

Adiponectin, a Fat Cell Product, Influences the Earliest Lymphocyte Precursors in Bone Marrow Cultures by Activation of the Cyclooxygenase-Prostaglandin Pathway in Stromal Cells¹

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Adiponectin, an adipocyte-derived hormone, is attracting considerable interest as a potential drug for diabetes and obesity. Originally cloned from human s.c. fat, the protein is also found in bone marrow fat cells and has an inhibitory effect on adipocyte differentiation. The aim of the present study is to explore possible influences on lymphohematopoiesis. Recombinant adiponectin strongly inhibited B lymphopoiesis in long-term bone marrow cultures, but only when stromal cells were present and only when cultures were initiated with the earliest category of lymphocyte precursors. Cyclooxygenase inhibitors abrogated the response of early lymphoid progenitors to adiponectin in stromal cell-containing cultures. Furthermore, PGE₂, a major product of cyclooxygenase-2 activity, had a direct inhibitory influence on purified hematopoietic cells, suggesting a possible mechanism of adiponectin action in culture. In contrast to lymphopoiesis, myelopoiesis was slightly enhanced in adiponectin-treated bone marrow cultures, and even when cultures were initiated with single lymphomyeloid progenitors. Finally, human B lymphopoiesis was also sensitive to adiponectin in stromal cell cocultures. These results suggest that adiponectin can negatively and selectively influence lymphopoiesis through induction of PG synthesis. They also indicate ways that adipocytes in bone marrow can contribute to regulation of blood cell formation. *The Journal of Immunology*, 2003, 171: 5091–5099.

The fraction of bone marrow that is occupied by fat is substantial and increases with age (1, 2). Fat cells derive from the same mesenchymal stem cells that give rise to the hematopoiesis supporting stromal cells in that organ and have long been suspected to influence blood cell formation (1, 3). Indeed, many fat cell products, including type 1 IFNs, PGs, leptin, and sex steroids are known modulators of lymphohematopoiesis (4–9). A positive correlation between the ability of stromal cells to undergo adipocyte differentiation and the ability to support hematopoiesis in culture has been repeatedly reported (10, 11). However, patterns of cytokines made by mature adipocytes and preadipocyte stromal cells differ substantially (12). There is a growing appreciation that fat cells in other tissues function as part of an intricate endocrine network, responding to and producing hormone-like substances (13). Therefore, it is reasonable to assume that adipocytes within bone cavities functionally interact with cells that surround them.

Adiponectin, also known as Acrp30, AdipoQ, and GBP28, was recently discovered as an abundant protein made exclusively by fat cells (14–17). The molecule is a homotrimer that is similar in size and overall structure to complement protein C1q, with particularly

high homology in the C-terminal globular domain (16). Solution of the adiponectin crystal structure revealed additional high similarity between the same domain and TNF- α (18). Adiponectin synthesis corresponds to adipocyte differentiation in culture and is inhibited by TNF- α (19).

The normal biologic activities of adiponectin are poorly understood, but provocative findings suggest potential involvement in obesity, cardiovascular disease, and diabetes. Production and circulating protein concentrations are suppressed in obese mice and humans (15, 20). Low plasma levels may be a risk factor in coronary heart disease and concentrations are also significantly reduced in type 2 diabetes (21, 22). Injections of recombinant intact or fragmented adiponectin reduce blood glucose, overcome insulin tolerance, decrease fatty acids, and can cause weight loss in obese mice (23–25). An inverse relationship between insulin tolerance and adiponectin levels in plasma was also shown in adiponectin knockout mice (26). Metabolic changes in muscle and hepatocytes may account for these systemic changes. Human aortic endothelial cells directly respond to adiponectin with modulated NF- κ B-mediated signals, and this leads to reduced adhesiveness for monocytes (21, 27).

Hematopoietic cells and the microenvironment that supports their differentiation are also potential adiponectin targets. We recently determined that adiponectin is made by fat cells within human bone marrow and blocks differentiation of marrow preadipocytes through a paracrine mechanism (28). Furthermore, the protein suppresses myelomonocytic progenitor growth and macrophage functions in culture (29). More complex long-term bone marrow cultures (LTBMCs)³ were exploited in this study to explore adiponectin influences on other lymphohematopoietic cells.

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³ Abbreviations used in this paper: LTBMC, long-term bone marrow culture; SCF, stem cell factor, FL, flk2/flt3 ligand; COX, cyclooxygenase; Lin[−], lineage marker negative; W/W, Whitlock-Witte culture; ELP, early lymphoid progenitor; CB, cord blood; CFU, colony-forming units.

We now report that although early lymphohematopoietic progenitors are not directly responsive, their differentiation is influenced through adiponectin-induced changes in stromal cells. The findings suggest new mechanisms for functional interactions between fat cells and the surrounding hematopoietic tissue within bone marrow.

Materials and Methods

Animals and cell sources

BALB/c mice were obtained at 4–8 wk of age from the Oklahoma Medical Research Foundation Laboratory Animal Resource Center or Charles River Breeding Laboratories (Wilmington, MA) and used for culture experiments. Human fresh cord blood (CB) samples were obtained from placentas of healthy newborns at the Oklahoma University Hospital (Oklahoma City, OK). The murine bone marrow stromal cell line, MS-5, was generously provided by Dr. Mori (Niigata, Japan) and maintained in α -MEM medium supplemented with 10% FCS.

Reagents

We used highly purified recombinant human adiponectin and the ANOC 9103 murine mAb reactive with adiponectin (20). Briefly, a 693-bp adiponectin cDNA encoding a peptide leader-deficient protein was subcloned into the pET3c expression vector and was used to transform host *Escherichia coli*, BL21(DE3)pLysS. Synthesis of recombinant adiponectin was induced by isopropyl- β -D-thiogalactoside. Bacterial extracts were prepared using standard methods and recombinant human adiponectin was purified by DEAE-5PW ion-exchange HPLC (Toso, Japan) as previously described (28). Effect of adiponectin was evaluated at 10 μ g/ml throughout this report. Potential endotoxin contamination was <0.07 endotoxin U/ml as determined by Limulus Amebocyte Lysate Pyrogen Plus (BioWhittaker, Walkersville, MD). In some experiments, recombinant GST was also prepared from the same strain of *E. coli* and used as a control. The mAb ANOC 9103 was raised against recombinant human adiponectin and used at 30 μ g/ml.

Recombinant murine IL-7 was purchased from Endogen (Woburn, MA). Recombinant murine stem cell factor (SCF), recombinant murine flk2/flt3 ligand (FL), recombinant human SCF, and recombinant human G-CSF were purchased from R&D Systems (Minneapolis, MN).

Cyclooxygenase (COX) inhibitors (Dup-697, SC-58125, NS-398, 2-acetoxyphenylhept-2-ynyl sulfide (APHS), SC-560) and PGE₂ were purchased from Cayman Chemicals (Ann Arbor, MI). Tested concentrations in each inhibitors are described in Fig. 4 and Table II (see below).

Antibodies

Regarding mAbs for murine Ags, anti-CD19 mAb (1D3) was purified from the culture supernatant of hybridoma cells grown in our laboratory. Anti-CD45RA (14.8) mAb developed in our laboratory and the anti-Mac-1/CD11b (M1/70) mAb were used as culture supernatants of the respective hybridomas. Purified anti-erythroid (TER-119) and anti-Ly-6G (Gr-1) and Ly-6C (RB6-8C5) mAbs, FITC-conjugated anti-CD2 (RM2-5), anti-CD3 (145-2C11), anti-CD8 (53-6.7), anti-CD19 (1D3), anti-CD45R/B220 (RA3/6B2), anti-Mac-1 (M1/70), and anti-Ly-6G and Ly-6C mAbs, PE-conjugated anti-CD2 (RM2-5), anti-CD19 (1D3), anti-TER-119, anti-CD45R/B220 (RA3/6B2) and anti-Sca-1 (Ly6A/E; D7) mAbs, biotinylated anti-IL-7R α (B12-1) and anti-VCAM-1 (429 MVCAM.A) mAbs, and allophycocyanin-conjugated anti-c-kit (2B8), and anti-CD45R (RA3/6B2) mAbs were all purchased from BD Pharmingen (San Diego, CA). Regarding mAbs for human Ags, FITC-conjugated anti-CD13 (TUK1), anti-CD33 (4D3), and PE-conjugated anti-CD19 (SJ25-C1) mAbs were purchased from Caltag Laboratories (Burlingame, CA).

Long-term bone marrow cultures

LTBMCs of lymphoid cells (Whitlock-Witte (W/W) cultures) were initiated and maintained according to published methods (30). Bone marrow cells (8×10^6) derived from BALB/c mice (4-wk-old) were cultured in 25-cm² flasks in 5% CO₂ at 37°C. The medium consisted of RPMI 1640 supplemented with 50 μ M 2-ME and 5% FCS. LTBMCs of myeloid cells (Dexter cultures) were initiated and maintained by published methods (31). Bone marrow cells (12×10^6) from BALB/c mice (6–8-wk-old) were cultured in 25-cm² flasks in 5% CO₂ at 33°C. The medium consisted of α -MEM supplemented with 100 nM hydrocortisone and 20% horse serum (HyClone Laboratories, Logan, UT). Both cultures were maintained by changing one-half of their medium once a week. They were treated with adiponectin or BSA beginning at culture initiation and thereafter weekly.

Colony-forming cell assay

Bone marrow or spleen cells were prepared and suspended in 1 ml of assay medium as described (32). The semisolid agar colony-forming units (CFU) assay for lymphoid clones responsive to IL-7 used 1 ng/ml recombinant mouse IL-7. The CFU assay for B lymphocyte clones responsive to LPS used 25 μ g/ml LPS. Both colony assays used 35-mm dishes and were incubated at 37°C for 6 days.

Cell sorting

Mouse bone marrow cells were collected from BALB/c mice (6–8-wk-old) and suspended with Hanks' medium supplemented with 3% FCS. Cells were incubated with Abs to lineage markers (Gr-1 and Mac-1 for myeloid cells, anti-CD19 and anti-CD45RA for B lineage cells, and TER-119 for erythroid cells), followed by incubation with goat anti-rat IgG-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells attached to beads were removed with a magnetic separator using negative selection columns. The lineage-depleted bone marrow cells were then incubated with a mixture of labeled Abs to the lineage markers (FITC-conjugated anti-CD3, anti-CD8, anti-Gr-1, anti-Mac-1 PE-conjugated TER-119, anti-CD2, and anti-CD45R) and allophycocyanin-conjugated anti-c-kit Ab to sort lineage marker negative (Lin[−]) c-kit^{low} or Lin[−] c-kit^{high} populations. In some experiments, a PE-labeled anti-Sca-1 Ab and a biotinylated anti-IL-7R α Ab were also used to isolate Lin[−] IL-7R α [−] c-kit^{high} Sca-1⁺ or Lin[−] IL-7R α [−] c-kit^{high} Sca-1[−] cells. In this case, PE-labeled TER-119 was eliminated and FITC-conjugated anti-CD45R and anti-CD2 Abs were used instead of PE-conjugated Ab. Streptavidin-RED613 (Life Technologies, Rockville, MD) was used as the secondary reagent for biotinylated anti-IL-7R α . Stained cells were subjected to sorting on a MoFlo (Cytomation, Fort Collins, CO).

Human CB mononuclear cells were separated over Ficoll/Hypaque (Lymphocyte Separation medium; Cellgro-Mediatech, Herndon, VA). Enrichment for CD34⁺ cells from CB mononuclear cells was performed following manufacturer's instructions using the Direct CD34 Isolation kit (Miltenyi Biotec). CD34⁺-enriched cells were washed in PBS with 3% FCS, stained with CD34-FITC and CD38-PE for 30 min at 4°C. Sixty wells of a 96-well flat-bottom plate containing pre-established MS-5 stromal cell layers were plated with 10 or 20 cells each using the Automated Cell Deposition unit of the MoFlo.

Serum-free, stromal cell-free cultures

Murine Lin[−] c-kit^{low} (5000 cells/well) or Lin[−] c-kit^{high} (2000 cells/well) cells were cultured in 24-well culture plates (Costar, Cambridge, MA) with X-VIVO15 medium (BioWhittaker) containing 1% detoxified BSA (Stem-Cell Technologies, Vancouver, BC, Canada), 50 μ M 2-ME, 2 mM L-glutamine, 1 ng/ml recombinant murine IL-7, 100 ng/ml recombinant murine FL, and 20 ng/ml recombinant murine SCF as previously described (33). Each culture was fed every 4 days and maintained for 7–9 days or 12–14 days, respectively. At the end of culture, cells were harvested and counted with a hemocytometer. The proportion of B cells, myeloid lineage, or primitive cells was determined by flow cytometry using anti-CD19, CD45R, and Mac-1 mAbs. Our criteria for each lineage is CD19⁺ Mac-1[−] for B lymphoid lineage, Mac-1⁺ for myeloid lineage and CD19[−]CD45R[−] Mac-1[−] for primitive cells.

Coculture assay

Sorted Lin[−] c-kit^{low} (3000 cells/well for 7–9 days) or Lin[−] c-kit^{high} (1000 cells/well for 10–12 days) cells were cocultured with MS-5 cells in 24-well plates. The α -MEM medium contained 10% FCS, 1 ng/ml recombinant murine IL-7, 100 ng/ml recombinant murine FL and 20 ng/ml recombinant murine SCF. Lin[−] IL-7R α [−] c-kit^{high} Sca-1⁺ (500 cells/well) or Lin[−] IL-7R α [−] c-kit^{high} Sca-1[−] cells (1000 cells/well) were cultured for 10–12 days under the same conditions. At the end of culture, cells were counted on a hemocytometer excluding stromal cells and then subjected to flow cytometric analysis. We used a biotinylated anti-VCAM-1 mAb in addition to CD19, CD45R, and Mac-1 mAbs to exclude potential contamination of VCAM-1⁺ MS-5 cells in the analyzed populations.

Human CB CD34⁺CD38[−] cells were cultured with MS-5 cells in α -MEM medium containing 10% FCS, 100 ng/ml recombinant human SCF, and 10 ng/ml recombinant human G-CSF (34). They were directly sorted into wells of 96-well flat-bottom plates preseeded with MS-5 cells, and then cultured for 6 wk. One-half the medium was replaced twice a week with fresh medium containing the same concentration of cytokines. Wells containing hematopoietic cell foci were determined with an inverted microscope. The B or myeloid lineage growth in each well was then determined by flow cytometry using mouse anti-human CD19, CD13 and CD33 mAbs.

Single cell culture assay

Murine Lin⁻ IL-7R α ⁻ c-kit^{high} Sca-1⁺ or Lin⁻ IL-7R α ⁻ c-kit^{high} Sca-1⁻ cells were sorted at a concentration of one cell per well into 96-well plates preseeded with MS-5 cells. Each well had 100 μ l of α -MEM medium containing 10% FCS, 1 ng/ml recombinant murine IL-7, 100 ng/ml recombinant murine FL, and 20 ng/ml recombinant murine SCF. Wells with clonal growth were scored after 14 days of culture. Individual clones were analyzed by flow cytometry using anti-CD19, CD45R, Mac-1, and VCAM-1 mAbs as previously described, and divided into four groups according to their lineage potential. The categories included primitive clones (all recovered cells were CD19⁻CD45R⁻ Mac-1⁻), myeloid-lymphoid bi-potential clones (both CD19⁺/CD45R⁺ Mac-1⁻ and Mac-1⁺ cells were present), unipotential lymphoid clones (CD19⁺/CD45R⁺ Mac-1⁻ but no Mac-1⁺ cells were recovered), and myeloid unipotential clones (Mac-1⁺ but no CD19⁺/CD45R⁺ Mac-1⁻ cells were recovered).

Results

Adiponectin inhibits B lymphopoiesis in LTBMCS

In an earlier study, we showed that human stromal cells contained adiponectin protein (28). RT-PCR analysis revealed that the adherent layers of cultures established from murine bone marrow also contained transcripts for adiponectin (data not shown). We then investigated the influence of adiponectin on lymphohematopoiesis using two types of LTBMCS. W/W culture conditions support formation of B lineage lymphocytes, while myeloid and stem cells are maintained in Dexter type cultures (30, 31). Although lymphocyte production commenced in control W/W cultures after 3 wk, lymphocytes were not detected in adiponectin-treated flasks even after 6 wk (Fig. 1, left). This dramatic response was not observed when the protein was added to pre-established W/W cultures. Neither the numbers nor the phenotype of B lineage cells, which are mainly Mac-1⁻ CD45R⁺CD19⁺CD24⁺ BP-1⁺ surface-IgM⁻ pro-B/pre-B cells, changed during 6–12 wk of continuous adiponectin treatment of cultures that were prepared 6 wk previously (data not shown). Thus, the factor is not toxic for lymphocyte precursors and only abrogates a critical phase in the establishment of LTBMCS. The specificity of adiponectin was also reflected in the results of experiments using Dexter type culture conditions. Myelopoiesis was not significantly inhibited even when adiponectin was present from the time of culture initiation

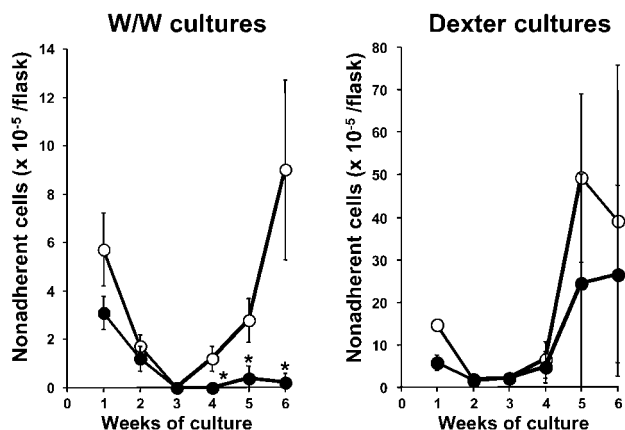


FIGURE 1. Adiponectin inhibits B lymphopoiesis in LTBMCS. Adiponectin inhibits the production of B lymphocytes in W/W cultures, but not the production of myeloid cells in Dexter cultures. Cultures were prepared and maintained in the continuous presence of adiponectin (●) or BSA (○). Numbers of nonadherent cells collected at weekly intervals are expressed as the mean per flask (four flasks in each experiment). Lineage-restricted cell production in such LTBMCS is normally established after 2 or 3 wk of culture. Significant differences from control values are indicated (*, $p < 0.05$). Similar results were obtained in three independent experiments.

(Fig. 1, right). The protein appeared to have very selective activity on lymphoid progenitors before the pro-B stage.

B cell precursors do not directly respond to adiponectin

The resistance of established W/W bone marrow cultures to adiponectin indicated that B lineage lymphocytes might be less sensitive beyond a certain point in their differentiation. As another possibility, the protein might indirectly influence them through effects on the environment, a point more thoroughly addressed with additional cultures. Clonal assays for mitogen responsive B cells (CFU-B) were unaffected by addition of recombinant adiponectin (Fig. 2A). Colony formation of IL-7 responsive pro-B cells (CFU-IL-7) appeared to be slightly suppressed in the presence of adiponectin, but it was not statistically significant ($p = 0.06$). Defined stromal cell-free, serum-free cultures that contained only recombinant cytokines were used to assess the potential influence of this protein on early B cell precursors (33). The Lin⁻ c-kit^{high} population of bone marrow generates myeloid and lymphoid cells under these conditions, while the Lin⁻ c-kit^{low} population is highly enriched with respect to prolymphocytes. Adiponectin had no suppressive effect on the generation of CD45R⁺CD19⁺ cells from either population (Fig. 2B).

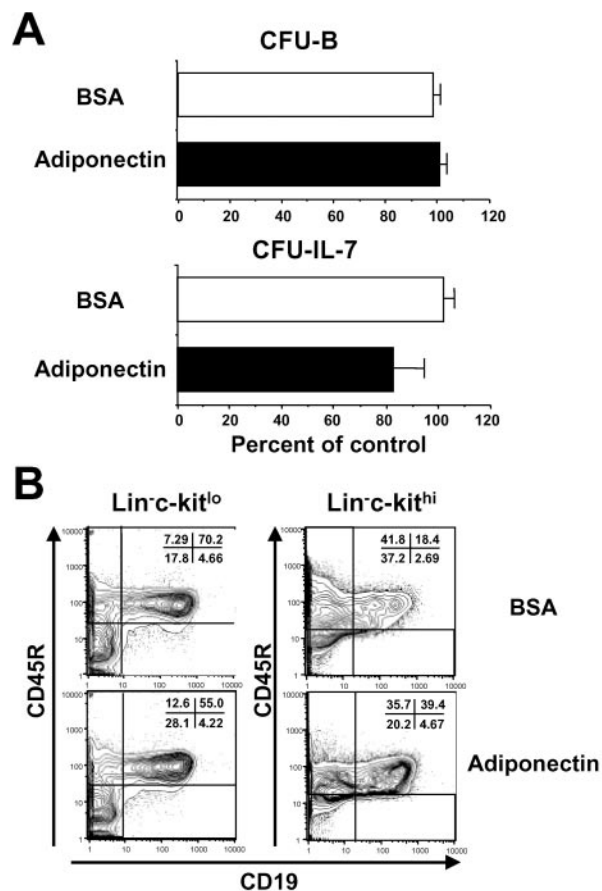


FIGURE 2. Adiponectin has no direct inhibitory effect on B lymphopoiesis. **A**, Adiponectin or BSA was added to colony assays of CFU-B and CFU-IL-7. Data are shown as the mean \pm SD percentage of control values from five independent experiments in which each experiment was set up with triplicate cultures. **B**, Sorted Lin⁻ c-kit^{low} (5000 cells/well) or Lin⁻ c-kit^{high} (2000 cells/well) were cultured in serum-free, stromal cell-free cultures in the presence of adiponectin or BSA. Growth of B lineage in each culture was evaluated at day 7 or at day 13, respectively. The percentages of four fractions are shown in each box. Data represent one of three similar experiments for each.

The same was true when FCS-containing medium was used (data not shown). It is noteworthy that increased numbers of Mac-1⁺ CD19⁺ myeloid cells were recovered from adiponectin-containing cultures initiated with the Lin⁺ c-kit^{high} population. The same was true for CD45R⁺CD19⁺ cells in three of four independent experiments. These results suggested that although no cells in the B lymphocyte lineage are directly suppressed, their differentiation might be altered and even stimulated because of adiponectin-induced changes in neighboring cells.

Adiponectin inhibits B lymphopoiesis only when stromal cells are present along with early B lineage precursors

Cloned MS-5 marrow stromal cells and enriched populations of early bone marrow precursors were then used for coculture experiments. A variety of evidence suggests that prolymphocytes in the Lin⁺ c-kit^{low} fraction of bone marrow are more differentiated than early lymphoid progenitors (ELP) in the Lin⁺ c-kit^{high} category (33, 35–37). For example, prolymphocytes efficiently give rise to CD19⁺ B lineage cells in less than 1 wk, and the fraction has a greatly reduced incidence of myeloid progenitors. Generation of CD19⁺CD11b/Mac-1⁺ lymphocytes from this Lin⁺ c-kit^{low} population was unaffected by adiponectin (Fig. 3A, top panels). Production of the less B lineage-restricted category of CD45R⁺CD19⁺CD11b/Mac-1⁺ cells (36, 38) was also unaffected (Fig. 3A, lower panels). In contrast, adiponectin significantly suppressed the formation of both CD19⁺CD11b/Mac-1⁺ cells and CD45R⁺CD19⁺CD11b/Mac-1⁺ cells from Lin⁺ c-kit^{high} precursors under these conditions (Fig. 3, B and C). Percentages and absolute numbers of primitive CD19⁺CD45R⁺CD11b/Mac-1⁺ cells were significantly suppressed, whereas numbers of CD11b/Mac-1⁺ remained relatively intact (Fig. 3, B and C). In fact, a slight increase

in numbers of myeloid cells was observed in each of five similar, but independent experiments. The specificity of these responses was further investigated by use of ANOC 9103 adiponectin-specific mAb. This reagent reversed the inhibitory effect of adiponectin on B lymphopoiesis to a substantial degree and blocked the tendency for myelopoiesis to be enhanced (Fig. 3D). Furthermore, CD19⁺CD11b/Mac-1⁺ cells were produced normally in the presence of a control recombinant GST protein (see *Materials and Methods*) (data not shown).

It was important to learn whether the recombinant human adiponectin also influenced human B cell precursors. Fortunately, the murine MS-5 stromal cell clone used in the experiments previously described also supports formation of human B lineage lymphocytes in culture (34). The hematopoietic stem cell-enriched CD34⁺CD38⁺ fraction was isolated from umbilical CB, and two cell concentrations were used to initiate cocultures on MS-5 monolayers (Table I). Hematopoietic cell growth was recorded in most of the culture wells regardless of starting cell number or whether adiponectin was present. However, the protein markedly reduced the fraction of wells that supported growth of B lineage cells. Thus, selective suppression of B lymphopoiesis is not restricted to murine cells. Stromal cell-free culture conditions have not been developed for human stem cells. However, these observations indicate that the survival or differentiation of human very early lymphocyte precursors is also sensitive to adiponectin when stromal cells are present in the same cultures.

A potential role for PGs in adiponectin-mediated responses

We recently found that adiponectin directly blocks the differentiation of preadipocytes through induction of COX-2 and synthesis

FIGURE 3. Adiponectin inhibits B lymphopoiesis from early progenitors when stromal cells are present. Sorted Lin⁺ c-kit^{low} (3000 cells/well) cells (A) or Lin⁺ c-kit^{high} (1000 cells/well) cells (B) were cocultured with MS-5 cells in the presence of adiponectin or BSA. Data represent one of five similar experiments. C, The absolute number of B lymphocyte, myeloid, or primitive cells recovered from coculture of Lin⁺ c-kit^{high} with MS-5 cells. The data represent the mean ± SD values from triplicate cultures. Significant differences from control values are indicated by an asterisk (*p* < 0.05). Similar results were obtained in five independent experiments. D, Lin⁺ c-kit^{high} (1000 cells/well) cells were cocultured with MS-5 cells in medium containing adiponectin or BSA with or without anti-adiponectin mAb 9103.

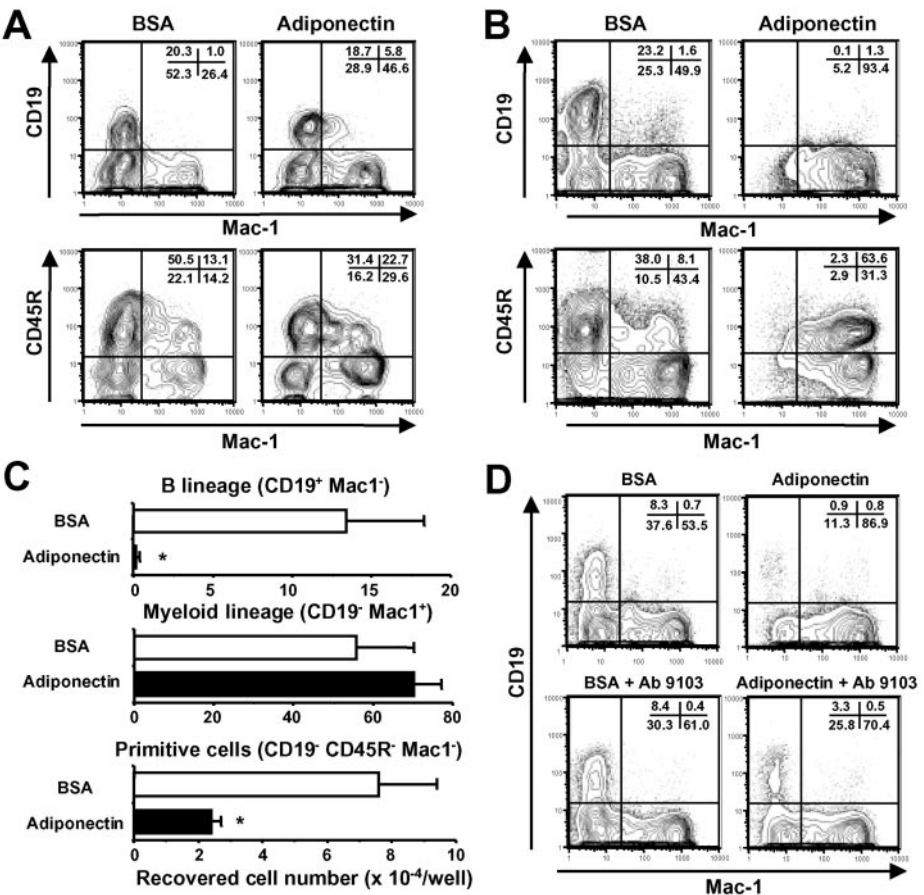


Table I. Adiponectin inhibits human B lymphopoiesis in stromal cell coculture^a

	Cultures with Growth		B Lineage Positive		Myeloid Lineage Positive	
No. of CD34 ⁺ CD38 [−] cells/well	10	20	10	20	10	20
Control Positive/Total (%)	58/60 (97)	60/60 (100)	7/12 (58)	11/18 (61)	12/12 (100)	18/18 (100)
Adiponectin Positive/Total (%)	51/60 (85)	57/60 (95)	1/12 (8)	7/18 (39)	12/12 (100)	18/18 (100)

^a Human CB CD34⁺CD38[−] cells were cultured at 10 or 20 cells per well in 96-well plates with MS-5 cells in the presence or absence of adiponectin. B and myeloid lineage growth in randomly chosen colony-positive wells from each plate was evaluated by flow cytometry using mouse anti-human CD19, CD13, and CD33 Abs. The data are representative of data obtained in two similar experiments.

of prostanoids (28). PGs are known to induce apoptosis in immature lymphocytes (6, 39, 40) and it seemed possible that they participate in the inhibitory responses previously discussed. Indeed, PGE₂ at a concentration of 10^{−6} M completely inhibited B lymphopoiesis when added to MS-5 stromal cell cocultures initiated with the Lin[−] c-kit^{high} fraction of bone marrow (Fig. 4). Dup-697 has been described as a selective inhibitor of COX-2 (41), and we determined that this material abrogates the inhibitory effect of adiponectin on B lymphopoiesis in stromal cell cocultures (Fig. 4A). Dup-697 itself did not enhance B lymphopoiesis and countered adiponectin effectively even at the very low concentration of 10^{−10}

M (Fig. 4, A and B). Furthermore, it partially prevented the enhancement of myelopoiesis normally observed in adiponectin-containing cultures (Table II). In addition to Dup-697, the SC-58125 and NS-398 COX-2 selective inhibitors (42, 43) as well as the APHS dual COX-1/COX-2 inhibitor (44) restored B lymphopoiesis in adiponectin-contained cultures (Table II). Interestingly, SC-560, a selective inhibitor for COX-1 (IC₅₀ = 9 nM; COX-2, IC₅₀ = 6.3 μM), (45) also abrogated adiponectin activity at 10^{−7} M (Table II).

Additional experiments were performed to determine whether primary stromal cells are responsive to adiponectin and to learn why lymphopoiesis was not arrested when the factor was added to established W/W cultures. Stromal cells were recovered from 8-wk-old W/W cultures and recharged with fresh Lin[−] c-kit^{high} bone marrow cells. These cocultures were suppressed by adiponectin and fully protected by the addition of Dup-697 (data not shown). Therefore, normal bone marrow cells are similar to cloned stromal cells with respect to adiponectin responsiveness. Also, lymphoid cell populations must eventually become PG insensitive in LTBMcs.

PGE₂ concentrations in stromal culture supernatants increased from 0.5 × 10^{−9} to 1.2 × 10^{−9} M after 24 h of adiponectin treatment (28). The level was much lower than the PGE₂ concentrations tested in previous studies, and it was unclear whether changes of this magnitude would be physiologically significant and whether the action of PGs would be sufficiently restricted to lymphoid progenitors. Therefore, we performed titration experiments with PGE₂ in defined serum-free stromal cell-free cultures initiated with Lin[−] c-kit^{low} or Lin[−] c-kit^{high} bone marrow cells (Fig. 5). Under these conditions, production of CD19⁺ B lineage lymphocytes from both categories, as well as that of primitive CD19[−]CD45R[−]CD11b/Mac-1[−] cells from the Lin[−] c-kit^{high} category, was strongly blocked by concentrations >10^{−9} M. In contrast, absolute numbers of CD11b/Mac-1⁺ myeloid cells were unaffected unless very high PG concentrations (~10^{−6}–10^{−5} M) were added. Indeed, it was rather up-regulated at low concentrations of ~10^{−10}–10^{−9} M. These findings strongly suggest that COXs participate in the responses of early lymphohematopoietic progenitors to adiponectin by enhancing prostanoid synthesis. In addition, PGE₂ at particular concentrations can very differently influence B lymphopoiesis and myelopoiesis in culture.

Adiponectin influence on early myeloid progenitors

Granulocyte-macrophage progenitors that can be detected in stromal cell-free clonal assays are suppressed by adiponectin (29). It was therefore a surprise to find modestly increased numbers of myeloid lineage cells in bone marrow cultures containing this protein. This was the case regardless of whether stromal cells were present and whether or not lymphopoiesis was suppressed. We explored this phenomenon further by culturing highly enriched categories of early hematopoietic cells on MS-5 stromal cells (Fig. 6). Production of myeloid cells from the stem cell containing Lin[−] IL-7Rα[−] c-kit^{high} Sca-1⁺ fraction (Fig. 6B) was enhanced in the

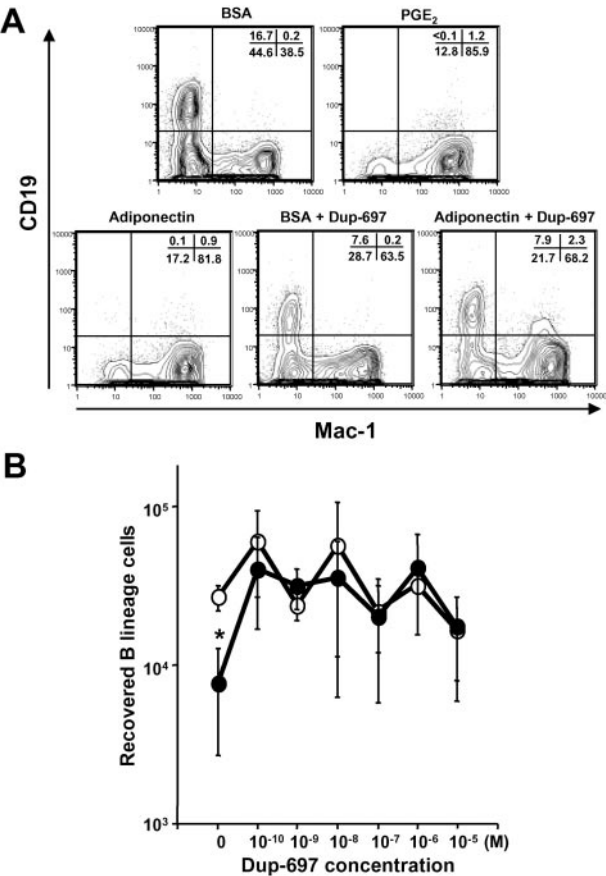


FIGURE 4. The COX-2-prostanoid pathway may contribute to adiponectin suppression of B lymphopoiesis. *A*, Sorted Lin[−] c-kit^{high} (1000 cells/well) cells were cocultured with MS-5 cells in the presence of BSA (10 μg/ml, upper left), PGE₂ (10^{−6} M, upper right), or adiponectin (10 μg/ml, lower left). Dup-697, a specific inhibitor of COX-2, was added at a concentration of 10^{−6} M to either BSA (bottom middle) or adiponectin-treated (bottom right) cultures. *B*, Dup-697 was added to adiponectin-treated cocultures (Lin[−] c-kit^{high} cells with MS-5) at the indicated concentrations. A significant difference between adiponectin-treated culture values (●) and control values (○) is indicated by an asterisk (*p* < 0.05). The data are representative of that obtained in two similar experiments.

Table II. Cyclooxygenase antagonists block adiponectin-mediated suppression of lymphopoiesis in culture

COX Inhibitor ^a	Without Adiponectin		With Adiponectin	
	No. of Recovered Cells (× 10 ⁴) Mean ± SD			
	B Lymphoid	Myeloid	B Lymphoid	Myeloid
None	5.2 ± 2.4	22.0 ± 7.1	1.0 ± 0.3	58.3 ± 3.4
Dup-697	5.7 ± 1.5	20.9 ± 8.2	11.9 ± 5.3	39.6 ± 9.4
SC-58125	4.6 ± 3.8	18.9 ± 7.8	8.8 ± 2.4	36.9 ± 5.7
NS-398	4.9 ± 3.8	17.4 ± 4.5	7.9 ± 8.5	41.2 ± 9.3
APHS	3.1 ± 1.8	23.6 ± 5.9	11.8 ± 11.2	60.0 ± 10.6
SC-560	3.2 ± 2.8	21.4 ± 7.9	6.7 ± 5.9	35.0 ± 12.6
SC-560	3.8 ± 2.5	15.3 ± 2.3	12.1 ± 7.7	39.1 ± 7.5

^a COX inhibitors were added to cocultures of Lin[−] c-kit^{high} cells (1000 cells/well) and MS-5 cells with or without adiponectin. The concentrations were: Dup-697, 10^{-8} M; SC-58125, 10^{-8} M; NS-398, 10^{-5} M; APHS, 10^{-5} M; and SC-560, 10^{-7} M (upper) and 10^{-6} M (lower). Absolute numbers of B or myeloid lineage cells recovered per well were calculated, and data shown as the mean \pm SD values from triplicate cultures. Similar results were obtained in two independent experiments.

presence of adiponectin. The same was true when single cell cultures were prepared from this primitive population (Fig. 6C). In contrast, myelopoiesis was not stimulated, rather slightly reduced in cultures initiated with the more differentiated Lin[−] IL-7R α [−] c-kit^{high} Sca-1[−] fraction that contains most progenitors that would be detectable with conventional methylcellulose colony assays (Fig. 6, B and C). Very small numbers of restricted lymphoid progenitors, and lymphomyeloid progenitors were detected in these experiments. As might be expected from the results previously shown, lymphocyte production was always reduced in these stromal cell cocultures when adiponectin was present. Thus, the influence of adiponectin on myelopoiesis may be a complex function of how differentiated the progenitors are and whether or not stromal cells are present.

Discussion

This study was inspired by the abundance of fat in normal bone marrow and previous findings that a fat cell product inhibited the differentiation of granulocyte-macrophage progenitors in clonal assays. Surprisingly, recombinant adiponectin inhibited lymphopoiesis rather than myelopoiesis in more complex LTBMCs. This

inhibitory response was only seen when stromal cells were present and only when the cultures were initiated with the earliest categories of lymphocyte precursors. Human B lymphopoiesis was also sensitive to adiponectin. Drug treatment experiments suggested that COX-dependent production of prostanoids might be involved. These findings support the notion that adipocytes are functional components of bone marrow, with the potential to regulate some of the earliest events in blood cell formation.

Responsiveness of hematopoietic precursors to a given substance can be influenced by what other signals the cells are receiving at the same time. Myeloid progenitors are directly sensitive to adiponectin in stromal cell-free colony assays, and the protein inhibits macrophage production of TNF- α (29). In contrast, we found no suppression of myelopoiesis in adiponectin treated Dexter type cultures and enhanced myeloid cell production in stromal cell cocultures initiated with an early hematopoietic cell fraction (Figs. 1 and 6). Paracrine responses are even possible in semisolid agar cultures where physical contact between cells is minimal (4). The cellular complexity and possibility of close cellular interactions is much greater in long-term cultures and even those models do not reproduce the situation within bone marrow. Early stages in B lymphopoiesis became the focus of our studies after finding that adiponectin completely inhibited establishment of W/W cultures.

Addition of adiponectin to bone marrow cultures appeared to favor myeloid cell production at the expense of B lymphopoiesis. It is tempting to speculate that adiponectin and/or an adiponectin-induced stromal cell product can influence the lineage decision of early lymphohematopoietic progenitors. However, myelopoiesis was slightly enhanced even in stromal cell-free cultures, where lymphopoiesis was not suppressed. Although our single cell experiments were conducted with highly purified fractions of early marrow progenitors, the populations are still heterogeneous. For example, the Lin[−] IL-7R α [−] c-kit^{high} Sca-1⁺ category includes stem cells, multipotential progenitors, and ELP (45, 46). It is quite possible that adiponectin selects individual cells in a positive or negative way after lineage choice decisions have been made. Although it is not possible to conclude that fat cell products influence lymphoid vs myeloerythroid differentiation decisions, they certainly have the potential to differentially regulate progression in these lineages.

B lymphopoiesis was totally blocked by adiponectin in W/W cultures, and in circumstances where early progenitors were in contact with stromal cells. In contrast, clonal proliferation of lymphocytes in semisolid agar cultures was not significantly affected. Furthermore, the lymphoid differentiation potential of highly purified hematopoietic cells in defined, serum-free, stromal cell-free

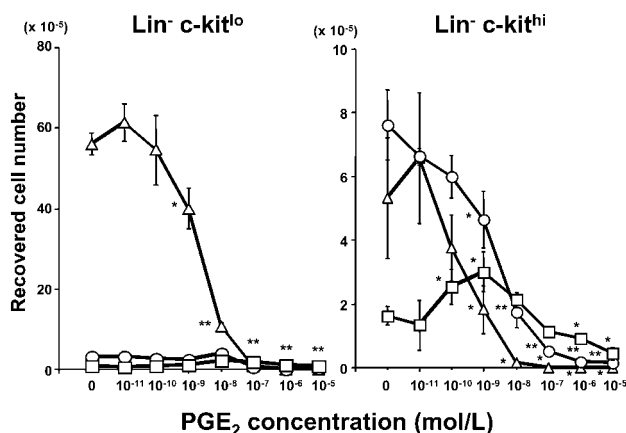


FIGURE 5. PGE₂ inhibits B lymphopoiesis from early progenitors in serum-free, stromal cell-free cultures. Sorted Lin[−] c-kit^{low} (5000 cells/well) or Lin[−] c-kit^{high} cells (2000 cells/well) were cultured in serum-free, stromal cell-free cultures with the indicated concentration of PGE₂. Absolute numbers of each population recovered were calculated and plotted (B lineage, ▲; myeloid lineage, □; and primitive cells, ○ as the mean \pm SD values from triplicate cultures. Significant differences from control values (at PGE₂ 0 M) are indicated by an asterisk ($p < 0.05$) or two asterisks ($p < 0.01$). Similar results were obtained in two independent experiments.

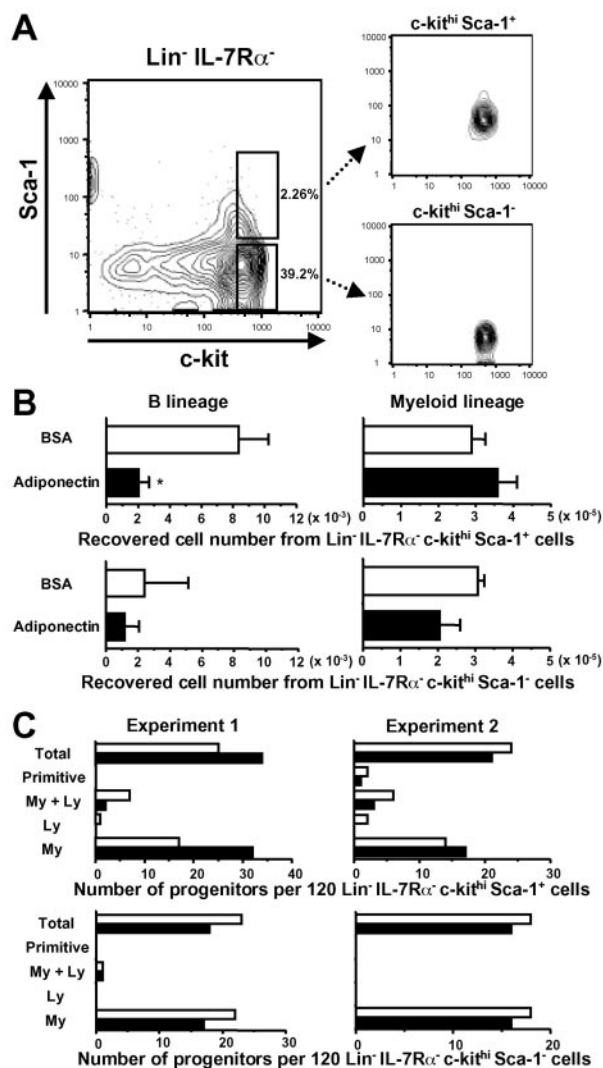


FIGURE 6. Adiponectin selectively reduces the lymphoid differentiation potential of early hematopoietic progenitors. Lin⁻ IL-7Rα⁻ c-kit^{hi} Sca-1⁺ or Lin⁻ IL-7Rα⁻ c-kit^{hi} Sca-1⁻ cells were sorted from bone marrow and their purity is shown in *A*. Lin⁻ IL-7Rα⁻ c-kit^{hi} Sca-1⁺ (500 cells/well) or Lin⁻ IL-7Rα⁻ c-kit^{hi} Sca-1⁻ cells (1000 cells/well) were then cultured on MS-5 cells in the presence (■) or absence (□) of adiponectin (*B*). Absolute numbers of B (CD19⁺ Mac-1⁻) or myeloid (Mac-1⁺) lineage cells were calculated and shown as the mean ± SD values from triplicate cultures. Similar results were obtained in three independent experiments. Cultures were also initiated with single cells and the resulting progeny were typed as primitive, myeloid-lymphoid bipotential (My + Ly), lymphoid unipotential (Ly), or myeloid unipotential (My) as described in *Materials and Methods* (*C*). Data are shown from two independent experiments.

cultures was not adiponectin sensitive (Fig. 2). These findings suggested that this fat cell product might indirectly inhibit lymphopoiesis via effects on additional cell types. Potential mechanisms were suggested by our recent finding that adiponectin can induce COX-2 and PG synthesis in marrow stroma-derived preadipocytes (28).

Indomethacin was initially found to have no influence on responses to adiponectin in bone marrow cultures (Ref. 29 and data not shown). However, this well-known PG synthesis inhibitor has complex dose dependent effects and can promote fat cell differentiation. For example, high concentrations of indomethacin induce peroxisome proliferator-activated receptor $\alpha\gamma$, whereas lower amounts block PG synthesis by inhibition of COXs (47). All of

five COX antagonists protected lymphopoiesis in adiponectin-treated cultures (Table II). Three of these drugs, Dup697, SC58125, and NS398 are COX-2-specific, whereas APhS inhibits COX-1 and COX-2 (41–44). The compound SC-560 was also active in our cultures, even though it is said to be a more potent inhibitor of COX-1 than COX-2 (48). None of these drugs influenced lymphomyelopoiesis when added to control, adiponectin-free cultures. Therefore, adiponectin has the potential to induce synthesis of multiple prostanoids through COX-dependent mechanisms. For example, COX-2 mediates the conversion of arachidonic acid into PGH₂, which is subsequently converted to arachidonate metabolites that include PGE₂, prostacycline, PGF_{2α}, PGJ₂, and thromboxane A₂ (49). PGE₂ was of particular interest because it is detectable in supernatants of adiponectin treated stromal cell cultures, and can suppress immature lymphoid cells (6, 28, 39, 40).

It was important to learn whether a prostanoid such as PGE₂ could selectively inhibit lymphopoiesis and we established that is the case in under highly defined culture conditions. Production of primitive CD19⁻CD45R⁻CD11b/Mac-1⁻ cells, as well as CD19⁺ B lineage lymphocytes was completely blocked by PGE₂ concentrations ≥10⁻⁷ M in stromal cell-free cultures initiated with Lin⁻ c-kit^{hi} bone marrow cells (Fig. 5). Although this amount is two logs more than that previously measured in culture supernatants, (28) the important point is that myelopoiesis was spared. Additionally, hematopoietic cells beneath or in close proximity to stromal cells might be exposed to much higher concentrations and active prostanoids besides PGE₂ could be produced. Thus, adiponectin-induced PG synthesis could account for its selective suppression of lymphocyte formation in stromal cell containing cultures. However, the question arises why adiponectin treatment of pre-established W/W cultures did not inhibit lymphopoiesis (data not shown). It is possible that prostanoid production is different under those conditions, and we note previous observations that stromal cell contact can protect lymphoid progenitors from apoptosis inducing agents (50). However, stromal cells from 8-wk-old cultures were capable of delivering a suppressive stimulus to freshly prepared progenitors, suggesting that the insensitivity of established long-term marrow cultures results from changes in the lymphocyte populations. In any case, the present observations extend previous studies that demonstrated the preferential sensitivity of immature lymphoid cells to PGE₂ (40). The Lin⁻ c-kit^{hi} fraction of bone marrow contains the earliest known progenitors of B, T, and NK lineage lymphocytes (46). These ELP have very little myeloid differentiation potential and may be direct targets of prostanoids.

The survival, expansion, and differentiation of hematopoietic cells are dependent on cytokines and substantial progress has been made in their identification. For example, early stages of B lymphopoiesis can be observed in defined cultures containing only SCF, FL, and IL-7 (33). However, lymphocyte production in normal bone marrow probably reflects the net activity of positive and negative regulators. A case can be made that sex steroids regulate lymphopoiesis under steady-state circumstances, whereas IFNs, TGF- β , and PGs may only be important during disease circumstances (51). In this regard, it is noteworthy that bone marrow lymphocyte populations are normal in adiponectin knockout mice (K. Oritani, unpublished observations). One of the phenotypes observed in the knockout model is dysregulation of TNF- α production induced by a high-calorie diet or LPS treatment, however the mice appear healthy under the steady conditions (Ref. 26 and K. Oritani, unpublished observations). It is interesting that all of these negative regulators can potentially be made by the fat cells that normally reside within bone marrow. Further study is required to precisely understand if and how adiponectin influences stromal

cells to produce these inhibitors, but some can be excluded as likely candidates with available information. Expression of IFNs, TNF- α , and TGF- β did not increase in adiponectin-treated MS-5 cells (28). In addition, those cytokines suppress nonlymphoid lineages as well as pro-B/pre-B cells (52). Very early lymphocyte precursors are affected by the adiponectin-induced substance(s) and it is interesting that the same cells express functional receptors for sex steroids (Refs. 34, 37, 53 and H. Igarashi, unpublished observations). It has been reported that adipocytes in human bone marrow can express cytochrome P450 aromatase, a key enzyme in sex steroid biosynthesis (54). Adiponectin could also control local production of estrogen within bone marrow in addition to PGs if it regulates aromatase expression. This possibility will be explored in future studies.

Adipocytes produce substances that can influence the formation of additional fat in positive and negative ways (28, 55). In addition to adiponectin, they include PGs, TNF- α , leptin, angiotensin II, and agouti. Adiponectin is attracting considerable attention as a fat cell product that can regulate levels of glucose in the circulation, overcome insulin resistance and cause weight loss. These properties have been demonstrated by injection of mice with recombinant forms of the protein and analysis of adiponectin deficient mice (23–26). Adiponectin may directly influence the metabolism of cells in muscle and liver, whereas cardiac endothelial cells and pre-adipocytes are also targets (21, 23–25, 27, 28). Modulation of fat in marrow could influence lymphohematopoiesis because adipogenesis alters the expression of the extracellular matrix, membrane proteins, and cytokines by stromal cells (1). A full understanding of its biological activities is essential for predicting potential side effects of therapy. Additionally, we may learn that the protein normally mediates functional responses involving hematopoietic cells within bone marrow.

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