glucocorticoids have been extensively exploited for studies of the thymus and the present results suggest strategies for studying events in fetal tissues and adult marrow. Furthermore, regeneration of the immune system following transplantation and chemotherapy could be compromised by treatment with anti-inflammatory steroids and/or stress-induced endogenous hormones. Therefore, glucocorticoid antagonist drugs might have protective effects in some circumstances. Lymphoid progenitors may represent a unique target organ for glucocorticoids, utilizing particular mechanisms for hormone responsiveness. If that is the case, synthetic agonist compounds that have tissue-specific activity could be advantageous as anti-inflammatory drugs.

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Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>CBMNC</td>
<td>cord blood mononuclear cells</td>
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<td>DMSO</td>
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<td>ELP</td>
<td>early lymphoid progenitor</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>Lin</td>
<td>lineage marker</td>
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References
Glucocorticoid sensitivity of lymphoid progenitors in marrow


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