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Inflammation Research

Cytokine production profile of splenocytes derived from zymosan A-treated SKG mice developing arthritis

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Abstract. *Objective:* SKG mice have a point mutation of the zeta-associated protein of 70 kD (ZAP-70) and spontaneously develop a severe polyarthritis in the conventional condition, whereas they are healthy under the specific pathogen free (SPF) condition. The purpose of this study was to investigate the cytokine production from splenocytes in SKG mice developing arthritis under the SPF condition.
Material: SKG and BALB/c mice were intraperitoneally injected with zymosan A under the SPF condition. Spleen was isolated 1, 2 or 8 weeks after the intraperitoneal injection of saline or zymosan A. Splenocytes were cultured with concanavalin A. Cytokine production and proliferation were measured 48 and 72 h after the culture.
Results: An intraperitoneal injection of zymosan A induced severe polyarthritis with increased levels of rheumatoid factor and interleukin 6 (IL-6) only in SKG mice. Splenocytes from SKG mice did not proliferate well maybe because of less productivity of IL-2. The IL-4 production from splenocytes of SKG mice was higher, while interferon-γ production was lower than those of BALB/c mice. An injection of zymosan A reduced the IL-4 production only in SKG mice.
Conclusions: SKG mice do not develop arthritis under the SPF condition possibly because of a low proliferative activity of T cells and Th2-predominance.

Key words: Arthritis – T cell and cytokine

Introduction

A single spontaneous point mutation of the gene encoding a src homology domain-2 of zeta-associated protein of 70 kD (ZAP-70), a key signal transduction molecule in T cells, causes chronic arthritis in SKG mouse similar to rheumatoid arthritis (RA) [1]. Altered signal transduction from the T-cell receptor (TCR) through the aberrant ZAP-70 changes the thresholds of T cells to thymic selection, leading to positive selection of otherwise negatively selected autoimmune T cells. We previously reported that RA-like disease in SKG mice is an autoimmune disease mediated by apparently joint-specific CD4+ T cells, in accord with the infiltration of CD4+ T cells to subsynovial tissue. The thymus in SKG mice is continuously generating arthritogenic autoimmune T cells. Bone marrow cells give rise to such arthritogenic T cells through the normal thymic environment, indicating that the arthritogenic abnormality in SKG mice is T cell-intrinsic. However, under the specific pathogen free (SPF) condition, SKG mice do not develop arthritis [2]. These results suggest that some kind of stimulation related with pathogen is necessary for the development of arthritis in addition to the genetic background. We previously reported that not only zymosan A, a crude fungal β-glucan, but also purified β-glucans such as curdlan and laminarin developed arthritis under the SPF condition [3].

RA is a systemic autoimmune disease characterized by the progressive chronic inflammation of multiple joints. The incidence of RA is close to 1 % worldwide, but its etiology is not yet known. Several characteristics of RA, such as hyper-γ-globulinemia, autoantibody production, genetic linkage with the HLA-DR locus and infiltration of T cells into the synovium, have suggested that immunological dysfunction play crucial roles in the pathogenesis of this disease [4]. In the majority of synovial infiltrating T cells, interferon-γ (IFN-γ) is produced much more than IL-4 [5, 6]. A markedly elevated Th1/Th2 ratio in the synovial fluid correlates with disease activity [7]. These results suggest that upregulation of Th1 cytokines and downregulation of Th2 cytokines play an important role for the development of RA. The major purpose of this study is to investigate the cytokine production from splenocytes in SKG mice. To control the induction of arthritis and make the analysis easier, SKG mice were treated with zymosan A under SPF condition.

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Materials and methods

Animals

SKG mice have been established from the closed breeding colony of BALB/c mice [1]. SKG mice were maintained at a temperature of 22 ± 3 °C and a humidity of 50 ± 20 %. Food and water were provided *ad libitum*. BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). The study protocol for the animal experiment was approved by Animal Care Committee of Kyowa Hakko Kogyo Co., Ltd.

Reagents

Zymosan A was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Sources of other chemicals were as follows: concanavalin A (Roche Diagnostics, Basel, Switzerland), pristane (2,6,10,14-tetramethylpentadecane, Wako Pure Chemical, Osaka, Japan), Freund's complete adjuvant (Difco, Detroit, MI, USA), type II collagen (Cosmo Bio Co., Ltd., Tokyo, Japan), heat killed mycobacterium tuberculosis H37RA (Difco), dinitrophenylated keyhole limpet haemocyanin (Calbiochem, Bad Soden, Germany), serum amyloid P (SAP: Calbiochem), rabbit anti-mouse SAP (Calbiochem), peroxidase labeled goat anti-rabbit IgG antibody (Wako Pure Chemical), Mouse-lgM Rheumatoid factor ELISA KIT (Shibayagi, Shibukawa, Japan), ELISA kits for each mouse cytokine: IL-6 and IL-1β (BioSource International, Camarillo, CA, USA), TNF-α (R&D Systems, Minneapolis, MN, USA), IL-2 and IFN-γ (PIERCE, Rockford, IL, USA), IL-4 (BD Bioscience, San Diego, CA, USA) and IL-12 (Genzyme, Cambridge, MA, USA), RPMI 1640 tissue culture medium (Sigma-Aldrich), fatal bovine serum (Intergen, Purchase, NY, USA), penicillin-streptomycin (Life Technologies, Rockville, MD, USA), 2-melcaptethanol (Wako Pure Chemical), [^{3}H] thymidine (25 Ci/mmol: Amersham Biosciences, Tokyo, Japan).

Induction of arthritis

Zymosan A suspended in saline was kept in boiling water for 10 min. Zymosan A solution (0.5 mL/head) or saline was intraperitoneally injected into 7 or 8 week-old mice. Concanavalin A (1 mg/kg in saline) or pristane (0.5 mL/head) was intraperitoneally injected. Heat killed *mycobacterium tuberculosis* H37RA (1 mg/head) or type II collagen (0.15 mg in 0.05 mL saline/head) emulsified with the equal volume of Freund's complete adjuvant was intradermally injected.

Scoring of joint swelling

Joint swelling was scored by macroscopic observation as follows: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist or ankle; 1.0, severe swelling of wrist or ankle [1]. Scores for all fingers of forepaws and hind paws, wrists and ankles were totaled for each mouse.

Analyses of serological parameters

Blood was collected 8 or 14 weeks after the injection of zymosan A. Concentrations of total immunoglobulins and an anti-type II collagen antibody titer in the serum 8 weeks after the treatment with zymosan A were measured by an enzyme linked immunosorbent assay (ELISA) [1, 8]. Serum amyloid P (SAP) was measured as previously described with some modifications [9]. Briefly, Microwell plate was coated with 10 μg/mL of dinitrophenylated keyhole limpet haemocyanin. Standard SAP and 1:300 diluted serum samples were added to the plate and incubated for 2 h. Bound SAP was detected with rabbit anti-mouse SAP followed by peroxidase-labeled goat anti-rabbit IgG antibody. Rheumatoid factor was measured by Mouse-lgM Rheumatoid factor ELISA KIT.

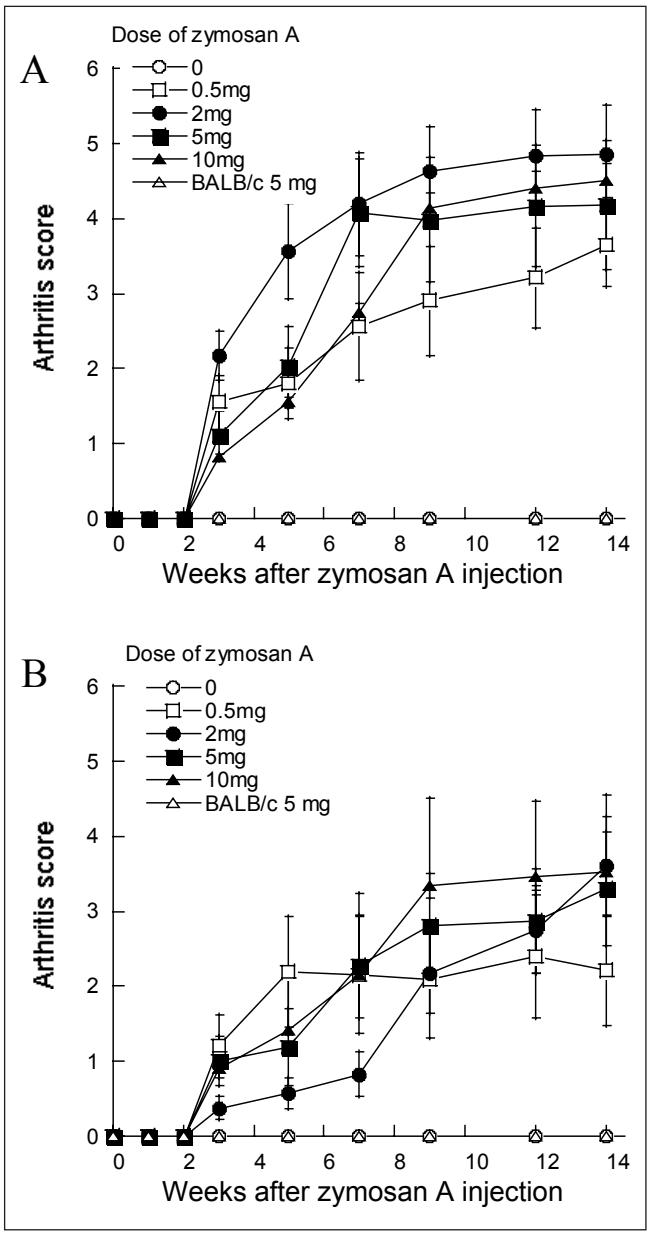

Fig. 1. Arthritis score in the zymosan A-treated SKG mice. SKG mice (7 week-old) were intraperitoneally injected with saline (open circle) or 0.5 mg (open square), 2 mg (closed circle), 5 mg (closed square) or 10 mg (closed triangle) of zymosan A at the point of week 0. BALB/c mice (7 week-old) were intraperitoneally injected with 5 mg of zymosan A at the point of week 0 (open triangle). Female (**A**) and male (**B**) mice were used. Each point represents mean ± SE of 6–10 mice.

Concentrations of IL-6, IL-1β and TNF-α in the serum 14 weeks after the treatment with zymosan A were measured by ELISA kits.

Histological analysis

All animals were sacrificed 14 weeks after the treatment with zymosan A. Ankle joints were immersed in 10 % neutral buffered formalin. These joints decalcified in 10 % ethylenediaminetetraacetic acid (EDTA), embedded in paraffin and sectioned at 5 μm thickness were stained with

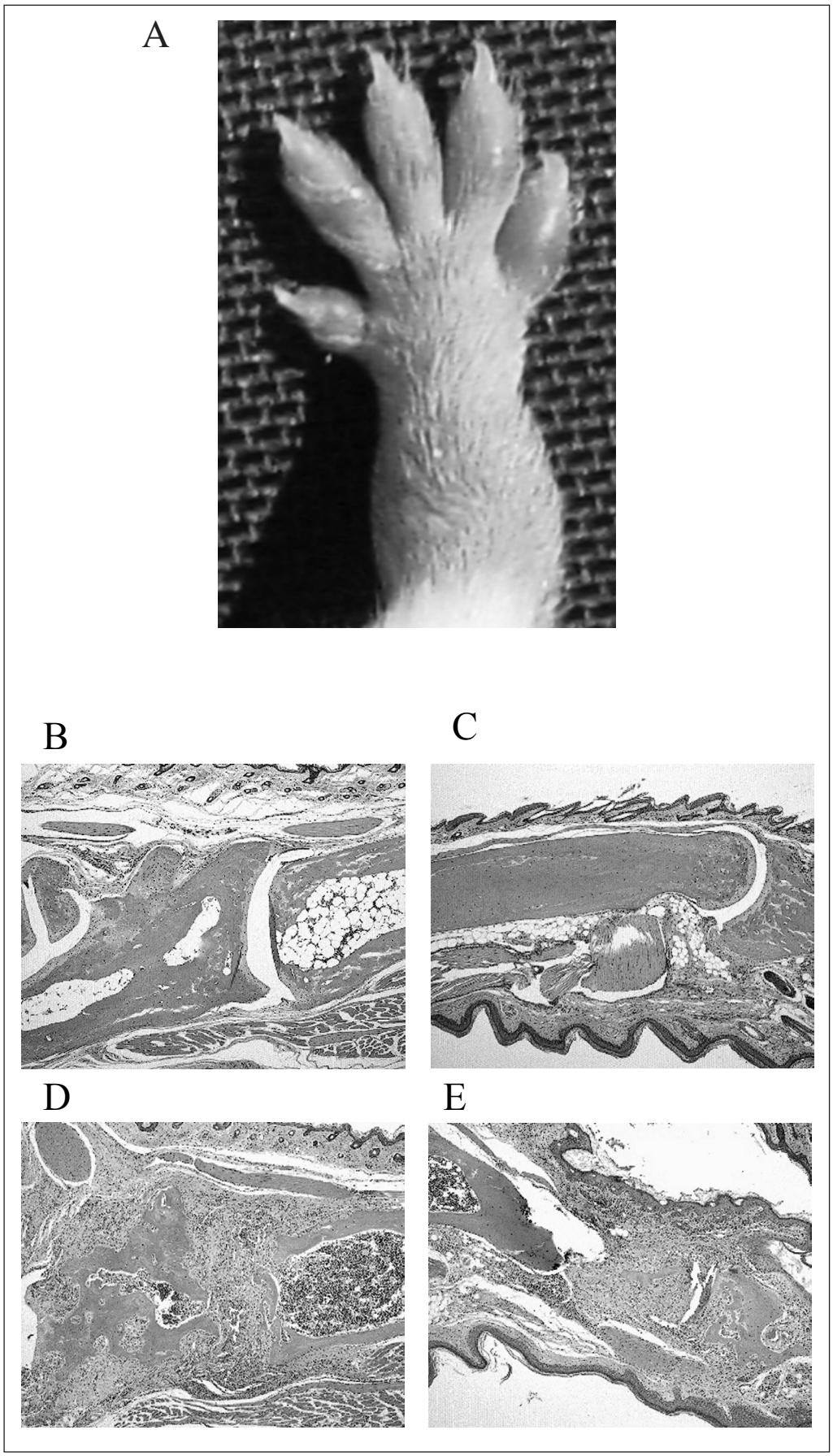

Fig. 2. Arthritis in SKG mice. **A,** A representative swelling of forepaw fingers in a SKG mouse 14 weeks after the intraperitoneal injection of zymosan A. **B–E,** Hematoxylin eosin stained paraffin sections of hind limb tarsus and phalangeal joints. Any pathological change was not observed at the hind limb tarsus joints **(B)** and the hind limb phalangeal joints **(C)** in a SKG mouse 14 weeks after saline treatment. Treatment with 2 mg of zymosan A showed synovial hyperplasia and erosion of articular cartilage and bone in hind limb tarsus joints **(D)** and phalangeal joints **(E)** 14 weeks after the zymosan A treatment. Original magnification: X 40.

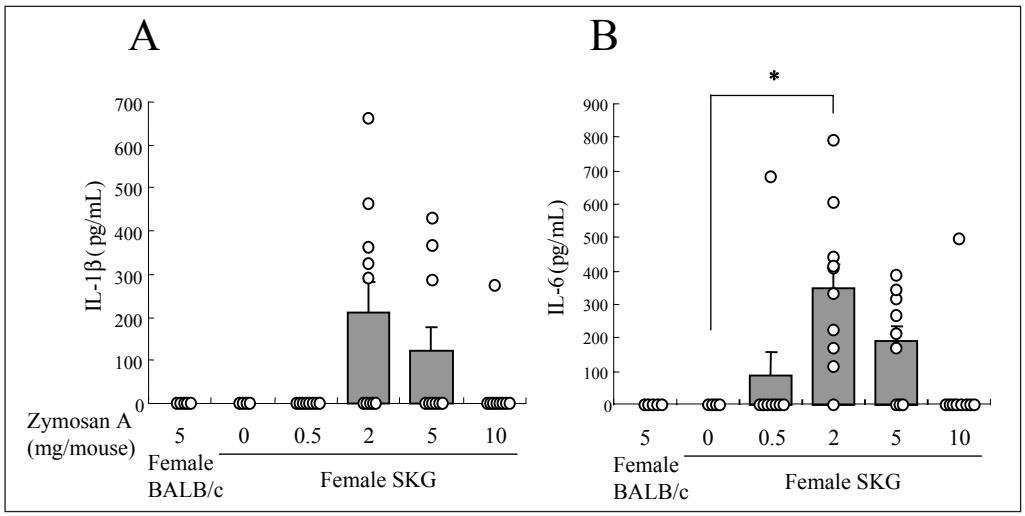

Fig. 3. Concentration of IL-1β and IL-6 in serum 14 weeks after the treatment with indicated dose of zymosan A. Serum concentration of IL-1β (**A**) and IL-6 (**B**) in BALB/c or SKG mice. Each data point represents 1 animal. Results are shown as the mean +SE of 4 to 9 mice. * = $P < 0.05$.

Strain	Treatment	Serum amyloid P (μg/mL)	Total immunoglobulins (mg/mL)	Rheumatoid factor (U/mL)
BALB/c	Saline	11.7 ± 0.9	0.47 ± 0.09 **	26.0 ± 4.8
BALB/c	Zymosan A	11.3 ± 2.2	0.90 ± 0.06 ##	21.4 ± 3.2
SKG	Saline	13.0 ± 1.6 *	1.1 ± 0.1 **	20.9 ± 4.9 *
SKG	Zymosan A	95.5 ± 22.3	5.4 ± 0.9	35.9 ± 3.9

Table 1. Serological parameters in zymosan A-induced arthritis.

Serum was collected 8 weeks after zymosan A treatment. The serological parameters were measured by ELISA. Results were shown as the mean ± SE. ##: $P < 0.01$ between the two strains. *, **: $P < 0.05$ and 0.01 between saline- and zymosan A-treated groups.

hematoxylin and eosin [8].

Proliferation and cytokine production of splenocytes

Single cell suspensions were prepared by triturating spleens between the ends of sterile frosted slides and filtrating through nylon mesh. Splenocytes (2×10^5 cells) were suspended in 200 μL of RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated fatal bovine serum, 50 unit/mL penicillin-50 μg/mL streptomycin and 50 μmol/L 2-melcaptethanol and were cultured in the presence or absence of 2 μg/mL of concanavalin A for 64 h at 37 °C followed by 8 h culture in the presence of [^{3}H] thymidine. The incorporation of [^{3}H] thymidine was counted by a liquid scintillation counter. Cytokine production from splenocytes stimulated with concanavalin A was determined 48 h after the stimulation. Splenocytes (1×10^6 cells/200 μL) were cultured in the presence or absence of concanavalin A (2 μg/mL) for 48 h. IL-4, IL-2, IL-12 and IFN-γ concentration in the supernatants were measured by ELISA kits for mouse cytokines.

Statistical analyses

Statistical analyses were performed by Student's *t* test or Aspin-Welch test. Alternatively, Steel test for multiple comparison was carried out. P values less than 0.05 were considered to be statistically significant.

Results

Development of arthritis in SKG mice under the SPF condition

A single intraperitoneal injection of zymosan A induced severe polyarthritis in SKG mice, especially in female (Fig. 1). Arthritis was not induced by other stimulants such as concanavalin A (1 mg/kg i. p.), pristane (0.5 mL i. p.), heat killed *mycobacterium tuberculosis* H37RA (1 mg, intradermal) or type II collagen (0.15 mg, intradermal) emulsified with equal volume of Freund's complete adjuvant (data not shown). SKG mice began to develop arthritis from 2 to 4 weeks after the zymosan A injection. No development of arthritis was found in saline-treated SKG mice or zymosan A-treated BALB/c mice. Massive swelling was observed even in the fingers (Fig. 2A). Histological analysis revealed the synovial hyperplasia in the hind limb tarsus joints (Fig. 2D) and the hind limb phalangeal joints (Fig. 2E) 14 weeks after the zymosan A injection. Erosions of both articular cartilage and bone were shown in the tarsus and the hind limb phalangeal joints. Pathological changes were not observed in saline-treated SKG mice (Figs. 2B, C). The deformation of bone and osteoporosis were observed in zymosan-treated SKG mice by radiography (data not shown). Vasculitis, pneumonitis and alopecia were also observed in zymosan A-treated SKG mice, which had been reported in spontaneously developed arthritis under the conventional condition [1].

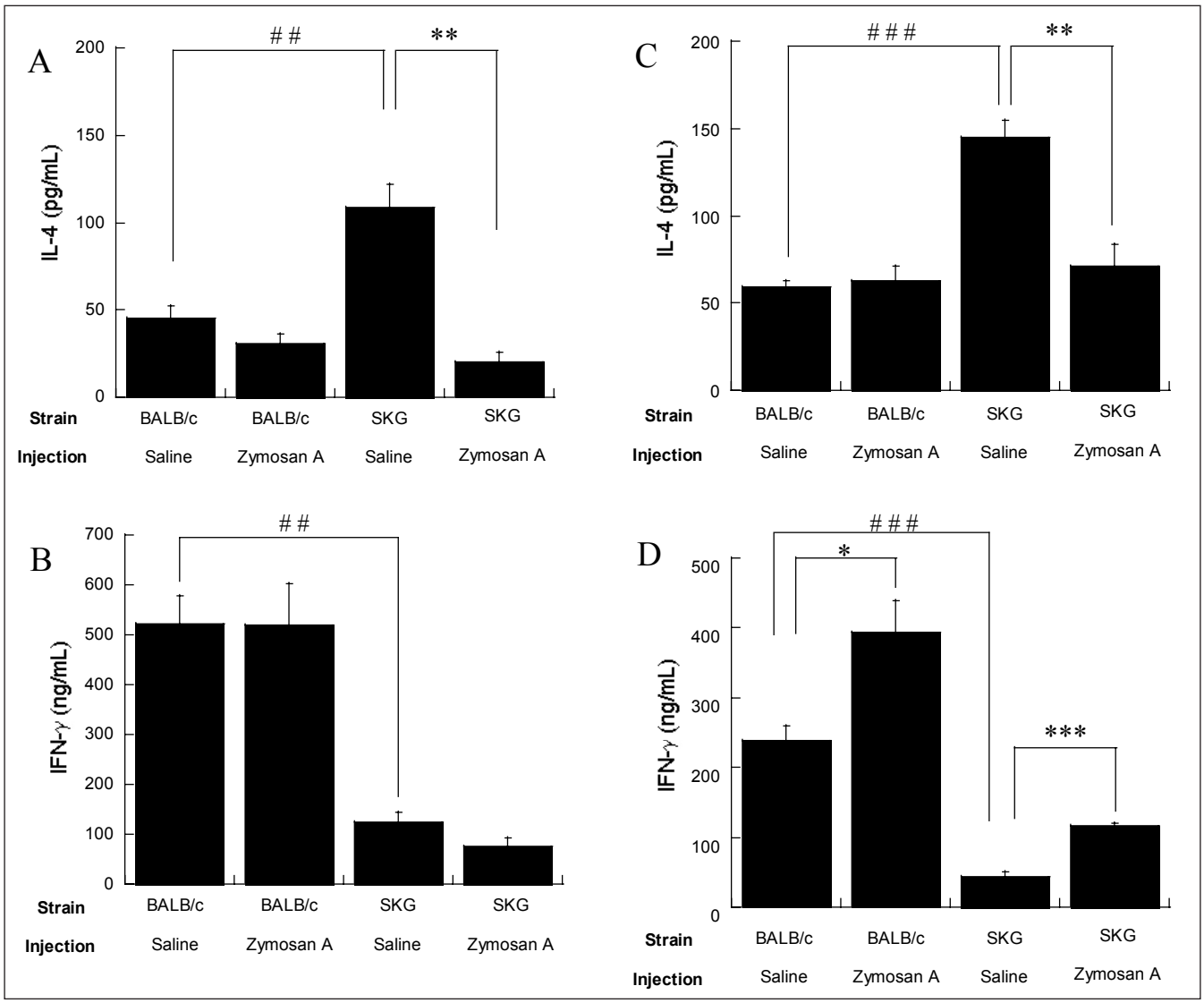

Fig. 4. IL-4 and IFN-γ production from concanavalin A-stimulated splenocytes 1 or 8 weeks after the zymosan A injection (2 mg/head). Splenocytes isolated 1 week (**A**, **B**) or 8 weeks (**C, D**) after the injection of saline or zymosan A, were stimulated with concanavalin A (2 μg/mL) for 48 h. Concentrations of IL-4 (**A, C**) and IFN-γ (**B, D**) in the culture supernatants were determined. Results are shown as the mean ±SE of 3 to 5 mice. ##, ###: $P < 0.01$ and 0.001, between the groups. *, **, ***: $P < 0.05$, 0.01 and 0.001 between saline- and zymosan A-treated mice.

Changes in serological parameters

Serum concentrations of IL-1β were elevated in some mice 14 weeks after the treatment with zymosan A (Fig. 3A). Serum concentrations of IL-6 were significantly elevated in the group treated with 2 mg of zymosan A ($p < 0.05$, Fig. 3B). Serum concentrations of tumor necrosis factor-α (TNF-α) were under the detection limit (<7.5 pg/mL) in all animals. All of the cytokines measured were under the detection limit in BALB/c mice treated with zymosan A. Serum amyloid P, a typical acute phase protein, was significantly increased only in SKG mice 8 weeks after the treatment with zymosan A ($p < 0.05$, Table 1). The concentration of total immunoglobulins in the serum was higher in the saline-treated SKG mice than that in the saline-treated BALB/c mice ($p < 0.01$, Table 1). Zymosan A significantly increased the concentration of total immunoglobulins in both mice ($p < 0.01$). The concentrations of rheumatoid factor in serum was significantly higher in the zymosan A group compared to the saline group in SKG mice ($p < 0.05$), whereas no significant difference was seen between the two groups in BALB/c mice (Table 1). Anti-type II collagen antibody titers were increased by the treatment with zymosan A in SKG mice, but not statistically significant (data not shown).

The role of cytokines and the proliferation of T cells in the development phase and the progression phase of arthritis

Splenocytes isolated 1 week (the development phase) or 8 weeks (the progression phase) after the injection of saline or zymosan A were stimulated with concanavalin A for 48 h. IL-4 production in the culture supernatants of splenocytes from the saline-treated SKG mice was significantly higher

Table 2. Concanavalin A-stimulated IL-2 production and proliferation of splenocytes 1 week after the zymosan A injection

Strain	Treatment	IL-2 (pg/mL) [§]		Proliferation [&]	
BALB/c	Saline	8540 ± 2120	**	215888 ± 4871	###
BALB/c	Zymosan A	90258 ± 15342	#	235780 ± 7784	
SKG	Saline	343 ± 33		42360 ± 13990	
SKG	Zymosan A	399 ± 125		28929 ± 7186	

[§] Splenocytes were isolated 1 week after the injection of saline or zymosan A. Splenocytes were cultured in the presence of concanavalin A (2 μg/mL) for 48 h. IL-2 concentration in the supernatants was measured by ELISA. [&]Proliferation of splenocytes was measured by culturing in the presence of 2 μg/mL of concanavalin A for 72 h. The incorporation of [^{3}H] thymidine was counted. Results are shown as the mean ±SE. #, ###: $P < 0.05$ and 0.001 between the two strains. **: $P < 0.01$ between saline- and zymosan A-treated BALB/c mice.

Table 3. Spontaneous IL-12 production from splenocytes

Strain	Treatment	IL-12 (pg/mL) 1 week [§]		IL-12 (pg/mL) 2 week [§]
BALB/c	Saline	1855 ± 110	**	1635 ± 81
BALB/c	Zymosan A	3136 ± 318		1924 ± 92
SKG	Saline	1624 ± 111		1742 ± 78
SKG	Zymosan A	2001 ± 116		1920 ± 259

[§] Splenocytes were isolated 1 or 2 week after the injection of saline or zymosan A. Splenocytes were cultured for 48 h. Spontaneous IL-12 (p70) production was measured. Results are shown as the mean ±SE. **: $P < 0.01$ between saline- and zymosan A-treated BALB/c mice.

than that from the saline-treated BALB/c mice in both phases ($p < 0.01$ at 1 week, $p < 0.001$ at 8 weeks, Figs. 4A, C). An injection of zymosan A markedly reduced the IL-4 production in SKG mice in both phases ($P < 0.01$), although it did not affect the IL-4 production in BALB/c mice. IFN-γ production in the saline-treated SKG mice was significantly lower than that of the saline-treated BALB/c mice in both phases ($p < 0.01$ at 1 week, $P < 0.001$ at 8 weeks, Figure 4B, D). The treatment with zymosan A did not alter the production of IFN-γ in both mice in the development phase, but increased it in the progression phase ($p < 0.05$ in BALB/c mice, $p < 0.001$ in SKG mice). A larger amount of IL-2 was produced in the saline-treated BALB/c mice than that in the saline-treated SKG mice ($p < 0.05$, Table 2). The concanavalin A-stimulated IL-2 production was markedly potentiated by the treatment with zymosan A in BALB/c mice ($P < 0.01$), whereas the induction was not observed in SKG mice by the treatment with zymosan A (Table 2). The proliferation of concanavalin A-stimulated splenocytes was also depressed in SKG mice ($P < 0.001$). It was not affected by the treatment with zymosan A in both mice (Table 2).

To evaluate the activation of innate immunity, spontaneous IL-12 production from splenocytes was measured (Table 3). An injection of zymosan A increased a spontaneous production of IL-12 from splenocytes in BALB/c mice when cells were obtained 1 week after the treatment ($P < 0.01$). The increase in the IL-12 production disappeared 2 weeks after the treatment. In contrast, zymosan A did not affect the spontaneous IL-12 production from splenocytes in SKG mice.

Discussion

A single intraperitoneal injection of the small amount of zymosan A, a crude fungal β-glucan, induced the development of severe polyarthritis in SKG mice under the SPF condition. It was reported that an intraperitoneal injection of high dose of zymosan A (25 mg/mouse) induced an increase in the serum TNF-α concentration and ultimately led to the multi organ failures and death within a week in C57BL/6 mice [10]. Several SKG mice treated with high dose of zymosan A (10 mg/mouse) died in our experiments. No correlation was found between the severity of arthritis and death by the treatment with zymosan A. A low dose of zymosan A (2 mg/mouse) effectively induced severe arthritis in all mice. Concentrations of IL-1β and IL-6 in the serum were higher in the group treated with 2 mg of zymosan A than those with higher dose of zymosan A. These data suggested that IL-1β and IL-6 might be important for the development of zymosan A-induced arthritis in SKG mice. The reason was not clear, but TNF-α was not detected in the serum. However, TNF-α has been reported to play a pivotal role in the pathogenic mechanisms of RA (11).

The phenotype of arthritis in SKG mice looks similar to that in human RA. SKG mice are suffered from a pannus formation followed by lymphocytes infiltrations into the articular joints, destructions of bone and cartilage, bilateral development of arthritis in each paw, chronic inflammation leading to irreversible stiffness of joints, involvements of rheumatoid factor, serum amyloid P and hyper-γ-globulinemia. Because of these similarities, SKG mice can be considered as a disease model of RA. The arthritis induced by zymosan A in SKG mice under the SPF condition was also quite similar to that spontaneously developed in the conventional condition, especially bilateral development, histology of the swollen joints and serological characteristics such as the production of IgGs and rheumatoid factor [1]. The onset of zymosan A-induced arthritis was relatively earlier than that of spontaneously developed arthritis. All of SKG mice developed arthritis within 6 weeks after the zymosan A injection under the SPF condition, compared to 6 months under the conventional condition without any treatments [1]. These results suggest that this arthritis model is useful to clarify the onset of human RA in terms of the similarity with RA and the shortness of evaluation period.

Splenocytes of SKG mice showed a Th2 phenotype, different from those of BALB/c mice. Concanavalin A ligates the T cell receptors, which could mimic the ligation of TCR with antigen [12]. It has been reported that an association of TCR-ζ and ZAP-70 in Th2 is weaker than that in Th1 cells [13, 14], which means the mutation in ZAP-70 could affect on Th1 cells more strongly than Th2 cells. Actually, pharmacological inhibition of the association of ZAP-70 with TCR-ζ shifted TCR-mediated response to the Th2 skewing [15]. Theses reports support the evidence that SKG mice are originally Th2 skewing mice. The decrease in IL-4 production by the zymosan A injection was also supported by the

report that a Toll-like receptor 2 (TLR2) ligand suppressed the differentiation of T cells into Th2 cells and consequently inhibits IL-4 production [16]. As zymosan A is the one of the TLR2 ligands, it might inhibit the differentiation of T cells into Th2 cells and allow autoreactive Th1 cells to activate. Because IL-4 has been reported to inhibit various models of arthritis [17, 18], a decrease in IL-4 production may trigger the development of arthritis in SKG mice. Zymosan A upregulated the IFN-γ production only in the progressional phase of arthritis in SKG mice. An increase in the IFN-γ production was also observed in BALB/c mice, which did not develop arthritis. However, upregulation of IFN-γ production from splenocytes has also been reported in some chronic arthritic models including collagen-induced arthritis [19, 20]. Therefore, we do not think that IFN-γ is involved in the development of arthritis but, we think that IFN-γ should be partially involved in the progression and continuation of chronic arthritis.

The exact mechanism how zymosan A suppressed the IL-4 production is still unclear. Zymosan A can activate innate immune cells including macrophages, monocytes, and neutrophils, resulting in the stimulated secretion of inflammatory products including TNF-α, IL-1ɣ IL-12, hydrogen peroxide, and arachidonic acid which are important factors in the pathogenesis of arthritis. Therefore, zymosan A may trigger/exacerbate arthritis via direct activation of innate immunity. Zymosan A has been reported to stimulate IL-12 release from dendritic cells in spleen [21]. We observed an increase in spontaneous IL-12 production from splenocytes, which is considered to reflect the activation of innate immunity *iin vivo*. However, IL-12 production was not induced in SKG mice. This result suggests that innate immunity may not be involved in the development of arthritis in SKG mice.

In conclusion, we characterized the zymosan A-induced chronic polyarthritis in SKG mice. This model is useful to elucidate the mechanism of the onset of RA. The long lasting suppression of IL-4 and the upregulation of IFN-γ production may play an important role for the incidence and the maintenance of chronic arthritis. Although there are some differences between SKG mice and human RA, this is a very interesting model for analyzing the mechanism of rheumatoid arthritis.

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