Inflammatory Biomarker, Neopterin, Suppresses B Lymphopoiesis for Possible Facilitation of Granulocyte Responses, Which Is Severely Altered in Age-Related Stromal-Cell–Impaired Mice, SCI/SAM

AKIHIRO MINAMI,* ISAO TSUBOI,*,[1] TOMONORI HARADA,* TOSHITAKA FUKUMOTO,* MASAKI HIRAMOTO,* MORIMICHI KOSHINAGA,* YOKO HIRABAYASHI,† JUN KANNO,† TOHRU INOUE,‡ AND SHIN AIZAWA*

**Department of Anatomy, Nihon University School of Medicine, Tokyo 173-8610, Japan; †Division of Cellular and Molecular Toxicology, Center for Biological Safety and Research, National Institute of Health Science, Tokyo 158-8501, Japan; and ‡Center for Biological Safety and Research, National Institute of Health Science, Tokyo 158-8501, Japan*

Neopterin is produced by monocytes and is a useful biomarker of inflammatory activation. We found that neopterin enhanced *in vivo* and *in vitro* granulopoiesis triggered by the stromal-cell production of cytokines in mice. The effects of neopterin on B lymphopoiesis during the enhancement of granulopoiesis were determined using the mouse model of senescent stromal-cell impairment (SCI), a subline of senescence-accelerated mice (SAM). In non-SCI mice (a less senescent stage of SCI mice), treatment with neopterin decreased the number of colonies, on a semisolid medium, of colony-forming units of pre–B-cell progenitors (CFU-preB) from unfractionated bone marrow (BM) cells, but not that from a population rich in pro-B and pre-B cells without stromal cells. Neopterin upregulated the expression of genes for the negative regulators of B lymphopoiesis such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and transforming growth factor-β (TGF-β) in cultured stromal cells, implying that neopterin suppressed the CFU-preB colony formation by inducing negative regulators from stromal cells. The intraperitoneal injection of neopterin into non-SCI mice resulted in a marked decrease in the number of femoral CFU-preB within 1 day, along with increases in TNF-α and IL-6 expression levels. However, in SCI mice, *in vivo* and *in vitro* responses to B lymphopoiesis and the upregulation of cytokines after neopterin treatment were less marked than those in non-SCI mice. These results suggest that neopterin predominantly suppressed lymphopoiesis by inducing the production of negative regulators of B lymphopoiesis by stromal cells, resulting in the selective suppression of *in vivo* B lymphopoiesis. These results also suggest that neopterin facilitated granulopoiesis in BM by suppressing B lymphopoiesis, thereby contributing to the potentiation of the inflammatory process; interestingly, such neopterin function became impaired during senescence because of attenuated stromal-cell function, resulting in the downmodulation of the host-defense mechanism in the aged. Exp Biol Med 232:134–145, 2007

Key words: neopterin; B lymphopoiesis; stromal-cell–impaired mouse; subline of senescence-accelerated mice; aging

Introduction

Neopterin is a metabolite of guanosine triphosphate that is produced in the biopterin synthetic pathway (1). *In vitro,* large amounts of this metabolite are generated by "monocytes and macrophages" in response to interferon-γ (IFN-γ) (2). Because increased neopterin levels accompany immune responses *in vivo* and *in vitro,* there has been considerable interest in measuring neopterin levels as a biomarker of immunologic activation (2–4).

The possible immunologic relevance of neopterin pertinent to inflammatory processes has been considered on the basis of observations that neopterin induces apoptosis in rat alveolar epithelial cells L2 (5) and inhibits NADPH-oxidase in peritoneal macrophages (6). Moreover, neopterin has been found to inhibit erythropoietin gene expression (7)

This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan for the promotion of industry-university collaboration at Nihon University and also by a Nihon University Joint Research Grant for 2004.

[1] To whom correspondence should be addressed at Department of Anatomy, Nihon University School of Medicine, 30–1 Ohyaguchi-kamimachi, Itabashiku, Tokyo 173-8610, Japan. E-mail: tsuboi@med.nihon-u.ac.jp

Received January 29, 2006.
Accepted May 26, 2006.

1535-3702/07/2321-0134$15.00

and induce the expression of genes for proto-oncogene c-*fos* (8) and nitric oxide synthase (9). Furthermore, because the gene expression of the gene for inducible nitric oxide synthase (iNOS) and the subsequent nitric oxide (NO) release from vascular smooth muscle cells (VSMCs) following incubation with neopterin have been documented, neopterin is supposed to play a role as a modulator in gram-negative endotoxemia that contributes to the excessive production of NO (9, 10).

Neopterin, on the other hand, increases the number of colony-forming units of granulocyte-macrophage progenitor cells (CFU-GM) in a semisolid culture system (11), and intraperitoneal neopterin injection into mice stimulates granulopoiesis (12). This stimulation of *in vitro* and *in vivo* granulopoiesis is due not to a direct action of neopterin on hematopoietic stem cells, but rather to an indirect effect mediated by the stromal-cell production of hematopoietic growth factors, such as granulocyte-macrophage colony-stimulating factor and interleukin-6 (IL-6) (11). Thus, regarding the most interesting characteristics of neopterin, we would like to clarify how this biosynthetic compound functions not only in immune responses but also in hematopoietic anti-inflammatory responses.

To answer the above question, SAM/P-1, a subline of senescence-accelerated mice (SAM) exhibiting a unique stromal-cell impairment after 30 weeks of age, was used, because in SAM the numbers of splenic cells and splenic hematopoietic progenitor cells start to decrease (13–15). Furthermore, senescent SAM/P-1 mice show the simultaneous downregulations of interleukin-7 (IL-7) and transforming growth factor-β (TGF-β) caused by the suppression of B lymphopoiesis in senescence (16). Interestingly, the murine system possesses a markedly low level of neopterin in the circulation in the steady state; thus, the SAM/P-1 mouse model may be a useful tool for elucidating the possible interaction of neopterin and stromal cells during B lymphopoiesis. Thus, we investigated whether neopterin affects B lymphopoiesis using stromal-cell–impaired (SCI) mice, a subline of SAM/P-1 mice.

In this study, we suggest the functions of neopterin in the mechanisms underlying the suppression of B lymphopoiesis, consequent facilitation of potentiation of granulopoiesis, and further functional impairment during senescence.

Materials and Methods

Mice. A subline of SAM, SAM/P-1 (13), is a senescent stromal-cell impairment substrain (SCI mice), derived from AKR mice (Jackson Laboratory, Bar Harbor, ME), and established by Dr. Toshio Takeda, Professor Emeritus of the Chest Disease Research Institute, Kyoto University, Japan. The mice were bred and maintained in an experimental facility at the National Institute of Health Sciences under pathogen-free conditions. SAM/P-1 exhibits stromal-cell impairment after 30 to 36 weeks of age. In this study, male SAM/P-1 mice designated as non-SCI mice (8 to 12 weeks old) and SCI mice (30 to 36 weeks old) were compared. These ages were selected because the numbers of splenic cells and splenic hematopoietic progenitor cells start to decrease significantly at approximately 30 weeks of age (15, 16). The study was approved by the Institutional Animal Care and Use Committee at the National Institute of Health Science guidelines for animal care.

Neopterin. D(+) neopterin was obtained from Sigma (St. Louis, MO). Neopterin was dissolved in 1 *N* HCl at 1 mg/ml and diluted tenfold with Dulbecco's phosphate-buffered saline (PBS).

Neopterin Administration. The mice were injected intraperitoneally with neopterin (0.35 or 3.5 mg/kg body weight) for single-dose administration or on three consecutive days. The doses chosen here correspond to those for humans determined in the laboratory; however, the doses chosen are highly biased because the steady-state background dose of neopterin in rodents, in general, is significantly lower than those in humans and nonhuman primates; that is, lower than one n*M* per liter (12,925 and 85 n*M*, 30 and 60 mins, respectively, after 3.5 mg/kg body weight neopterin administered). The mice were evaluated, in the case of a single dose, 0.5, 1, 2, 3, 6, and 24 hrs after the administration, and, in the case of three doses, 1, 7, and 14 days after the last injection. As the control, a group of mice was injected with the same volume of PBS with the same amount of HCl as that used as the vehicle for neopterin. Three mice per group were examined for each data point.

Bone Marrow (BM) Cells. BM cells were isolated by repeatedly flushing cells from femurs using Iscove-modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, CA) or RPMI 1640 medium (Invitrogen). The BM cells were suspended by trituration using a 23-gauge hypodermic needle.

Preparation of Pro–B/Pre–B-Cell–Rich Populations. To obtain populations rich in pro-B cells and pre-B cells without stromal-cell components, the bulk culture of pooled BM cells from the non-SCI and SCI mice was stimulated with recombinant interleukin-7 (rIL-7) as described previously (16, 17). Briefly, BM cells were cultured at 1×10^6 cells/ml in RPMI 1640 supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT), 20 μ*M* 2-mercaptoethanol, 1% L-glutamine, and 2 ng/ml murine rIL-7 (Genzyme, Cambridge, MA) in six-well tissue culture plates (Falcon 3046; Beckton-Dickinson, Franklin Lake, NJ). Nonadherent cells were harvested after 4 days. This bulk culture provides a 10 times richer source of IL-7–responsive $B220^+/CD43^+/IgM^-$ pro-B/pre-B cells (16), which provide a functionally homogeneous large number of fractions requiring only gentler and easier detachment of B lymphocytes from stromal cells.

***In Vitro* Colony Assays.** Colony formation by colony-forming-unit pre-B cells (CFU-preB cells) was assayed using a semisolid medium containing rIL-7 (16).

Femoral BM cells from three mice per group were pooled and assayed in 35-mm plastic Petri dishes containing 1 ml of MethoCult M 3630 medium (Stem Cell Technologies Inc., Vancouver, Canada), which consisted of IMDM composed of 1% methylcellulose, supplemented with 30% FBS, 0.1 m*M* 2-mercaptoethanol, 2 m*M* L-glutamine, and 10 ng/ml rIL-7. Triplicate culture plates containing equal amounts of CFU-preB cells estimated were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. After 7 days, aggregates of 50 or more cells were counted as colonies.

Preparation of Cultured Stromal Cells. Stromal-cell monolayers were prepared by culturing whole BM cells derived from non-SCI or SCI mice at 1×10^6/ml cells in 10-cm diameter cell culture dishes (Corning Incorporated, Corning, NY) or 24-well flat bottom plates (Falcon 3047) in 30 ml or 1 ml of IMDM supplemented with 10% FBS. Confluent adherent layers were formed after 10 days of culture. The supernatant was removed and cultured dishes with confluent adherent layer were rinsed with IMDM twice to eliminate nonadherent cells. A fresh culture medium containing 4 μ*M* or 400 n*M* neopterin was then added to 10-cm diameter cell culture dishes or 24-well flat bottom plates, and the cells were further incubated. As for 10-cm diameter cell culture dishes, after 1, 3, and 9 hrs of incubation, the culture medium was removed completely and stromal cells were subjected to RNA extraction. As for 24-well flat bottom plates, after 24 hrs of incubation, the culture medium was collected and was used for determination of the levels of tumor necrosis factor-α (TNF-α), IL-6, and TGF-β proteins produced by cultured stromal cells. The concentration of TNF-α, IL-6, or TGF-β in the culture medium was determined using TNF-α, IL-6, or TGF-β–specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according manufacturer's instructions. All the samples were assayed in triplicate.

Total RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR). Total RNA was extracted from BM cells using TRIzol (Invitrogen) according to the manufacturer's instructions. The isolated mRNA was reverse transcribed using Superscript (Life Technologies, Grand Island, NY) and Oligo-dT (Promega, Madison, WI). Next, quantitative real-time PCR was performed using a TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA) and specific primers and probes, with the Applied Biosystems 7900 sequence detection system, version 2.0. Specific primers and probes for murine IL-7, TNF-α, IL-6, TGF-β, and glyceraldehyde phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems (TaqMan Gene Expression Assays; IL-7, Mm00434291_m1; TNF-α, Mm00443281_m1; IL-6, Mm 00446190_m1; TGF-β, Mm00441724 _m1; GAPDH, Mm99999915_g1). PCR conditions and data analysis were performed according to the instructions in the Sequence Detection System, version 2.0. In this study, total RNA extraction and expression of GAPDH were compared between non-SCI and SCI mice (data are shown in Appendix). All reactions were performed in triplicate. According to the manufacturer's instructions, cytokine-specific signals were normalized by the GAPDH signal using the formula $2^{-\Delta Ct} = 2^{-[Ct(GAPDH) - Ct(cytokine)]}$, and the relative level of cytokine gene expression was calculated using $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ for neopterin treatment} - \Delta Ct \text{ for control})}$, where Ct is the threshold cycle.

Statistical Analyses. Data were subjected to analysis of variance (ANOVA). Values were considered significantly different at $P < 0.05$.

Results

Effects of Neopterin on *in Vitro* CFU-PreB Colony Formation from Unfractionated BM Cells and Populations Rich in Pro-B/Pre-B Cells: Comparison Between Non-SCI and SCI. To determine whether neopterin directly affects the proliferation or differentiation of pre–B-cell progenitors, we cultured whole (unfractionated) BM cells from non-SCI and SCI mice in a semisolid medium system containing rIL-7 and in the presence of various concentrations of neopterin and measured the number of colonies formed (Fig. 1). In the colonization assays without neopterin, the absolute numbers of colonies from the non-SCI and SCI mice were significantly different, that is 137 ± 10 and 37 ± 1 per 2×10^5 cells, respectively. In Figure 1, each value is expressed as 100% of control, revealing the *in vitro* effects of neopterin on CFU-preB colonies in whole BM cells from non-SCI and SCI mice. When graded increasing doses of neopterin from 4 n*M* to 4 μ*M* were administered, the number of CFU-preB colonies from non-SCI BM cells decreased in a dose-dependent manner compared with that of the nontreated control groups, and except for the lowest dose at 4 n*M,* the other three higher dose groups all showed statistically significant decreases (10.5%, 24.2%, 26.9%, and 35.7% for 4 n*M,* 40 n*M**, 400 n*M**, and 4 μ*M**, respectively; *$P < 0.05$). Interestingly, these decreases in the number of CFU-preB colonies were observed to be smaller in those from senescent SCI BM cells. Namely, there were no significant decreases observed in the number of CFU-preB cells from SCI BM cells except at the highest dose of 4 μ*M* (0%, 5.0% , 17.2%, and 28.5%, for 4 n*M,* 40 n*M,* 400 n*M,* and 4 μ*M* [$P < 0.05$], respectively); thus, it may be concluded that the groups treated with neopterin at the three lower concentrations did not show the suppression of B-cell colonization of BM cells in the senescent SCI groups as compared with those of the young non-SCI groups. When the reduction ratios at the same dose of neopterin treated groups were compared between non-SCI and SCI, a statistically significant difference was observed for solely the dose at 40 n*M* ($P < 0.05$).

However, the above suppression of CFU-preB colony formation was not observed when BM cells were cultured, under IL-7 stimulation, with the population rich in pro-B and pre-B cells isolated and seeded prior to neopterin

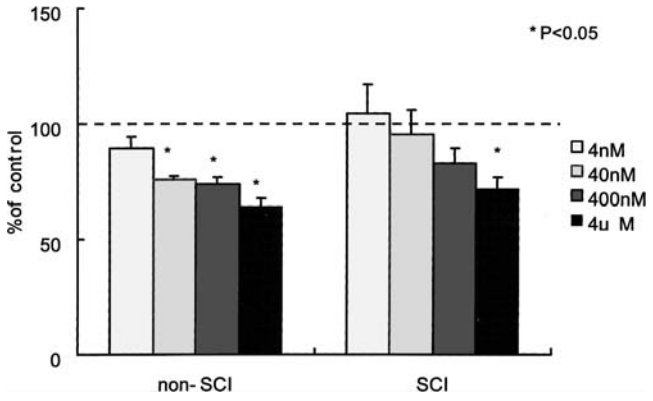


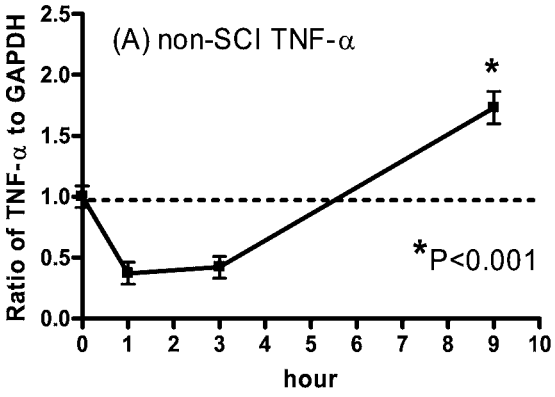
Figure 1. CFU-preB colony formation of whole BM cells from non-SCI and SCI mice in the presence of neopterin. Unfractionated BM cells from the non-SCI and SCI mice were incubated in a semisolid culture system containing interleukin-7 (IL-7) in the presence or absence of neopterin. The number of colonies was determined after 7 days. The absolute numbers of colonies in cultures without neopterin for the non-SCI and SCI mice were significantly different (137 ± 10 and 37 ± 1 per 2×10^5 cells, respectively; $P < 0.001$; data not shown). The reduction ratios following neopterin treatment are expressed as percentage relative to the nontreated control of each group, namely, non-SCI and SCI mice. Each bar represents the mean $\pm$ standard deviation (SD) from triplicate experiments ($^*P < 0.05$ vs. each control). The comparison of reduction ratios between non-SCI and SCI showed statistical significance only at the lowest dose, that is 40 n*M* ($P < 0.05$).

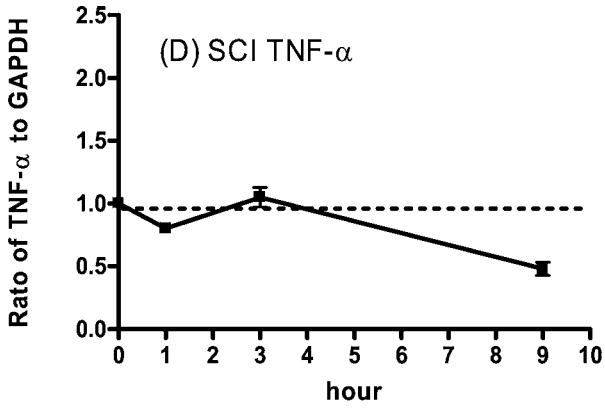
treatment regardless of the use of non-SCI and SCI mouse BM cells (data not shown). Thus, the above data imply that the suppression of the colonization of CFU-preB progenitor cells by neopterin treatment is presumably due to a stromal-cell–mediated effect.

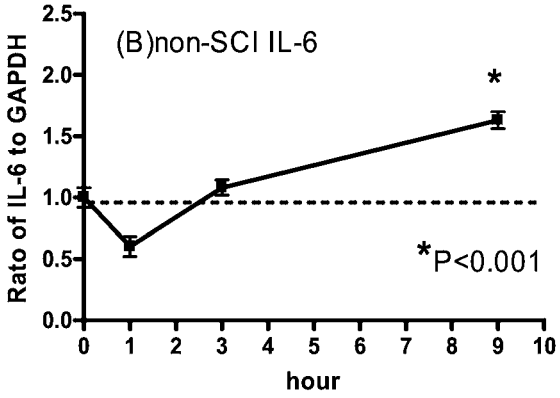
Gene Expressions and Protein Productions of TNF-α, IL-6, and TGF-β in Cultured Stromal Cells Induced by Neopterin: Comparison Between Non-SCI and SCI. To elucidate the mechanism underlying the suppression of CFU-preB-cell progenitor colony formation, which is presumably induced by stromal cells, messenger RNAs of principal cytokine genes for the negative regulation of the pro-B/pre-B fraction were evaluated, namely, TNF-α, IL-6, and TGF-β. Figure 2 shows the time course of cytokine gene expression levels in stromal cells cultured in the presence of 4 μ*M* neopterin. The results were expressed as a ratio to each cultured non-SCI or SCI fraction without neopterin. In non-SCI stromal cells (Fig. 2A–C), neopterin transiently and modestly suppressed the expressions of TNF-α, IL-6, and TGF-β genes from 1 to 3 hrs and then continuously upregulated the gene expressions until 9 hrs. The gene expression levels of TNF-α, IL-6, and TGF-β in cultured stromal cells 9 hrs after neopterin treatment increased by 1.63-, 1.93- and 2.07-fold (asterisks), respectively, compared with those in the control stromal cells without neopterin treatment ($P < 0.001$). In contrast to non-SCI cultured stromal cells, neopterin did not increase the gene expression levels of TNF-α, IL-6, and TGF-β in cultured SCI cells compared with those in SCI cells without neopterin treatment (Fig. 2D–F). Therefore, it is clear that the gene expression levels of TNF-α, IL-6, and TGF-β in cultured SCI cells and non-SCI cells show significant differences (A vs. D, $P < 0.001$; B vs. E, $P < 0.005$; and C vs. F, $P < 0.001$, respectively). After 24 hrs commercially available ELISA kits showed essentially comparable data, although 400 n*M* neopterin elicited much higher responses (23 ± 3 to 76 ± 11 pg/ml [$P < 0.05$] for non-SCI and 22 ± 8 to 38 ± 8 pg/ml for SCI, nontreated with 400 n*M* for TNF-α; 191 ± 25 to 455 ± 92 pg/ml [$P < 0.05$] for non-SCI and 155 ± 14 to 184 ± 11 pg/ml for SCI, nontreated with 400 n*M* for IL-6; 220 ± 15 to 298 ± 28 pg/ml [$P < 0.05$] for non-SCI and 68 ± 24 to 115 ± 43 pg/ml for SCI, nontreated with 400 n*M* for TGF-β; Table 1).

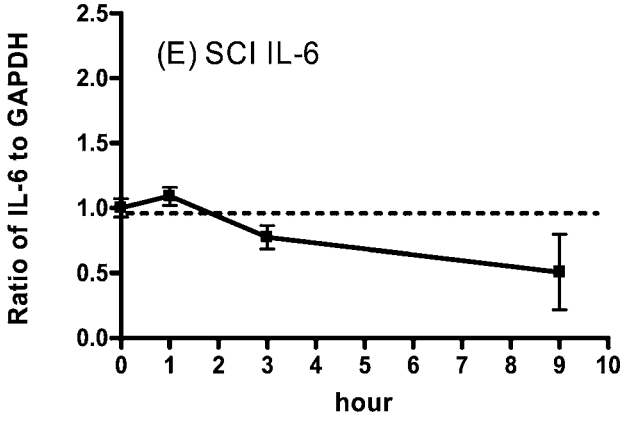
Neopterin-Induced B-Lymphopoietic Regulation in BM *in Vivo:* Comparison Between Non-SCI and SCI. Neopterin at doses 0.35 mg/kg body weight and 3.5 mg/kg body weight was intraperitoneally injected into non-SCI mice and SCI mice, respectively, to determine the stromal effect of neopterin on B lymphopoiesis (data not shown). The absolute numbers of CFU-preB progenitor cells observed in the femur of non-SCI mice and SCI mice were 9438 ± 177 and 3140 ± 57, respectively. As expected, on Day 1 after the three doses of daily treatment with neopterin, significant decreases in the number of CFU-preB progenitor cells in the femoral BM were observed in both non-SCI mice and SCI mice (43.1% [$P < 0.001$] and 22.9% [$P < 0.001$] for non-SCI mice, whereas 82.9% [$P > 0.05$] and 45.1% [$P < 0.005$] for SCI mice; relative to those of the control at 0.35 mg/kg body weight and 3.5 mg/kg body weight, respectively); thus, the suppression was markedly milder in the senescent SCI mice than in the non-SCI mice. To determine the effects of neopterin at the *in vivo* level, the same three daily treatments at 3.5 mg/kg body weight were carried out on both non-SCI mice and SCI mice, and the number of femoral CFU-preB progenitor cells was evaluated 1, 7, and 14 days after the last treatment with neopterin. As observed in Figure 3, the number of femoral CFU-preB progenitor cells shows a prominent oscillation, that is, a statistically significant decrease in number on Day 1 (22.9% of that from the nontreated non-SCI mice), followed by a marked increase on Day 7 (209% that from the nontreated non-SCI), and then returns to the nontreated control level of the non-SCI mice. Very interestingly, not only the number of CFU-preB progenitor cells in the nontreated group but also the oscillatory extent was smaller in the SCI mice. In this figure, the marked overshoot increase in the number of CFU-preB progenitor cells from the SCI mice is smaller than that from the nontreated non-SCI mice (number in nontreated non-SCI mice, 9438 ± 177 vs. number on Day 7 in treated SCI mice, 5134 ± 292).

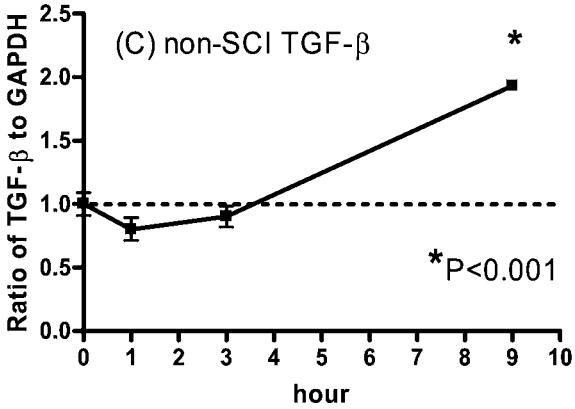
Gene Expressions of IL-7, TNF-α, IL-6, and TGF-β in BM *in Vivo* During Neopterin-Induced Pre–B-Cell Suppression: Comparison Between Non-SCI and SCI. To elucidate the mechanism underlying the oscillation presumably induced by stromal cells, the

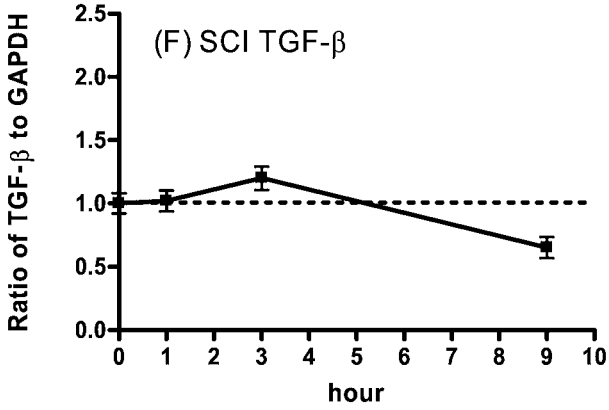

(A) non-SCI TNF-α
Ratio of TNF-α to GAPDH
*P<0.001
hour


(D) SCI TNF-α
Rato of TNF-α to GAPDH
hour


(B)non-SCI IL-6
Rato of IL-6 to GAPDH
*P<0.001
hour


(E) SCI IL-6
Ratio of IL-6 to GAPDH
hour


(C) non-SCI TGF-β
Ratio of TGF-β to GAPDH
*P<0.001
hour


(F) SCI TGF-β
Ratio of TGF-β to GAPDH
hour

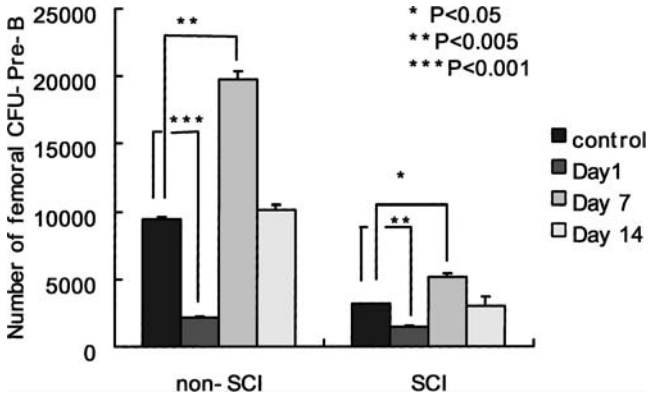


Figure 3. Time courses of numbers of CFU-preB in BM cells from non-SCI and SCI mice after neopterin treatment. Femoral BM samples of the non-SCI and SCI mice were obtained from three non-SCI mice and SCI mice 1, 7, and 14 days after the intraperitoneal injection of neopterin (3.5 mg/kg) for three consecutive days. The changes in the number of CFU-preB cells were determined. Each bar represents the mean ± SD obtained from triplicate experiments. $^*P < 0.05$, $^{**}P < 0.005$,$^{***}P < 0.001$ vs. each control.

messenger RNAs of principal cytokine genes for the positive and negative regulations of the pro-B/pre-B fraction were evaluated, namely, IL-7, TNF-α, IL-6, and TGF-β. In Figure 4, the short-term effects of neopterin on cytokine gene expression levels in BM cells were evaluated 30 min and 1, 2, 3, 6, and 24 hrs after the single-dose neopterin administration because the half-life of neopterin is 90 min (18). In each panel, the results are expressed as a treatment normalized by GAPDH expression level, respectively. Absolute GAPDH expression levels in non-SCI or SCI mice did not show significant differences from each other (see Appendix). In the non-SCI mice, the gene expression levels of the negative regulators TNF-α and IL-6 increased markedly up to sixfold at 1 hr followed by a mild increase up to 6 hrs, although that of TGF-β showed an increase throughout, although not significant. During the course of the experiment, the positive regulator IL-7 showed a very mild increase from 3 to 6 hrs. Regardless of being positive and negative regulators, the expression levels of IL-7, TNF-α, IL-6, and TGF-β all decreased or came back to the level of nontreated control by 24 hrs. In the SCI mice, the negative regulator TNF-α, IL-6, or TGF-β was not expressed at all, except that a milder and delayed peak was observed for IL-6 at 2 hrs. The positive regulator IL-7 showed a milder increase in the same manner as that in non-SCI mice from 3 to 6 hrs. The suppression of cytokine gene expression in the SCI mice at 24 hrs was not observed for all the positive and negative regulators; specifically for TGF-β, the expression levels were slightly higher than those in the nontreated control SCI mice (44.0%, 28.0%, 43.6%, and 92.4%, vs. 128.5%, 130.0%, 274.6%, and 138.5%, for IL-7, TNF-α, IL-6, and TGF-β, respectively).

Table 1. Concentrations of Cytokines in the Supernatant of Cultured Stromal Cells from Non-SCI and SCI Mice[a]

Concentration of neopterin	Non-SCI		SCI	
	None	400 n*M*	None	400 n*M*
TNF-α pg/ml)	23 ± 3	76 ± 11*	22 ± 8	38 ± 8
IL-6 pg/ml)	191 ± 25	455 ± 92*	155 ± 14	184 ± 11
TGF-β pg/ml)	220 ± 15	298 ± 28*	68 ± 24	115 ± 43

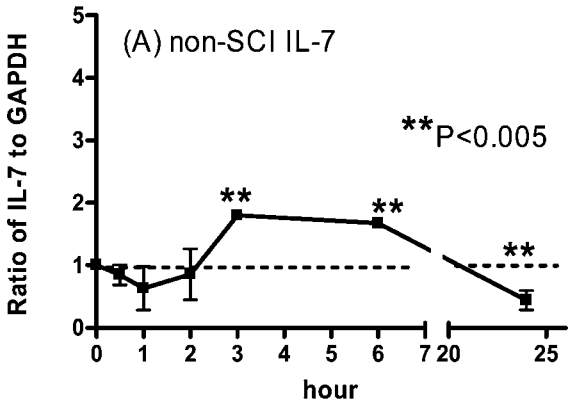
[a] Supernatant of cultured stromal cells was collected 24 hrs after treatment with 400 n*M* neopterin, and concentrations of cytokines in the supernatant was measured by ELISA methods. Each data point represents the mean ± SD obtained from triplicate experiments. $^*P < 0.05$ vs. each control.

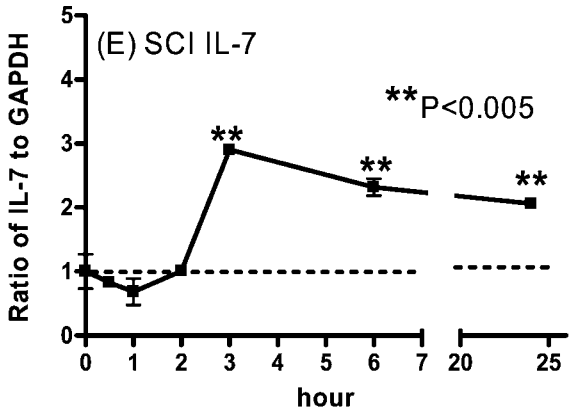
When neopterin was administered for 3 days, the prolonged effect of neopterins on cytokine gene expression was evaluated only 1 and 7 days after the treatment (Fig. 5). Again, the degrees of suppression of the genes expressions of IL-7, TNF-α, IL-6, and TGF-β on Day 1 in the non-SCI mice were 0.56, 0.78, 0.51, and 0.32 relative to those in the nontreated non-SCI mice considered as 1.0, respectively. All the gene expression levels returned to the control level on Day 7. Interestingly, again, the relative changes in these cytokine gene expressions in the SCI mice were essentially milder than those in the non-SCI mice except for IL-7 (0.47, 0.96, 0.81, and 0.84, relative to those of the nontreated SCI control considered as 1.0; senescent SCI mice for IL-7, TNF-α, IL-6, and TGF-β, respectively).

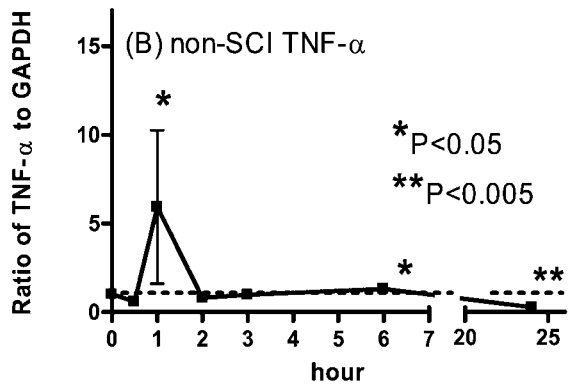
A significant decrease in the number of CFU-preB progenitor cells shown in Figures 4 and 5 may be supported by data shown in Figure 6, in which the steady-state contributions of the positive regulator IL-7 and the negative regulators TNF-α, IL-6, and TGF-β are shown with respect to their absolute expression levels relative to each of the non-SCI and SCI gene expression levels of GAPDH in each of the non-SCI and SCI control. As observed from the absolute cytokine level, the expressions of TGF-β and IL-6

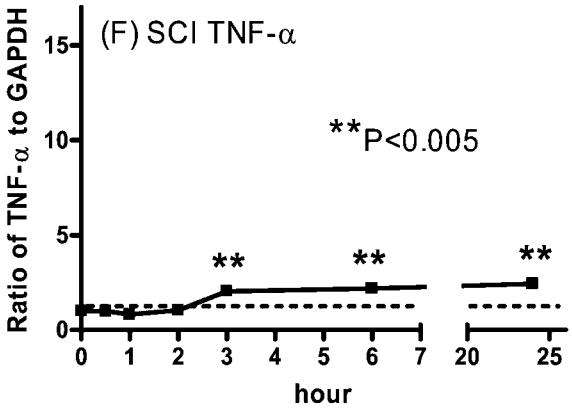
←

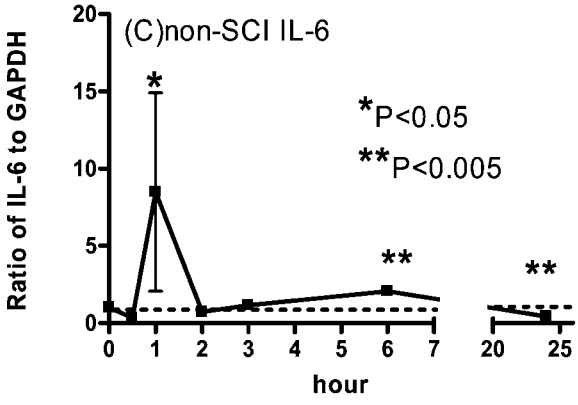
Figure 2. Time courses of TNF-α, IL-6, and TGF-β gene expression levels in stromal cells from young non-SCI and elderly SCI mice (A, B, C and D, E, F, respectively) cultured in the presence of neopterin. Stromal monolayers were prepared by culturing unfractionated BM cells derived from non-SCI or SCI mice. The supernatant was removed and the culture dishes with confluent adherent layer were rinsed with IMDM twice to eliminate nonadherent cells. A fresh culture medium containing 4 μ*M* neopterin was then added and the cells were incubated further. After 1, 3, and 9 hrs of incubation, the culture medium was removed completely and stromal cells were subjected to RNA extraction. Total RNA was extracted from the cultured stromal cells of the non-SCI and SCI mice with TRIzol (Invitrogen) according to the manufacturer's instructions. The reverse-transcribed complementary DNAs were amplified by real-time reverse transcription–polymerase chain reaction (real-time RT-PCR) using the TaqMan universal PCR master mix (Applied Biosystems) and specific primers for murine TNF-α, IL-6, TGF-β, and GAPDH. GAPDH gene expression levels are the same in non-SCI and SCI mice (see Appendix). The results are expressed as a ratio to the cultured non-SCI or SCI fraction without neopterin. Each bar represents the mean ± SD obtained from triplicate experiments ($^*P < 0.001$ vs. each control).

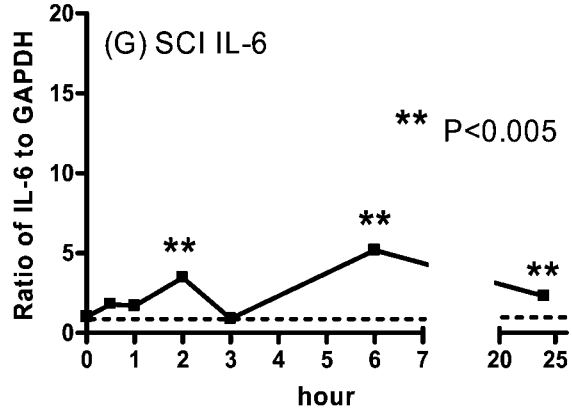

(A) non-SCI IL-7
Ratio of IL-7 to GAPDH
**P<0.005
hour

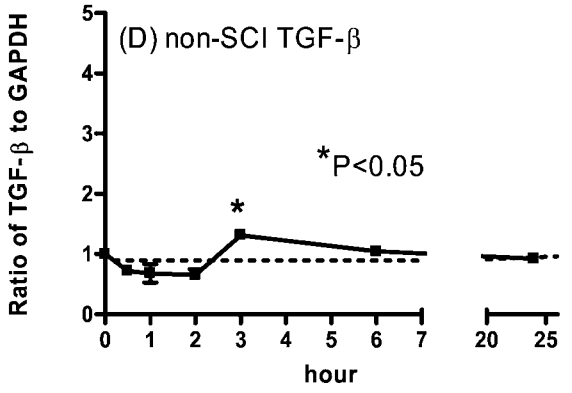

(E) SCI IL-7
Ratio of IL-7 to GAPDH
**P<0.005
hour

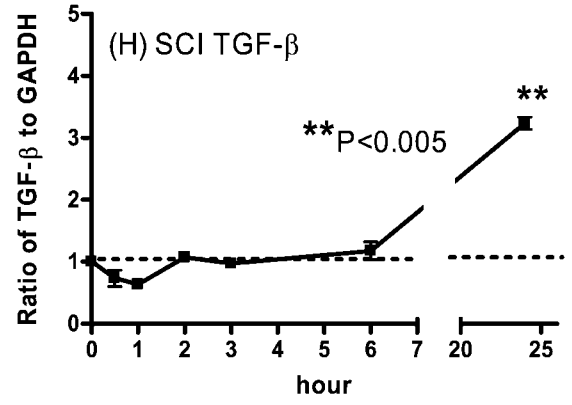

(B) non-SCI TNF-α
Ratio of TNF-α to GAPDH
*P<0.05
**P<0.005
hour


(F) SCI TNF-α
Ratio of TNF-α to GAPDH
**P<0.005
hour


(C)non-SCI IL-6
Ratio of IL-6 to GAPDH
*P<0.05
**P<0.005
hour


(G) SCI IL-6
Ratio of IL-6 to GAPDH
** P<0.005
hour


(D) non-SCI TGF-β
Ratio of TGF-β to GAPDH
*P<0.05
hour


(H) SCI TGF-β
Ratio of TGF-β to GAPDH
**P<0.005
hour

markedly predominate as compared with that of IL-7 (43.2, 60.3, and 657.5 relative to expression level IL-7 from nontreated non-SCI mice considered as 1.0, nontreated non-SCI mice for TNF-α, IL-6, and TGF-β, respectively).

Discussion

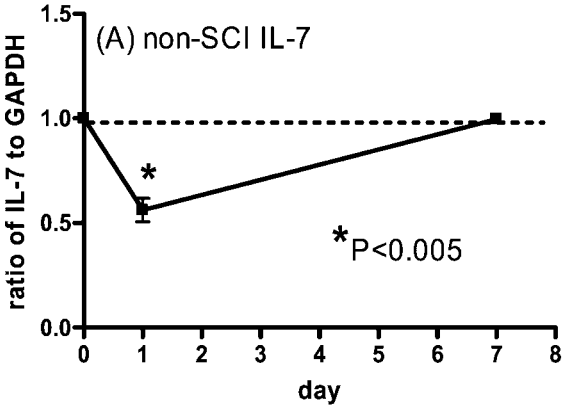
Neopterin, a guanosine derivative, is an inflammatory byproduct that stimulates the proliferation of granulocytes and their progenitor cells due to stromal-cell–derived cytokines, such as GM-CSF and IL-6 (11, 12). During the inflammatory process, the production of lymphocytes is occasionally suppressed when granulocyte proliferation is stimulated (19–21). Thus, whether lymphocyte proliferation is stimulated or suppressed by the inflammatory byproduct neopterin, during the suppurative inflammatory process is of interest. During the suppurative inflammatory process, T cells undergo apoptosis induced by 7,8-dehydroneopterin (22). Thus, in this study, we attempted to clarify the effect of neopterin on the B-cell lineage to determine whether such a suppressive mechanism also exists in this lineage.

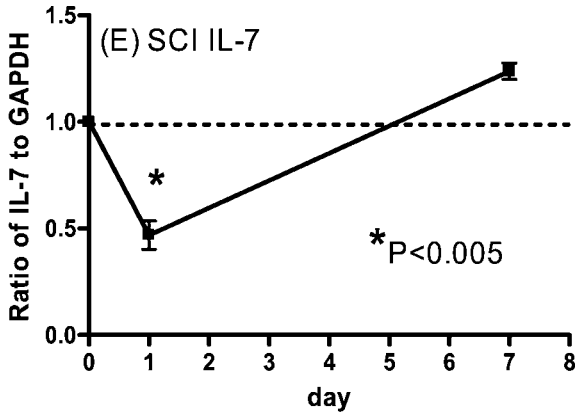
We conducted the experiment with the aim of determining the possible regulatory mechanism of inflammatory cytokines in the B-cell lineage. It should be noted that because the basal level of neopterin in rodents was lower than that in primates, including humans, the dose of neopterin used in the present study was markedly lower than those used in previous studies (5, 7, 9, 23, 24), and neopterin was still clearly detectable. Interestingly, when the B-cell lineage was treated with neopterin to determine whether B-cell suppression might occur, the suppressive effect was induced, presumably by different cytokines specific to B-cell lineages released from stromal cells as in the case of granulopoiesis observed previously (11, 12). Furthermore, we found that this suppressive effect was milder when stromal cells were derived from SCI mice, although this significant mildness in SCI mice compared with those in non-SCI mice was not always significant in terms of relative suppressive ratios between non-SCI and SCI, which was only significant at the dose of 40 n*M*. SAM/P-1 mice used in the experiment showed B-cell suppression earlier, in which both the positive and negative regulators of B cells were suppressed within a short time, and the mice underwent senescence that mimicked stromal-cell impairment. TGF-β is produced by stromal cells (25) and is also a negative regulator of B lymphopoiesis (26). Fernandez *et al*. have recently demonstrated that TNF-α has a direct inhibitory effect on IL-7 responses in $B220^{+}$ /IgM^{-} pre-B cells (27). Neopterin was found to amplify the secretion of TNF-α from peripheral blood mononuclear cells induced by the lipopolysaccharide (LPS), IFN-γ and IL-2 (28). In addition, in vascular smooth muscle cells, neopterin potentially stimulates TNF-α gene expression and protein release (29). Fernandez *et al*. have recently shown that IL-6 indirectly inhibits IL-7 responses in $B220^{+}$/IgM^{-} B-cell progenitors by inducing the stromal-cell production of an inhibitory factor (27). Furthermore, Nakamura *et al*. (30) and Maeda *et al*. (31) have demonstrated recently that IL-6 suppresses B-cell differentiation and causes excessive myeloid development in BM. When cultured non-SCI stromal cells were treated with neopterin, the negative regulators of the B-cell lineage such as TNF-α, IL-6, and TGF-β were all upregulated significantly, whereas the upregulations of the gene expressions of these cytokines were not observed in cultured senescent SCI stromal cells. Therefore, the milder suppression of B-cell proliferation in the SCI group is assumed to be caused by the age-related functional deterioration of stromal cells.

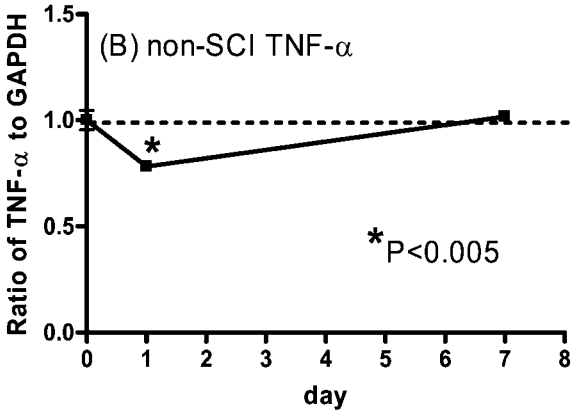
Next, we examined the effect of neopterin *in vivo* using non-SCI mice. The intraperitoneal administration of neopterin at two doses to the non-SCI mice for three consecutive days resulted in a dose-dependent decrease in the number of femoral CFU-preB cells on Day 1, a finding that was consistent with those of the *in vitro* experiments (Fig. 1). When the non-SCI mice were administered repeatedly with neopterin, the number of CFU-preB cells decreased on Day 1, then rapidly increased to 209% 7 days after the neopterin treatment, and then returned to the level before the treatment (Fig. 3). As a mechanism underlying the decrease in CFU-preB-cell number on Day 1, on the bases of cytokine levels, it can be observed that the negative regulators of B lymphocytes such as TNF-α and IL-6 were predominantly and markedly increased 1 hr after neopterin treatment (Fig. 4B, C). These increases in the levels of negative regulators indicate that TNF-α and IL-6 play key roles as major cytokines in the suppression of B lymphopoiesis *in vivo* 1 day after neopterin treatment. After three consecutive days of treatment, all the cytokine levels decreased after 24 hrs and then started to increase 7 days after the last treatment. As a mechanism underlying the subsequent rapid increase in CFU-preB-cell number, the most important factor contributing to the recovery up to Day 7 from B-cell suppression 1 day after neopterin treatment was the marked downregula-

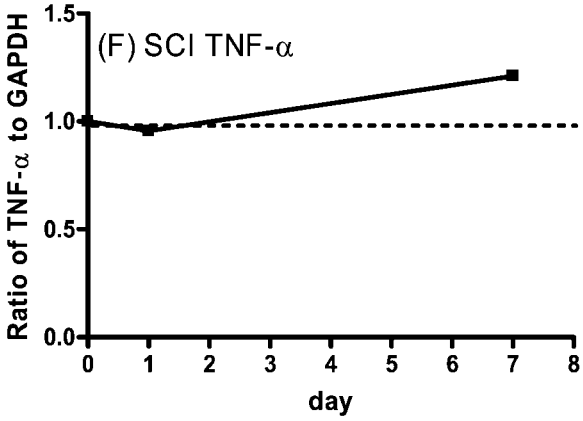
←

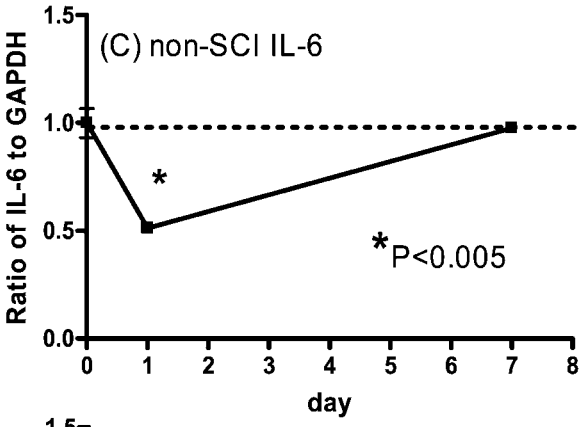
Figure 4. Time courses of IL-7, TNF-α, IL-6, and TGF-β gene expression levels in BM cells from non-SCI and SCI mice after single intraperitoneal injection of 3.5 mg/kg neopterin. Total RNA was extracted from BM cells of the non-SCI and SCI mice (A–D and E–H, respectively) at 30 mins, and 1, 2, 6, and 24 hrs after a single intraperitoneal injection of 3.5 mg/kg body weight neopterin using TRIzol (Invitrogen) according to the manufacturer's instructions. The extracted RNA was reverse-transcribed using Superscript (Life Technologies) and Oligo-dT (Promega). The reverse-transcribed complementary DNAs were then amplified by real-time polymerase chain reaction (real-time PCR) using TaqMan universal PCR master mix (Applied Biosystems) and specific primers for murine IL-7 (A vs. E), TNF-α (B vs. F), IL-6 (C vs. G), TGF-β (D vs. H) and GAPDH. GAPDH gene expression levels are the same in non-SCI and SCI mice (see Appendix). The results are expressed as a ratio to each value of the nontreated non-SCI and SCI group. Each bar represents the mean ± SD obtained from triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.005$ vs. each control.

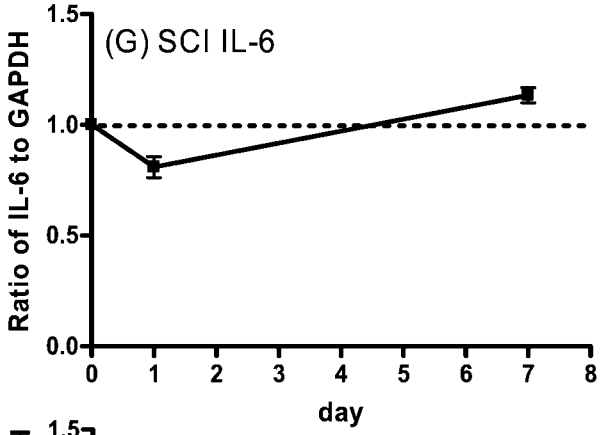

(A) non-SCI IL-7
ratio of IL-7 to GAPDH
1.5
1.0
0.5
0.0
*
*P<0.005
0 1 2 3 4 5 6 7 8
day

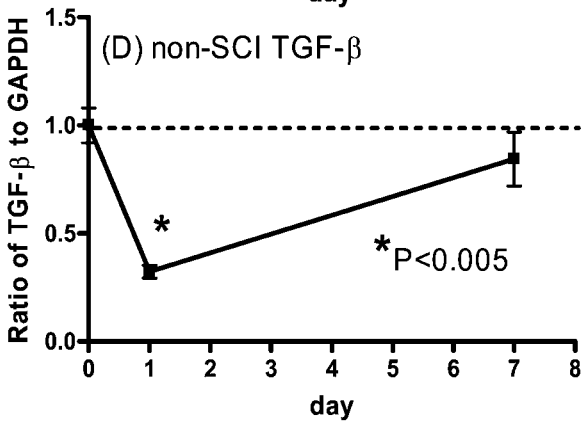

(E) SCI IL-7
Ratio of IL-7 to GAPDH
1.5
1.0
0.5
0.0
*
*P<0.005
0 1 2 3 4 5 6 7 8
day

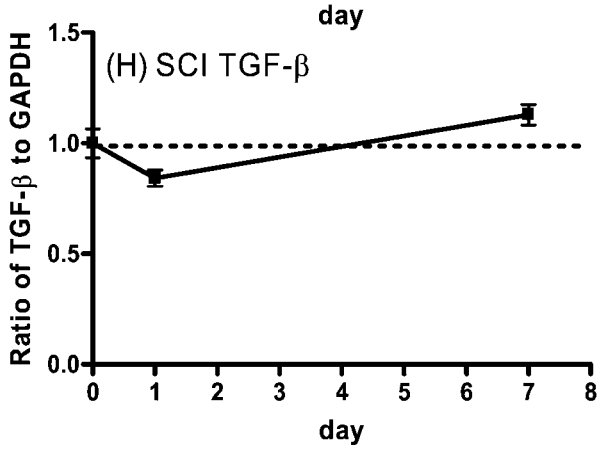

(B) non-SCI TNF-α
Ratio of TNF-α to GAPDH
1.5
1.0
0.5
0.0
*
*P<0.005
0 1 2 3 4 5 6 7 8
day


(F) SCI TNF-α
Ratio of TNF-α to GAPDH
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8
day


(C) non-SCI IL-6
Ratio of IL-6 to GAPDH
1.5
1.0
0.5
0.0
*
*P<0.005
0 1 2 3 4 5 6 7 8
day


(G) SCI IL-6
Ratio of IL-6 to GAPDH
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8
day


(D) non-SCI TGF-β
Ratio of TGF-β to GAPDH
1.5
1.0
0.5
0.0
*
*P<0.005
0 1 2 3 4 5 6 7 8
day


(H) SCI TGF-β
Ratio of TGF-β to GAPDH
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8
day

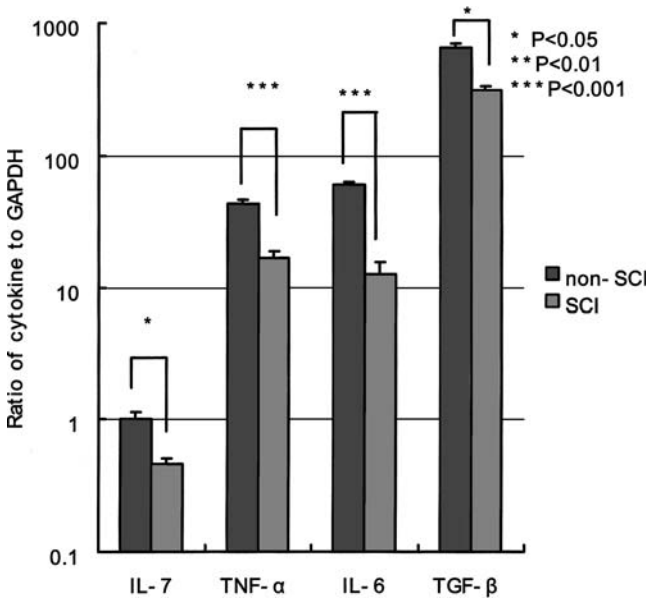


Figure 6. Relative expression levels of positive regulator IL-7, negative regulators TNF-α, IL-6, and TGF-β from non-SCI mice (dark columns) and SCI mice (light columns) (values relative to expression level of IL-7 from non-SCI considered as 1.0). Total RNA was extracted from BM cells of non-SCI and SCI mice using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The reverse-transcribed complementary DNAs were then amplified by real-time polymerase chain reaction (real-time PCR) using the TaqMan universal PCR master mix (Applied Biosystems) and specific primers for murine IL-7, TNF-α, IL-6, TGF-β, and GAPDH. The results are expressed as a ratio to the expression level of GAPDH in the cultured non-SCI or SCI fraction. Each bar represents the mean ± SD obtained from triplicate experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. non-SCI.

tion, on Day 1, of negative regulators, specifically TGF-β (Fig. 5), as compared with the steady-state value (Fig. 6), which could be a potential trigger for the recovery. Throughout the experiment, the expression levels of the genes in the stroma of the SCI mice and the negative regulator cytokines were lower than those in the non-SCI mice. It seems likely that stromal cells that have undergone senescence could not induce TNF-α and IL-6 production in the early phase of neopterin administration and could not suppress TGF-β production in the late phase of neopterin administration, resulting in milder changes in B lymphopoiesis in the SCI mice than in the non-SCI mice. The difference in anti-inflammatory resistances between non-SCI mice and SCI mice has not been tested, but according to references available, a senescent mouse is much more sensitive than a young mouse to a challenge with LPS and TNF-α (32, 33).

These results suggest that the suppression of B cells is milder in the SCI mice, indicating that response of granuloid lineages to suppurative inflammation is very weak. Thus, it is suggested that the hematopoietic regulations during the suppurative inflammatory process in senescence are based on the competitive bilateral regulation between the stimulation of granulopoiesis and the suppression of lymphopoiesis, which forms a hematopoietic environment for anti-inflammatory hematopoietic proliferation. In a senescent hematopoietic environment, it is noted that hematopoietic suppression is not based on the stimulation of the suppression system, but rather on both the positive and negative regulators that are downmodulated simultaneously. Cytokines in homeostasis are at lower functional levels. Therefore, the senescent-downmodulated homeostasis hardly recovers to the upper functional level by a simple administration of one-sided negative or positive regulator. Downmodulated homeostasis is, thus, an important subject of studies in future antisenescent hematologic programs.

Further studies are required to elucidate the mechanisms underlying the neopterin action, but our present findings taken together provide evidence that neopterin is a potent B-lymphopoietic regulatory factor and that the response of B lymphopoiesis to neopterin changes with senescence.

We thank Dr. Yukio Kodama, Ms. Erika Tachihara, Ms. Yoshiko Usami, and Ms. Minako Kenjoh for assistance in maintaining the SAM/P-1 mice in the experimental animal facilities, and Ms. Sonoko Araki and Ms. Sachiko Yuda for technical assistance.

APPENDIX

Glyceraldehyde phosphate dehydrogenase (GAPDH), a housekeeping gene expression in non-SCI (stromal-cell impairment) or SCI steady-state mice.

In the present study, cytokine genes were evaluated quantitatively by real-time PCR using the Taqman Universal PCR master mix and specific primers and probes (see Materials and Methods). Because the expression of each of the cytokines was evaluated as a ratio to the standard expression level of GAPDH from non-SCI mice and SCI mice, respectively, the steady-state expression of GAPDH in non-SCI mice was compared with compatible GAPDH expression level in SCI mice.

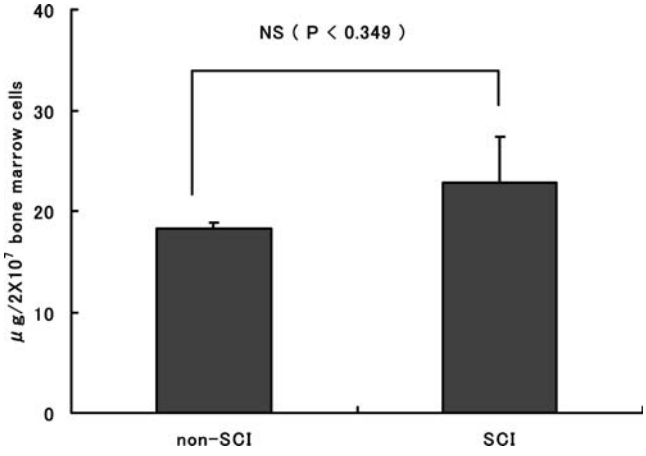
Total RNA was extracted from BM cells using TRIzol

←

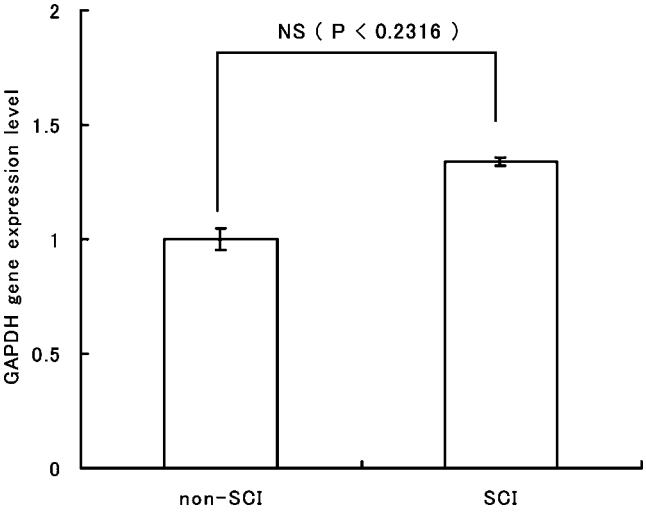
Figure 5. Time courses of IL-7, TNF-α, IL-6, and TGF-β gene expression levels in BM cells from non-SCI and SCI mice (A–D and E–H, respectively) after intraperitoneal injection of 3.5 mg/kg body weight neopterin for three consecutive days. Total RNA was extracted from BM cells of non-SCI and SCI mice 1 and 7 days after the intraperitoneal injection of 3.5 mg/kg body weight neopterin for three consecutive days using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The reverse-transcribed complementary DNAs were then amplified by real-time polymerase chain reaction (real-time PCR) using the TaqMan universal PCR master mix (Applied Biosystems) and specific primers for murine IL-7 (A vs. E), TNF-α B vs. F IL-6 (C vs. G), TGF-β (D vs. H) and GAPDH. GAPDH gene expression levels are the same in non-SCI and SCI mice (see Appendix). The results are expressed as a ratio to each value of the nontreated non-SCI or SCI group. Each bar represents the mean ± SD obtained from triplicate experiments ($*P < 0.005$ vs. each control).

(Invitrogen) according to the manufacturer's instructions. Amounts of RNA per unit cell number (18.3 ± 0.6 μg vs. 22.8 ± 4.5 μg, $P < 0.349$) from non-SCI and SCI mice did not differ significantly from each other when the same number of BM cells (2×10^7 cells) was compared. Appendix Figure 1 shows the amounts of total RNA from non-SCI and SCI mice extracted separately. All reactions were performed in triplicate. Then, total RNA extracted from non-SCI and SCI mice at the same amounts was transcribed with avian reverse transcriptase, Superscript (Life Technologies) and Oligo-dT primer (Promega). RT products were inactivated at 98°C for 10 min with a heat block, followed by quantitative real-time PCR performed using the TaqMan universal PCR master mix (Applied Biosystems) and specific primers and probes, with the Applied Biosystems 7900 sequence detection system, version 2.0. Specific primers and probes for GAPDH were purchased from Applied Biosystems (TaqMan Mm99999915_g1). PCR conditions and data analysis were performed according to the instructions in the sequence detection system, version 2.0.

Appendix Figure 2 shows the ratio of expression of GAPDH from senescent SCI mice expressed as those from non-SCI mice (three mice each) considered as 1.0. Both expressions were not significantly different from each other (1.0 vs. 1.3, respectively, $P < 0.2316$).



Appendix Figure 1. Relative amounts of RNA per unit cell number from non-SCI and SCI mice. Total RNA was extracted from the same number of BM cells (2×10^7) from the non-SCI and SCI mice using TRIzol (Invitrogen). Each bar represents the mean ± SD obtained from triplicate experiments.



Appendix Figure 2. Relative expression levels of GAPDH from non-SCI and SCI mice. RNA extracted from non-SCI mice and SCI mice at the same amount was reverse-transcribed using Superscript (Life Technologies) and Oligo-dT(Promega). The reverse-transcribed complementary DNAs were then amplified by real-time polymerase chain reaction using TaqMan PCR master mix (Applied Biosystems) and specific primer for murine GAPDH. Each bar represents the mean ± SD obtained from triplicate experiments.

1. Rembold H, Gyure WL. Biochemistry of the pteridines. Angew Chem Int Ed Engl 11:1061–1072, 1972.
2. Huber C, Batchelor JR, Fuchs D, Hausen A, Lang A, Niederwieser D, Reibnegger G, Swetly P, Troppmair J, Wachter N. Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. J Exp Med 160:310–316, 1984.
3. Niederwieser D, Huber C, Gratwohl A, Bannert P, Fuchs D, Hausen A, Reinbnegger G, Speck B, Wachter H. Neopterin as a new biochemical marker in the clinical monitoring of bone marrow transplant recipients. Transplantation 38:497–500, 1984.
4. Reibnegger G, Aulitzky W, Huber C, Margreoter R, Riccabona G, Wachter H. Neopterin in urine and serum of renal allograft recipients. J Clin Chem Biochem 24:770–775, 1986.
5. Schobersberger W, Hoffmann G, Hobisch-Hagen P, Bock G, Volkl H, Baier-Bitterlich G, Wirleitner B, Wachter H, Fuchs D. Neopterin and 7,8-dihydroneopterin induce apoptosis in the rat alveolar epithelial cell line L2. FEBS Lett 397:263–268, 1996.
6. Kojima S, Nomura T, Icho T, Kajiwara Y, Kitabatake K, Kubota K. Inhibitory effect of neopterin on NADPH-dependent superoxide-generating oxidase of rat peritoneal macrophages. FEBS Lett 329: 125–128, 1993.
7. Schobersberger W, Jelkmann W, Fandrey J, Frede S, Wachter H, Fuchs D. Neopterin-induced suppression of erythropoietin production *in vitro*. Pteridines 6:12–16, 1995.
8. Uberall F, Werner-Felmayer G, Schubert C, Grunicke HH, Wachter H, Fuchs D. Neopterin derivatives together with cyclic guanosine monophosphate induce c-*fos* gene expression. FEBS Lett 352:11–14, 1994.
9. Schobersberger W, Hoffmann G, Grote J, Wachter H, Fuchs D. Induction of inducible nitric oxide synthase expression by neopterin in vascular smooth muscle cells. FEBS Lett 377:461–464, 1995.
10. Kibourn RG, Traber DL, Szabo C. Nitric oxide and shock. Dis Mon 43: 277–348, 1997.
11. Aizawa S, Hiramoto M, Araki S, Negishi S, Kimura Y, Hoshi G, Kojima S, Wakasugi K. Stimulatory effects of neopterin on hematopoiesis *in vitro* are mediated by activation of stromal-cell function. Hematol Oncol 16:57–67, 1998.
12. Aizawa S, Hamamoto H, Araki S, Hoshi H, Kojima S, Wakasugi K. *In vivo* stimulatory effects of neopterin on hematopoiesis. Pteridines 9:13–17, 1998.
13. Takeda T, Hosokawa M, Takeshita S, Irino M, Higuchi K, Matsushita T, Tomita Y, Yasuhira K, Hamamoto H, Shimizu K, Ishii M, Yamamuro T. A new murine model of accelerated senescence. Mech Ageing Dev 17:183–194, 1981.
14. Izumi-Hisha H, Ito Y, Sugimoto K, Oshima H, Mori KJ. Age-related decrease in the number of hemopoietic stem cells and progenitors in senescence accelerated mice. Mech Ageing Dev 56:89–97, 1990.

15. Tsuboi I, Morimoto K, Horie T, Mori KJ. Age-related changes in various hemopoietic progenitor cells in senescence-accelerated (SAM-P) mice. Exp Hematol 19:874–877, 1991.
16. Tsuboi I, Morimoto K, Hirabayashi Y, Li GX, Aizawa S, Mori KJ, Kanno J, Inoue T. Senescent B lymphopoiesis is balanced in suppressive homeostasis: decrease in interleukin-7 and transforming growth factor-β levels in stromal-cells of senescence-accelerated mice. Exp Biol Med (Maywood) 229:494–502, 2004.
17. Riley RL, Kruger MG, Elia J. B cell precursors are decreased in senescent BALB/c mice, but retain normal mitotic activity *in vivo* and *in vitro*. Clin Immunol Immunopathol 59:301–313, 1991.
18. Fuchs D, Stahl-Hennig C, Gruber A, Murr C, Hunsmann G, Wachter H. Neopterin—its clinical use in urinalysis. Kidney Int Suppl 47:S8–S11, 1994.
19. Nagaoka H, Gonzalez-Aseguinolaza G, Tsuji M, Nussenzweig MC. Immunization and infection change the number of recombination activating gene (RAG)-expressing B cells in the periphery by altering immature lymphocyte production. J Exp Med 191:2113–2120, 2000.
20. Ueda Y, Yang K, Foster SJ, Kondo M, Kelsoe G. Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression. J Exp Med 199:47–58, 2004.
21. Ueda Y, Kondo M, Kelsoe G. Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. J Exp Med 201:1771–1780, 2005.
22. Wirleitner B, Obermoser G, Bock G, Neurauter G, Schennach H, Sepp N, Fuchs D. Induction of apoptosis in human blood T cells by 7,8-dihydroneopterin: the difference between healthy controls and patients with systemic lupus erythematosus. Clin Immunol 107:152–159, 2003.
23. Nemunaitis J, Tompkins CK, Andrews DF, Singer JW. Transforming growth factor beta expression in human marrow stromal-cells. Eur J Haematol 46:140–145, 1991.
24. Hoffman G, Schobersberger W, Frede S, Pelzer L, Fandrey J, Wachter H, Fuchs D, Grote J. Neopterin activates transcription factor nuclear factor-κB in vascular smooth muscle cells FEBS Lett 391:181–184, 1996.
25. Hoffman G, Kenn S, Wirleitner B, Deetjen C, Trede S, Smolny M, Rieder J, Fuchs D, Baier-Bitterlich G, Schobersberger W. Neopterin induces nitric oxide-dependent apoptosis in rat vascular smooth muscle cells. Immunobiol 199:63–73, 1998.
26. Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-beta. J Immunol 142:3875–3883, 1989.
27. Fernandez S, Knopf MA, Shankar G, McGillis JP. Calcitonin gene-related peptide indirectly inhibits IL-7 responses in pre-B cells by induction of IL-6 and TNF-alpha in bone marrow. Cell Immunol 226: 67–77, 2003.
28. Barak M, Gruener N. Neopterin augmentation of tumor necrosis factor production. Immunol Lett 30:101–106, 1991.
29. Hoffmann G, Frede S, Kenn S, Smolny M, Wachter H, Fuchs D, Grote J, Rieder J, Schobersberger W. Neopterin-induced tumor necrosis factor-alpha synthesis in vascular smooth muscle cells *in vitro*. Int Arch Allergy Immunol 116:240–245, 1998.
30. Nakamura K, Kouro T, Kincade PW, Malykhin A, Maeda K, Coggeshall KM. Src homology 2-containing 5-inositol phosphatase (SHIP) suppresses an early stage of lymphoid cell development through elevated interleukin-6 production by myeloid cells in bone marrow. J Exp Med 199:243–254, 2004.
31. Maeda K, Baba Y, Nagai Y, Miyazaki K, Malykhin A, Nakamura K, Kincade PW, Sakaguchi N, Coggeshall KM. IL-6 blocks a discrete early step in lymphopoiesis. Blood 106:879–885, 2005.
32. Saito H, Sherwood ER, Varma TK, Evers BM. Effect of aging on mortality, hypothermia, and cytokine induction in mice with endotoxemia or sepsis. Mech Ageing Dev 124:1047–1058, 2003.
33. Tateda K, Matsumoto T, Miyazaki S, Yamaguchi K. Lipopolysaccharide-induced lethality and cytokine production in aged mice. Infect Immun 64:769–774, 1996.