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Retinoids Accelerate B Lineage Lymphoid Differentiation[1]

Xinrong Chen,* Brandt L. Esplin,*† Karla P. Garrett,* Robert S. Welner,*† Carol F. Webb,* and Paul W. Kincade[2]*

Retinoids are known to have potent effects on hemopoietic stem cell integrity, and our objective was to learn whether they influence cells destined to replenish the immune system. Total CD19$^+$ B lineage cells increased substantially in the marrow and spleens of all-*trans* retinoic acid (ATRA)-treated C57BL6 mice, while lymphoid progenitors were reduced. All B lymphoid progenitors were targets of ATRA in culture and overall cell yields declined without reductions in proliferation. Remarkably, ATRA shortened the time required for primitive progenitors to generate CD19$^+$ cells. PCR analysis and a panel of retinoid acid receptor (RAR)/retinoid X receptor agonist treatments suggested that RARα mediates these responses. The transcription factors *EBF1* and *Pax-5* were elevated during treatment and ATRA had similar effects on human B cell differentiation. That is, it inhibited the expansion of human progenitor cells and accelerated their differentiation to B lineage cells. There may be previously unsuspected side effects of ATRA therapy, and the new findings suggest retinoids can normally contribute to the lymphopoietic environment in bone marrow. ***The Journal of Immunology,*** **2008, 180: 138–145.**

Metabolites of vitamin A (retinol) have diverse biological activities that include roles in vision, embryonic development, and cell proliferation, differentiation, and survival (1). All-*trans* retinoic acid (ATRA)[3] has been particularly well studied as a developmental morphogen, a regulator of gene transcription, and a substance that may normally regulate hemopoietic stem cells (HSC). Evidence for the latter initially came from findings that ATRA promotes retention of stem cell properties in culture (2, 3). More recently, it was discovered that numbers of HSC were low in mice with a targeted retinoic acid receptor (RAR) γ (*RAR*γ) gene, while hemopoietic progenitors were abnormally increased (4). Furthermore, the HSC in these animals were defective with respect to long-term reconstitution ability and appeared to exhaust with age.

ATRA has long been known to promote progression in the myeloid lineage, and this property has been effectively exploited for therapy in acute promyelocytic leukemia (1). Furthermore, the innate and adaptive arms of the immune system are both compromised in vitamin A-deficient animals (5). ATRA and 9-*cis* retinoic acid have been shown to block activation-induced apoptosis in thymocytes while two other metabolites, 14-hydroxy-retro-retinol and anhydroretinol, compete with each other in supporting lymphocyte viability and expansion (6). There is also a report that retinoids inhibit IL-7-driven growth of murine pre-B cells (7).

There are hundreds of retinoid-regulated genes, and only some of their promoters contain retinoic acid response elements (8). Furthermore, HSC and other potential targets of retinoid action are rare in bone marrow. Consequently, it has been difficult to describe mechanisms that account for retinoic acid effects on hemopoiesis. Genes that have been considered in connection with this issue include *c-Myc*, *Stra-13*, *C/EBP*ε, *Hoxb4*, $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and *Notch 1* (1, 4). The pre-B cell leukemic homeobox 1 (*PBX1*) gene is up-regulated, and the half-life of the PBX1 protein is extended by ATRA (9). The *PBX1* gene is translocated in 23% of pediatric pre-B leukemias such that a PBX1-E2A fusion protein is made (10). Importantly, a role for PBX1 in normal B lymphopoiesis has just been discovered (11). Thymic stromal lymphopoietin (TSLP) was originally discovered as a cytokine that accelerated B lymphopoiesis (12). Although it is now attracting interest as a master regulator of atopic dermatitis and asthma, TSLP drives production of the first wave of B cells, and especially B1 progenitors (13–15). It is therefore interesting that TSLP expression is induced by retinoids (16).

Collectively, these findings suggest possible roles for retinoids in development and maintenance of the immune system. However, information is needed concerning target cells and molecular mechanisms. We have now determined that B lymphopoiesis was accelerated when several types of progenitors were exposed to ATRA.

Materials and Methods

Reagents and treatments

All-*trans* retinoic acid, 9-*cis* retinoic acid, 13-*cis* retinoic acid ,and AC-41848 were purchased from Sigma-Aldrich. 4-((E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid (TTNPB) and AM-580 were from Biomol. RAG-1/GFP knock-in mice have been described (17). Heterozygous F_1 RAG-1/GFP mice were generated at the Oklahoma Medical Research Foundation's Laboratory Animal Resource Center (LARC). C57BL6 mice used in CFSE assays (CD45.2 alloantigen)

*Immunobiology and Cancer Program, Oklahoma Medical Research Foundation and †Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

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[2] Address correspondence and reprint requests to Dr. Paul W. Kincade, Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, 825 Northeast 13th Street, Oklahoma City, OK 73104. E-mail address: kincade@omrf.ouhsc.edu

[3] Abbreviations used in this paper: ATRA, all-*trans* retinoic acid; EBF1, early B cell factor 1; ELP, early lymphoid progenitor (Lin$^-$RAG-1/GFP$^+$Sca-1$^+$c-Kit^{high}); FL, Flt3 ligand; HSC, hemopoietic stem cell; LSK, Lin$^-$Sca-1$^+$c-Kit^{high}; Pax-5, paired box gene 5; PBX1, pre-B cell leukemic homeobox 1; Pro-L, prolymphocyte (Lin$^-$Sca-1$^+$c-Kit^{low}); RAR, retinoic acid receptor; rh, recombinant human; rm, recombinant murine; RXR, retinoic X receptor; SCF, stem cell factor; sIgM, surface IgM; TSLP, thymic stromal lymphopoietin; TTNPB, 4-((E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid.

were bred and maintained in the LARC. Twenty-one-day time-release pellets of all-*trans* retinoic acid (10 mg/pellet) were purchased from Innovative Research of America and implanted s.c. with a 10-gauge precision trocar. Assuming constant delivery, this would correspond to 15 mg/kg/day or 45 mg/m^2 (calculated using the online dose calculator provided by the Center for Drug Evaluation and Research, U. S. Food and Drug Administration; http://www.fda.gov/cder/cancer/animalframe.htm). This dose is used for human therapy in acute promyelocytic leukemia (18). After 1 wk, mice were killed and bone marrow, splenocytes, and thymocytes were isolated and analyzed by flow cytometry. All animal protocols and the use of human umbilical cord blood were approved by the appropriate institutional committees.

Antibodies

Regarding mAbs for murine Ags, anti-CD3, anti-CD8, anti-CD19 (clone 1D3), anti- CD45RA (clone 14.8), anti-FcRγ (clone 2.4G2), and anti-TER119 mAbs were purified from the cultured supernatant of hybridoma cells grown in our laboratory. Purified anti-Ly-6G (clone Gr-1) mAb; biotin-conjugated anti-CD3 (clone 145-2C11), anti-CD8 (clone 53-6.7), anti-CD19 (clone 1D3), anti-CD45R/B220 (clone RA3/6B2), anti-Mac-1 (clone M1/70), anti-Gr1 (clone RB6/8C5), anti-NK1.1 (clone PK136), anti-Ter119 (clone ly-76), and anti-CD43 (clone S7) mAbs; allophycocyanin-conjugated anti-c-Kit (clone 2B8); and anti-NK1.1 (clone PK136), allophycocyanin-Cy7-conjugated anti-CD19 (clone 1D3), anti-B220 (clone RA3-6B2), anti-CD4 (clone GK1.5), and anti-CD25 (clone PC61); PE-Cy5-conjugated anti-CD45RA/B220; PerCp-CY5.5-conjugated anti-CD45.2 (clone 104) and anti-IgM (R6-60.2); and PE-conjugated anti-IL7Rα (clone SB199), anti-CD11c (clone HL3), and anti-CD3 (clone 145-2c11) mAbs were all purchased from BD Pharmingen. PE-Cy5-conjugated anti-Sca-1 (clone D7), anti-CD24 (clone M1/169), anti-Gr-1 (clone RB6-8C5), and anti-CD44 (clone IM7); allophycocyanin-conjugated anti-CD93 (clone AA4.1); and PE-conjugated anti-IgM (clone eB121-15F9), anti-F4/80 (clone BM8), anti-CD21 (clone eBio8D9), and anti-CD11b (clone M1/70) were obtained from eBioscience. Allophycocyanin-conjugated anti-CD23 (clone 2G8) was obtained from Southern Biotech. PE-conjugated anti-CD23 (clone B3B4) was obtained from BD Pharmingen. PE-Texas red tandem-conjugated streptavidin, allophycocyanin-conjugated streptavidin, and PE-Cy5.5-conjugated streptavidin were purchased from Caltag Laboratories. Regarding mAbs for human Ags, FITC-conjugated anti-CD38 and allophycocyanin-conjugated anti-CD34 were obtained from BD Pharmingen, and FITC conjugated anti-CD14 and PE-conjugated anti-CD19, anti-CD14, anti-CD33, anti-CD56, anti-CD64 and anti-glycophorin A mAbs were purchased from Caltag Laboratories.

Immunofluorescence staining and cell sorting

Cells recovered from animals or cultures were treated with anti-FCRγ (FcγRIII/II, clone 2.4G2) Ab to minimize nonspecific binding. Cells were stained with Abs and flow cytometry analyses were performed on a BD LSR II flow cytometer using the BD FACSDiVa software (BD Biosciences).

Follicular B cells were defined as B220$^+$CD24^{int}CD21^{int} (where "int" is intermediate), T1 cells were defined as B220$^+$CD24^{high}CD21$^-$, T2 cells were defined as B220$^+$CD24^{high}CD21^{high}CD23$^+$, and marginal zone cells were defined as B220$^+$CD24^{high}CD21^{high}CD23$^-$, respectively (19). In another type of staining, mouse follicular B cells were defined as B220$^+$AA4.1$^-$sIgM^{low}CD23$^+$ (where "sIgM" is surface IgM), T1 cells were defined as B220$^+$AA4.1$^+$sIgM^{high}CD23$^-$, T2 cells were defined as B220$^+$AA4.1$^+$sIgM^{high}CD23$^+$, T3 cells were defined as B220$^+$AA4.1$^+$sIgM^{low}CD23$^+$, and marginal zone cells were defined as B220$^+$AA4.1$^-$sIgM^{high}CD23$^-$, respectively (20).

Mouse bone marrow cells collected from 6- to 12-wk-old mice were suspended in PBS buffer supplemented with 3% FCS. Cells were incubated with mAbs to lineage markers (Gr-1 and Mac-1 for myeloid cells, CD19 and CD45RA for B lineage cells, TER-119 for erythroid cells, CD3 and CD8 for T lineage cells, and NK1.1 for NK cells), followed by incubation with goat anti-rat IgG-coated magnetic beads (Miltenyi Biotec). Lineage cells attached to beads were removed with a magnetic separator. The lineage-depleted bone marrow cells were then incubated with a mixture of labeled Abs to the lineage markers PE-Cy5-anti-Sca-1 and allophycocyanin-anti-c-Kit. Cells were sorted on a MoFlo cell sorter (DakoCytomation) or a FACSAria cell sorter (BD Biosciences). The LSK fraction was defined as Lin$^-$GFP$^-$Sca-1$^+$c-Kit^{high}, early lymphoid progenitors (ELP) were defined as Lin$^-$GFP$^+$Sca-1$^+$c-Kit^{high}, and prolymphocytes (Pro-L) were defined as Lin$^-$Sca-1$^+$c-Kit^{low}. The purity of each sorted population was confirmed by postsorting analyses.

Human cord blood mononuclear cells were separated over Ficoll/Hypaque (lymphocyte separation medium; Cellgro-Mediatech). Enrichment for CD34$^+$ cells was performed using the Direct CD34 isolation kit (Miltenyi Biotec). Enriched CD34$^+$ cells were stained with anti-Lin-PE (CD33, CD14, and CD64 for monocytes, CD19 for B lineage, CD56 for NK cells, CD3 and CD8 for T cells, and glycophorin for erythrocytes), anti-CD34-allophycocyanin, and anti-CD38-FITC for 20 min at 4°C. One hundred CD34$^+$CD38$^-$ cells were sorted directly into 96-well flat-bottom plates containing pre-established MS-5 stromal cells using the automated cell sorting system of the MoFlo Cell Sorter.

Murine stromal cell cocultures

Double or triple sorted progenitor cells were cocultured with OP9 stromal cells in 96-well plates. The α-MEM medium (Cellgro-Mediatech) contained 10% FCS, recombinant murine (rm) stem cell factor (SCF) (20 ng/ml), rm Flt3 ligand (FL;100 ng/ml), rmIL-7 (1 ng/ml), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and 5×10^{-5} m 2-mE. One hundred progenitor cells were cocultured with 200 stromal cells up to 11 days without replating the stromal cell. At the end of culture, hemopoietic cells were counted excluding stromal cells and then subjected to flow cytometry. We also used anti-CD45.2 mAb to exclude potential contamination of stromal cells.

Human stromal cell cocultures

Human cell cultures were performed as described (21) with some minor modifications. Briefly, 1,000 cells per well of MS-5 stromal cells were plated in 96-well plates 1 day before the seeding of hemopoietic progenitor cells at a concentration of 100 cells per well. Cells were cultured in α-MEM medium and a combination of recombinant human (rh) SCF (20 ng ml^{-1}), rhG-CSF (10 ng ml^{-1}), and rhFL (10 ng ml^{-1}) with or without the presence of ATRA. Dup-697 (Cayman Chemical) was always included in human stromal cell coculture with a final concentration of 1×10^{-7} M to improve the production efficiency of the culture (22). The cultures were maintained for 4 wk and fed weekly by removing half of the medium and replacing it with fresh medium. Cytokines and ATRA were added with each feeding. Flow cytometry was performed at the indicated intervals.

Stromal cell-free, serum-free cultures

Double or triple sorted progenitor cells were put in the X-Vivo medium containing 1% detoxified BSA, 20 ng/ml SCF, 100 ng/ml FL, 1 ng/ml IL7, 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin as described (23). Cells were analyzed by flow cytometry or pre-B colony assay 4–11 days later.

Pro-B colony assays

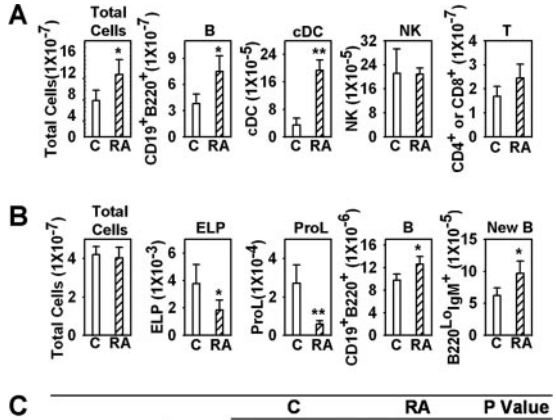
Progenitor cells derived from cocultured ELP were put in Iscove's MDM-based methylcellulose medium (Methocult GF 3630; StemCell Technologies) with an additional supplement of 20 ng/ml rmSCF and 100 ng/ml rmFL. After 7 days, colonies were enumerated under an inverted microscope. In some experiments, flow cytometry was performed to confirm the CD19$^+$ expression, and May-Grünwald/Giemsa staining was used to verify the B lineage lymphoid cell morphology.

Real-time PCR analysis of gene expression

Double sorted progenitor cells, including LSK, ELP, and Pro-L, were lysed and total RNA was isolated using an RNeasy mini kit (Qiagen). To analyze the gene expression pattern affected by ATRA, sorted LSK cells were treated with vehicle (0.1% ethanol) or ATRA in stromal cell-free culture for 1, 3, 5, or 7 days. The treated cells were washed, and total RNA was isolated. Total RNA was treated with DNase I (Invitrogen) to remove contaminating genomic DNA, and cDNA was made using random primers (Invitrogen Life Technologies) and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). The 20-μl real-time PCR amplification mixture contained template cDNA, 1 μl of predesigned TaqMan PCR primers (Applied Biosystems), and 10 μl of 2× TaqMan PCR master mix (Applied Biosystems). Reactions were conducted at 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative gene expression was calculated as $2^{-\Delta\Delta CT}$ (threshold cycle) values with eukaryotic 18S RNA used as an endogenous control. Real-time PCR was performed on an ABI PRISM 7500 (Applied Biosystems). Three repeats were run for each sample, and each experiment was performed three times. TaqMan predesigned PCR primers were purchased from Applied Biosystems.

CFSE labeling

LSK sorted from C57BL/6 mice (1×10^6 cells/ml) were labeled with 10 μM CFSE (Invitrogen Life Technologies catalog no. C34554) from a 5



C

		C	RA	P Value
BM	$CD19^+ CD43^-$	8.2 ± 0.8	11.5 ± 1.2	**0.004**
	$CD19^+CD43^+$	1.18 ± 0.42	0.73 ± 0.15	0.09
	cDC	0.21 ± 0.11	0.69 ± 0.19	**0.004**
	LSK	0.017 ± 0.005	0.014 ± 0.006	0.58
	CMP	0.048 ± 0.013	0.05 ± 0.014	0.80
	$NK1.1^+$	1.3 ± 0.3	0.8 ± 0.2	**0.03**
	$Mac1^+$	6.5 ± 0.7	6 ± 0.9	0.42
SPL	$CD19^+IgM^+$	25.5 ± 8.4	50.9 ± 10.6	**0.009**
	$Mac1^+$	3.8 ± 1.4	5.5 ± 1	0.10
THY	Total Cells	66 ± 29	51 ± 15	0.38
	$NK1.1^+$	0.4 ± 0.2	0.3 ± 0.1	0.68
	$CD8^+$	7.8 ± 3.4	6.2 ± 1.8	0.47
	$CD4^+$	2.9 ± 1.8	1.9 ± 0.7	0.34

FIGURE 1. ATRA depletes primitive lymphoid progenitors in bone marrow and increases B cells in the spleen. RAG-1/GFP mice were treated with ATRA (RA) or placebo pellets (C, control) for 7 days. Bone marrow (BM), splenocytes (SPL), and thymocytes (THY) were isolated, dead cells were excluded by trypan blue staining, flow cytometry was performed, and absolute numbers of cells in the spleen (*A* and *C*), one femur (*B* and *C*), and thymus (*C*) were calculated. Conventional dendritic cells (cDC) were defined as $B220^-CD11b^+CD11c^+$; NK cells were defined as $NK1.1^+$; ELP were defined as $Lin^-RAG\text{-}1^+Sca\text{-}1^+c\text{-}Kit^{high}$; Pro-L were defined as $Lin^-RAG\text{-}1^+Sca\text{-}1^+c\text{-}Kit^{low}$; LSK was defined as $Lin^-GFP^-Sca\text{-}1^+c\text{-}Kit^{high}$; (common myeloid progenitors (CMP) were defined as $Lin^-GFP^-Sca\text{-}1^-c\text{-}Kit^{high}$. All data are reported as mean ± S.D for $n = 4$. *, $p < 0.05$; and **, $p < 0.01$ (Student's t test). All numbers are given as millions of cells, and p values were given in boldface when $p < 0.05$ in *C*. The results shown are representative of five independent experiments.

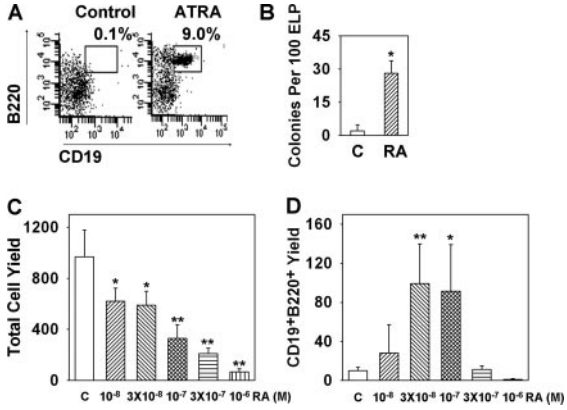


FIGURE 2. ATRA accelerates B lineage potential in culture. *A* and *B*, One hundred highly purified ELP were cocultured with OP9 stromal cells in 96-well flat-bottom plates for 4 or 7 days with ATRA (RA) or 0.1% ethanol diluents (C, control), and then the 4-day cultured cells were transferred to the pre-B colony medium (*B*) and the 7-day cultured cells were subject to flow cytometry to examine the production of $CD19^+B220^+$ cells (*A*). Total pre-B colony numbers were scored after 7 days of culture. *C* and *D*, Sorted ELP were treated with different concentrations of ATRA ranging from 10^{-8} to 10^{-6} M for 7 days, subjected to flow cytometry, and total cell yields (*C*) and B lineage lymphoid cell yields (*D*) were calculated. All treated groups were then compared with the control group using Student's t test. Data are reported as mean ± S.D for $n = 4-6$. *, $p < 0.05$; and **, $p < 0.01$. The results shown are representative of five independent experiments.

mM stock solution (in DMSO) for 10 min at 37°C. Cells were washed and put back in stromal cell-free culture and the intensity of the CFSE signal and the proliferation status of cultured cells were monitored at different times by flow cytometry.

Results

All-trans retinoic acid increases B cell numbers in vivo

As a first approach, time-release pellets containing ATRA or a placebo were implanted into RAG-1/GFP knock-in mice. One week later, spleens in the ATRA-treated mice were notably increased in size, and total nucleated cells were elevated 2-fold (Fig. 1*A*). Flow cytometry revealed that this resulted primarily from increases in B lymphocyte numbers while T cell and NK cell numbers were within the normal range. $CD11b^+$ myeloid cells were not substantially changed, but normally rare $B220^-CD11c^+CD11b^+$ cDC were elevated in response to ATRA treatment.

Two staining protocols were used to determine whether particular B cell subsets were preferentially increased in the spleens of ATRA-treated mice. Follicular B cells, T1 cells, T2 cells, and marginal zone cells increased by 55, 52, 89 and 82%, respectively (19). Follicular B cells, T1 cells, T2 cells, T3 cells, and marginal zone B cells were elevated by 70, 60, 65, 53, and 59%, respectively, with another type of analysis (20).

We then conducted a thorough analysis of hemopoietic cells in bone marrow and were surprised to find that the numbers of total nucleated cells were within the normal range (Fig. 1*B*). Further scrutiny of the B lineage showed that numbers of $Lin^-RAG\text{-}1/GFP^+Sca\text{-}1^+c\text{-}Kit^{high}$ ELP and $Lin^-Sca\text{-}1^+c\text{-}Kit^{low}RAG\text{-}1/GFP^+$ Pro-L were significantly reduced, while $CD19^+CD43^+$ pro-B/large pre-B cells were slightly decreased (Fig. 1*C* and data not shown). In contrast, the numbers of $CD19^+B220^+$ lymphocytes and $CD45R/B220^{low}IgM^+$ newly formed B cells were increased. These changes were both stage and lineage specific, because the larger LSK categories of stem/progenitors, $Lin^-Sca\text{-}1^-c\text{-}Kit^{high}$ $RAG\text{-}1/GFP^-$ common myeloid progenitors and $CD11b^+/Mac1^+$ myeloid cells, were all unchanged (Fig. 1*C*). As with the spleen, there was an increase in $CD11c^+CD11b^+$ myeloid dendritic cells, while $NK1.1^+$ NK/IKDC cells were somewhat decreased in bone marrow. In contrast to the spleen, the numbers of thymocytes were equivalent to those in placebo controls (Fig. 1*C*). These results demonstrate that retinoids can selectively increase the numbers of peripheral B lymphocytes. Reductions in primitive B lineage progenitors in bone marrow, together with increased numbers of B cells, might suggest accelerated differentiation or emigration from that site.

ATRA accelerates differentiation of B lineage progenitors in vitro

The in vivo results indicated that retinoids may normally contribute to the regulation of B lymphopoiesis, and culture experiments were performed to identify retinoid-responsive target cells. ELP were sorted to high purity and cultured for 4 days on OP9 stromal cells with or without 10^{-7} M ATRA. The cells were then harvested and examined by flow cytometry. ELP normally require 10 days to generate $CD19^+$ cells in culture (17). Therefore, we were surprised to find that $CD45R/B220^+CD19^+$ progenitors were produced from ELP in 7 day cocultures when the retinoid was present (Fig. 2*A*). As

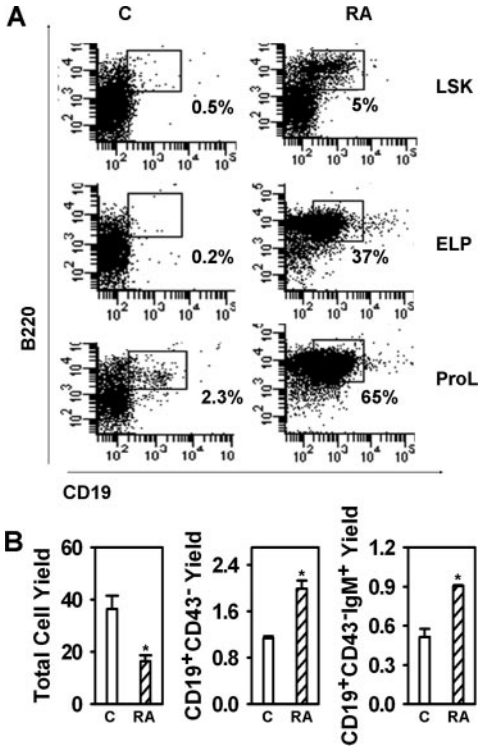


FIGURE 3. ATRA accelerates differentiation of four types of lymphoid progenitors in defined culture. *A*, LSK, ELP and Pro-L were sorted and put in serum-free, stromal cell-free culture for 7 days. Recovered cells were then counted and subjected to flow cytometry. Data shown are percentages of B lineage lymphoid cells recovered from LSK (*top panel*), ELP (*middle panel*), and Pro-L (*bottom panel*). *B*, Pre-B/pro-B cells ($Lin^-CD19^+CD43^+$) were sorted and were put in stromal cell-free culture for 7 days. Recovered cells were then counted and subject to flow cytometry. Data shown are total cell yields (*left panel*), $CD19^+CD43^-$ cell yields (*middle panel*), and $D19^+CD43^-IgM^+$ cell yields (*right panel*). The results shown are representative of three independent experiments. C, Control; RA, ATRA.

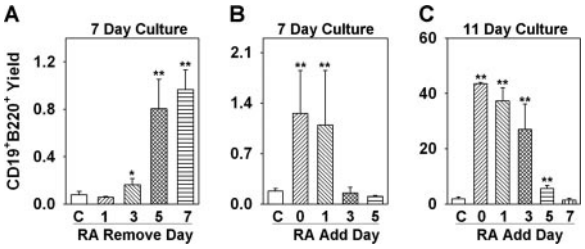


FIGURE 4. Time required for ATRA effects on B lymphopoiesis. *A*, Sorted LSK were treated with ATRA (RA) in stromal cell-free culture for 1, 3, 5, or 7 days, and the treated cells were washed and put back in culture. All of the cells were recovered on day 7 and flow cytometry was performed to examine B lineage lymphoid cell ($CD19^+B220^+$) production. *B*, Sorted LSK cells were put in stromal cell-free culture and ATRA was added at day 0, day 1, day 3, or day 5 and was kept in the culture until day 7. All of the cells were then recovered and flow cytometry was performed to examine the B lineage lymphoid cell ($CD19^+B220^+$) production. *C*, Sorted LSK were put in stromal cell-free culture and ATRA was added on day 0, day 1, day 3, day 5, or day 7 and was kept in the culture until day 11. All of the cells were recovered and flow cytometry was performed to examine the B lineage lymphoid cell ($CD19^+B220^+$) production. All data are reported as means ± S.D. for $n = 3$.*, $p < 0.05$; and **, $p < 0.01$ (Student's *t* test). Different *y*-axis scales were used to reflect the production of B lineage cells over time, and the results shown are representative of three independent experiments. C, Control.

another indication of maturity, precultured cells were tested for their ability to proliferate in response to IL-7 in Methocel cultures (Fig. 2*B*). This clonal assay normally measures the competence of $CD19^+$ lymphoid progenitors to respond to IL-7, and many colony-forming cells were generated in suspensions that had been pretreated for only 4 days with ATRA.

Experiments were then conducted to learn whether lymphopoiesis is affected by physiological concentrations of ATRA. As little as 10^{-8} M accelerated the differentiation of Pro-L (data not shown), and 3×10^{-8} M concentrations optimally stimulated B lineage differentiation from ELP (Fig. 2*D*). It should be noted that the numbers of total recovered cells were suppressed in a dose-dependent fashion (Fig. 2*C*).

Stromal cells could have been influenced by retinoids in the coculture experiments, and we sought additional information concerning target cells. Therefore, progenitors representing three successive stages of differentiation were used to initiate stromal cell-free, serum-free cultures (Fig. 3*A*). Stem cells, multipotent progenitors, and ELP are contained within the rare LSK fraction of bone marrow, and LSK generated $CD19^+$ cells in 7-day cultures containing ATRA. Even more $CD19^+$ progenitors arose in cultures initiated with highly purified ELP. Pro-L, a subset that includes common lymphoid progenitors, generate $CD19^+$ cells in 1-wk cultures, and this was augmented by the inclusion of ATRA.

The analysis was then extended to later stages of B lineage differentiation by initiation of cultures with $CD19^+CD43^+$ progenitors, a fraction that contains pro-B and large pre-B cells (Fig. 3*B*). Again, the numbers of total nucleated cells were depressed by the retinoid while there were marked increases in $CD19^+CD43^-$ pre-B cells as well as progression to $CD19^+CD43^-sIgM^+$ B cells.

These results show that most if not all stages of B lymphopoiesis are influenced by ATRA and that lymphoid progenitors are direct targets of the compound. At least some cells in each population responded by rapidly differentiating.

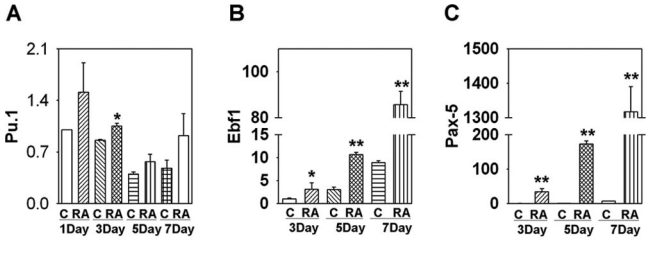
Time required for acceleration of B lymphopoiesis by ATRA

Additional information about target cells was then sought with a series of time course experiments. Defined, stromal cell-free conditions were again used for 7-day cultures initiated with RAG-1/GFP^- LSK. When ATRA was washed out after 5 days, the yield of $CD19^+$ cells was almost as high as when the drug remained in culture for the whole time (Fig. 4*A*). Differentiation was significantly augmented when ATRA was present for 3 or more days of the 7-day culture, but there were no changes with just 1 day of exposure.

The experimental design was then modified slightly to determine the minimum time required for retinoid responsiveness. Again, a 7-day culture interval was used, but with ATRA added on different days. Delay until the next day of culture was as effective as when the compound was present for all 7 days (Fig. 4*B*). However, there was no measurable influence when ATRA was added on day 3 or 5.

The total culture interval was then extended to 11 days (Fig. 4*C*) to allow more time for progenitors to differentiate. Total yields of $CD19^+$ lymphocytes were now very high when the drug was added at the beginning of culture (day 0), on day 1, or on day 3. A smaller but still significant increment was even seen when ATRA was added on day 5 for a total period of 11 days in culture.

These results complement those obtained when cultures were initiated with various types of progenitors (Fig. 3). That is, the



D

	PBX1	Bcl11a	IL7Rα	TSLP
1Day C	1.00 ± 0.06	1.00 ± 0.08	1.0 ± 0.68	1.000 ± 0.510
RA	0.39 ± 0.33	0.71 ± 0.12	1.9 ± 0.42	0.090 ± 0.010
3Day C	0.13 ± 0.01	0.51 ± 0.01	12 ± 1.89	0.012 ± 0.007
RA	0.22 ± 0.09	0.65 ± 0.05	31 ± 3.2	0.010 ± 0.009
5Day C	0.03 ± 0.01	0.43 ± 0.01	39 ± 0.45	0.004 ± 0.001
RA	0.07 ± 0.01	0.33 ± 0.06	29 ± 4.4	0.003 ± 0.001
7Day C	0.03 ± 0.01	0.65 ± 0.04	76 ± 5.0	0.009 ± 0.001
RA	0.14 ± 0.01	0.61 ± 0.06	105 ± 6.2	0.014 ± 0.006

FIGURE 5. ATRA dramatically increase *EBF1* and *Pax-5* expression. Double sorted LSK were treated with ATRA (RA) in stromal cell-free culture for 1, 3, 5, or 7 days, the treated cells were washed, total RNA was isolated, and RT-PCR was then performed to examine the mRNA expression of *Pu.1* (*A*), *EBF1* (*B*), *Pax-5* (*C*), *PBX1*, *Bcl11a*, *IL7Rα*, and *TSLP* (*D*). Expression was normalized to 18S RNA in each sample and given as $2^{-\Delta\Delta CT}$ (threshold cycle) values on the *y*-axes. *EBF1* and *Pax-5* results were calculated relative to the 3-day control (C) group, and the expressions of other genes were determined by comparison to the 1-day control group. All data are reported as means ± S.D. for $n = 3$. *, $p < 0.05$; and **, $p < 0.01$ compared with vehicle (0.1% ethanol)-treated cells within the same period of time. The results shown are representative of three independent experiments.

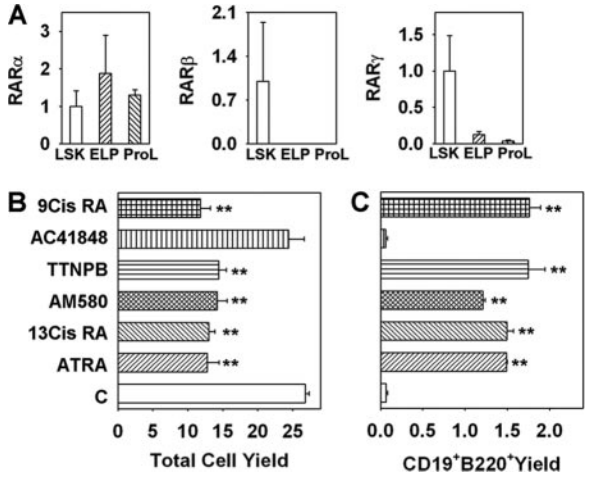


FIGURE 6. Retinoid receptors involved in B lymphopoiesis. *A*, Expression patterns of different RAR isoforms on lymphoid progenitors. Double sorted LSK, ELP, and Pro-L were subjected to real-time PCR. Results obtained for LSK with each primer set were artificially defined as 1, and numbers given on the *y*-axes were calculated as $2^{-\Delta\Delta CT}$ (threshold cycle) values. *B* and *C*, Sorted LSK cells were put in stromal cell-free cultures, treated with different retinoids for 7 days, subjected to flow cytometry, and total cell yield (*B*) and B lineage lymphoid cell yield (*C*) were calculated. ATRA is all *trans* retinoic acid, a ligand for RARα, RARβ and RARγ; 13Cis RA is 13-*cis* retinoic acid, a ligand specific for RARα and RARγ; AM580 is a ligand specific for RARα; TTNPB is a ligand for RARα, RARβ, and RARγ; AC41848 is a specific ligand for RARγ; 9Cis RA is 9-*cis* retinoic acid, a ligand for RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ. All data are reported as means ± S.D. for $n = 3$. **, $p < 0.01$ (Student's *t* test). The results shown are representative of three independent experiments.

short time required for a biological response suggests that primitive lymphoid progenitors are direct targets of ATRA. Exposure during the first 3 days was needed to achieve maximum responses.

Retinoids augment critical events in lymphopoiesis by ligating RARα receptor and do not inhibit proliferation

The early B cell factor 1 (Ebf1) and paired box gene 5 (Pax-5) transcription factors are needed to initiate and sustain B lymphopoiesis, respectively (24–27). Expression of these transcription factors in LSK was below the levels detectable by quantitative real-time PCR during the first day of culture (Fig. 5, *B* and *C*). However, both were measurable on day 2 and significantly increased in response to ATRA (Fig. 5 and data not shown). Levels of *Ebf1* mRNA rose with the duration of the culture, and the ratio of ATRA treated to controls increased from 3 on days 3 and 5 to 10 by day 7. The magnitude of change in *Pax-5* transcripts progressively increased with time such that ATRA-containing cultures were 34-, 133-, and 190-fold greater than control values on days 3, 5, and 7, respectively (Fig. 5*C*).

Critical levels of the PU.1 transcription factor are also needed for B lymphopoiesis and the *EBF1* gene is a potential target (28). Real-time PCR analyses revealed elevations in *PU.1* transcripts at all time points in retinoid-treated cultures (Fig. 5*A*). *PBX1* was of special interest because of its known responsiveness to retinoic acid, and *Bcl11a* is also important for the early stages of lymphopoiesis (11, 29). However, no consistent trends were found with *PBX1* or *Bcl11a* transcripts in ATRA-treated cultures (Fig. 5*D*). IL-7 is essential for the expansion of pro-B and pre-B populations in adult mice (30), and levels of *IL-7Rα* steadily increased with time in culture (Fig. 5*D*). This was slightly augmented by ATRA at some time points. TSLP is an IL-7 like cytokine and, like ATRA, can induce the production of IgM^+ cells (12). Furthermore, one report suggested that ATRA may induce TSLP in the skin (16). It is interesting that *TLSP* was detectable by quantitative real-time PCR in hemopoietic cells, because it might be involved in autocrine regulation of lymphopoiesis (Fig. 5*D*). However, the expression of *TSLP* was not consistently induced by ATRA, and levels were even reduced when ATRA was present for only 1 day.

Combinations of three RAR and three RXR receptor subunits are used by cells to recognize retinoids (31, 32). To determine what RAR subunits participate in B cell differentiation, we first determined the mRNA expression patterns of *RARα*, *RARβ*, and *RARγ* on all three lymphoid progenitor subsets including LSK, ELP, and Pro-L. Real-time PCR quantification indicated that LSK express all three isoforms of *RAR*, and this agrees with a recent report about *RAR* expression patterns on LSK (4). Both ELP and Pro-L express *RARα* and *RARγ*, but neither of them expresses *RARβ*. *RARα* was the most abundant subtype expressed on all three progenitors (Fig. 6*A*). To further explore mechanisms, the results of ATRA analyses were extended to related compounds. We compared five well-studied retinoids to ATRA for their potential influence on B lymphopoiesis (Fig. 6, *B* and *C*). With the exception of AC 41848, a known RARγ-specific ligand, all were effective. All other substances depressed total nucleated cell numbers and accelerated the generation of $CD19^+$ lymphocytes comparable to ATRA. Patterns of receptor use of these compounds are given in the figure legend.

It has been reported that retinoids inhibit the proliferation of lymphoid precursors (7, 33). However, analysis of CFSE label dilution from ATRA-treated cells suggests that this was not obvious for the cells that survived in our cultures (Fig. 7*A* and *B*).

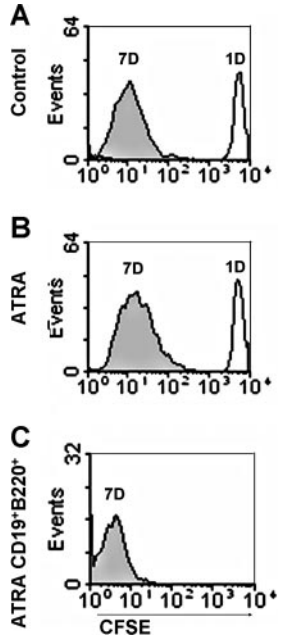


FIGURE 7. ATRA does not inhibit proliferation of B lineage cells. Sorted LSK were treated with 10 μM CFSE and the treated cells were put back in stromal cell-free cultures with or without the presence of ATRA. The intensities of CFSE signals were then monitored on day 1 and on day 7 by flow cytometry. *A*, Recovered total cell CFSE intensity of control group on day 1 (dark line) and day 7 (gray area). *B*, Recovered total cell CFSE intensity of ATRA-treated group on day 1 (dark line) and on day 7 (gray area). *C*, CFSE intensity of gated $CD19^+B220^+$ cells (as indicated in Fig. 3*A*, *top panel*) derived from 7-day stromal cell-free culture with ATRA. The results shown are representative of three independent experiments.

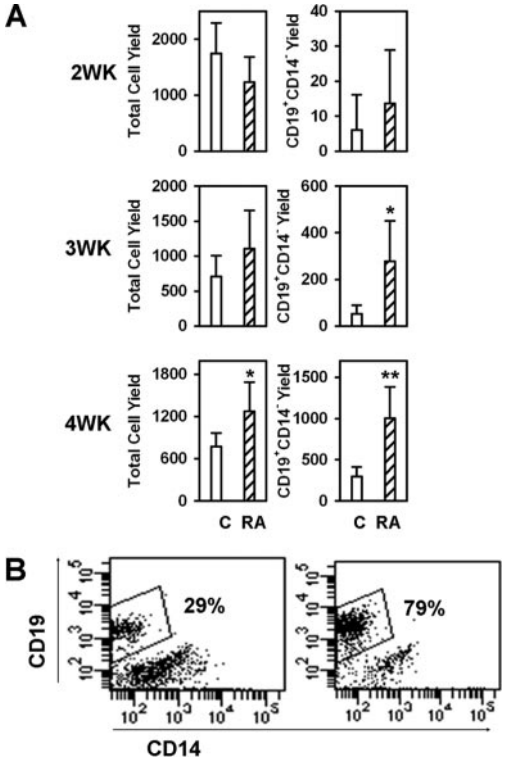


FIGURE 8. ATRA has a similar effect on human B cell development. *A*, $CD34^+CD38^-$ cells sorted from human cord blood were cocultured with MS-5 stromal cells with (RA) or without (C, control) the presence of ATRA for 2 wk (*top panel*), 3 wk (*middle panel*), or 4 wk (*bottom panel*) and subjected to flow cytometry. Total cell yields and B lineage lymphoid cell yields ($CD19^+CD14^-$) were calculated. All data are reported as means $\pm$ S.D. for $n = 5$–20. *, $p < 0.05$; and **, $p < 0.01$ (Student's t test). Different y-axis scales were used in the *right panels* to reflect production of B lineage cells over time, and the results shown are representative of three independent experiments. *B*, An example of flow cytometry results for 4-wk cultures is shown.

Indeed, $CD19^+$ lymphocytes had an even higher history of proliferation than other cells in the culture (Fig. 7*C*).

We conclude from these experiments that expression of two critical transcription factors is augmented by ATRA. PCR analysis indicates that all lymphoid progenitors express high levels of *RARα* and low levels of *RARγ*. The primitive LSK subset can recognize at least five retinoids. Absence of responsiveness to the drug AC 41848 suggests that RARγ is not important, so it is possible that RARα mediates the ATRA effect in B lineage cell differentiation. Contrary to previous reports, proliferation of B lineage progenitors was not inhibited.

Human lymphoid progenitors are also responsive to retinoic acid

Human B lineage differentiation in culture is much less efficient than that with murine progenitors, and it has not been achieved under defined conditions (34). However, human umbilical cord blood $CD34^+CD38^-$ cells differentiate into $CD19^+$ B lineage lymphocytes when cultured with MS-5 mouse stromal cells (35). Therefore, we used this model to determine whether retinoids influence B lymphopoiesis.

Similar to our experience with murine cells, the addition of ATRA reduced overall cell yields in 2-wk cultures, but the substance actually improved cell recoveries when the culture interval was extended to 4 wk (Fig. 8). ATRA was replenished at each weekly feeding, and the cells were not subcultured during this time. More importantly, the numbers of $CD19^+$ $CD14^-$ lymphocytes were significantly increased in ATRA-containing cultures maintained for 3 or more weeks. Although there was experiment to experiment variability, improved differentiation was seen in each of three independent experiments, as well as in another conducted with total $CD34^+$ cells.

Discussion

Retinoids are widely used as therapeutics, and there is evidence that vitamin A deprivation can cause immunodeficiency (5). Furthermore, there are numerous reports of an influence on hematopoiesis (1), and recent findings suggest that the RARγ receptor subunit is important for maintaining stem cell integrity (4). However, much less is known concerning roles in B lymphocyte formation. Our study began with the finding that ATRA treatment of mice caused profound changes in spleen B cell numbers. Subsequent analyses were designed to learn whether lymphoid progenitors or other cells are direct retinoid targets. We conclude that retinoids affect expression of two key transcription factors and accelerate progression through the B lymphocyte lineage. Therefore, retinoid gradients may normally provide rate limiting signals for B cell production.

The availability of reporter strains of mice, multiparameter flow cytometry, and cell culture innovations has permitted considerable progress to be made in identification of lymphohemopoietic cells in bone marrow. For example, we can isolate extremely rare $RAG\text{-}1^+$ ELP and know that it will take them ~10 days to yield $CD19^+$ lymphocytes in culture. The addition of ATRA significantly shortens this interval, and we found large numbers of $CD19^+$ cells within 1 wk in treated cultures. Oral treatment of

humans with 1 mg/kg/day is said to result in a mean plasma concentration of 400 ng/ml or 1.3×10^{-6} M. Importantly, we observed significant effects on lymphoid progenitors in culture with as little as 3×10^{-8} M concentrations. We consider this good evidence that retinoids can accelerate B lymphopoiesis.

Cultures initiated with the stem cell-enriched $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{high}$ LSK fraction of bone marrow was similar to those initiated with ELP in response to ATRA. That is, ATRA increased the numbers of $CD19^{+}$ cells generated in 1-wk cultures. Cultures started with more differentiated $Lin^{-}c\text{-}Kit^{low}RAG\text{-}1^{+}$ Pro-L normally generate lymphocytes in just 1 wk, and this was augmented by retinoid treatment. $CD43^{+}CD19^{+}$ pro-B/large pre-B cells gave rise to more $CD43^{-}CD19^{+}$ cells, including $sIgM^{+}$ B cells, when treated with this drug. Our experimental design used stromal cells for cultures of LSK and ELP, but no stromal cells were present when we assessed the effects of retinoids on Pro-L or pro-B cells. In that case, it was clear that the numbers of $sIgM^{+}$ B cells were substantially increased by retinoids. Therefore, these findings suggest that multiple stages in the B lineage are affected and that at least the more mature progenitors are responsive.

Alterations in our treatment protocols were then used to obtain further information about retinoid-responsive cells. As little as 3 days of ATRA exposure altered B lymphopoiesis, and 5 days were required for maximum effect. The kinetic information again suggests that retinoids may work across multiple stages of B lymphopoiesis. It also provided a basis for initiating further investigations concerning mechanisms.

Expression of many genes is altered by ATRA, even when the relevant promoters lack a retinoid acid response element (8). Also, cultures containing more $CD19^{+}$ cells would be expected to have more lymphocyte-related transcripts. Therefore, it is difficult to identify proximal events that determine retinoid effects on B lymphopoiesis, and we narrowed our search in two ways. Most of our measurements were not begun before 3 days of treatment because that interval is required for biological activity. In addition, we focused on genes that are essential for lymphopoiesis and/or are known to be retinoid regulated.

The PU.1 transcription factor is needed for B lymphopoiesis; it can act in a dose-dependent fashion, and levels were significantly higher at an early time point in cultures containing ATRA. In contrast, there was no consistent trend with Bcl11a. PBX1 levels fluctuated in our experiments even though the abundance and stability of its transcripts are increased by retinoids and conditional targeting in early progenitors selectively arrested the lineage (11). IL-7 is essential for the expansion of pro-B and pre-B populations in adult mice (30), but levels of *IL-7Rα* transcripts were only higher at some intervals of treatment. TSLP is an IL-7-like cytokine that can induce the production of IgM^{+} cells (12), and we were surprised to consistently find evidence for its production in hemopoietic cells. Also, there was a report that ATRA may induce TSLP in the skin (16). However, the PCR data do not suggest that TSLP accounted for the augmentation of B lymphopoiesis by ATRA in culture.

Detectable levels of *EBF1* and *Pax-5* transcripts were present within 3 days of culture. Quantitative real-time PCR analyses suggest that while both were elevated with ATRA treatment, the *Pax-5* response was particularly impressive. *EBF1* controls *Pax-5* transcription in a feed forward mechanism whereby Pax-5 augments *EBF1* transcription (36). Additionally, promoters of *PU.1, Ebf1*, and *Pax-5* genes have candidate retinoic acid response elements (data not shown). Direct regulation of these genes would represent a powerful rheostat for controlling lymphocyte production. In contrast, slight changes in cell surface markers were evident as early as 4 days after drug treatment, and transcript levels might just reflect the increased abundance of differentiating lymphocytes. Further study will be required to learn whether any of these critical genes are directly or indirectly regulated by retinoids.

It is possible that retinoids control events other than the time required for progenitors to differentiate in bone marrow. For example, the reductions in primitive lymphoid cells, together with increases in all spleen B cell subsets of ATRA-treated mice, could have resulted from increased proliferation, survival, and/or replenishment by B cells exported from bone marrow. It has been reported that retinoic acid can increase the expression of a gut-homing receptor and modulate both B and T migration in mucosal immunity (37, 38). If that is the case in the hemopoietic system, ATRA may contribute to B lineage cell exit from the bone marrow. It is easier to draw conclusions from experiments involving highly purified progenitors under defined conditions of culture. Then, at least, altered migration is excluded from consideration. It has been reported that ATRA inhibits the proliferation of B cell precursors (7, 33), and we always recovered fewer total cells from drug-containing cultures. However, CFSE labeling revealed that a subset of cells rapidly differentiated under the influence of ATRA and had a history of extensive proliferation. In addition, there was evidence for some proliferation by cells that lacked CD19 at the end of culture. Preliminary experiments with Bcl-2 transgenic mice and staining with annexin V did not suggest that retinoids increased apoptosis, so we are left with no clear explanation for the reductions in the numbers of total nucleated cells. The findings would be compatible with heterogeneity in progenitor populations or changes in proliferation rates at particular times.

Our studies were inspired in part by a report that RARγ has a critical role in stem cell maintenance (4). However, one agonist that is specific for this receptor subunit had no effect on B lymphopoiesis in culture. The testing of additional compounds and analysis of knockout mice might be informative in this regard.

In two preliminary experiments, murine LSK were held with ATRA for 24 h before transplantation. The numbers of donor type $CD19^{+}$ lymphocytes in bone marrow were increased (data not shown). Furthermore, human umbilical cord blood $CD34^{+}CD38^{-}$ progenitors were responsive to ATRA (Fig. 8). Consequently, one could imagine augmenting B cell production in some clinical settings.

In summary, our observations indicate the widely used drug ATRA has a previously undisclosed effect on B lymphopoiesis. The ability of retinoids to accelerate B lineage cell differentiation may be applied to improve humoral immune recovery following chemotherapy or bone marrow transplantation. Given the widespread therapeutic use of ATRA, it is important to learn more about the effects on human B cell development.

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Disclosures

The authors have no financial conflict of interest.

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