VOL. 56 NO. 2, FEB. 2003 THE JOURNAL OF ANTIBIOTICS pp. 62 — 67

FR252921, a Novel Immunosuppressive Agent Isolated from *Pseudomonas fluorescens* No. 408813

II. *In Vitro* Property and Mode of Action

KIYOTAKA FUJINE*, FUMIE ABE, NOBUO SEKI, HIROTSUGU UEDA, MOTOHIRO HINO and TAKASHI FUJII

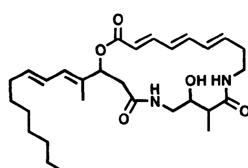
Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.,
5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan

(Received for publication October 18, 2002)

A novel immunosuppressive agent, FR252921 was isolated from the cultured broth of a species of *Pseudomonas fluorescens*. We have shown that FR252921 inhibited splenic proliferation stimulated with LPS, insensitive to calcinuerin inhibitor. In this study, FR252921 was found to inhibit IL-2 and IL-12 production as well as proliferaion of splenocyte. Analysis of transcription activity revealed that FR252921 inhibited activating protein-1 (AP-1). Exposures of antigen presenting cells (APC) to FR252921 attenuated proliferation supplemented by naïve T cells. Further, FR252921 strongly suppressed splenic dendritic cell proliferation stimulated with LPS and anti-CD40 mAb, while it did not inhibit purified T cell activation, including CD154 expression and IL-2 production. These results suggest that APC is dominant target cell population.

Discovery of cyclosporin A (CsA) and FK506, as metabolites from natural microorganisms has enabled to arrest acute rejection after transplantation in high success rate[1~3]. However, we have some problems to be resolved, including chronic rejection and side effect. To establish more effective therapy for rejection control, we need a novel immunosuppressant that has different target from calcinuerin (CN), target phosphatase of CsA and FK506. As described in preceding paper, we explored a new agent whose target is different from that of CN inhibitor using CN inhibitor-insensitive screening system[4]. And we found that a novel compound, FR252921 (**1**, Fig. 1) isolated from the cultured broth of *Pseudomonas fluorescens*, inhibited both FK506-sensitive (stimulated by anti-CD3 mAb) and FK506-insensitive (stimulated by lipopolysaccharide (LPS)) splenocyte proliferations.

Fig. 1. Structure of FR252921.



FR252921 (**1**)

In this paper, to obtain more information about mode of action of **1**, we examined the effects on T cell and APC activation including cell proliferation, cytokine production and expression of activation marker of lymphocytes. In reporter gene assay, we investigated whether **1** inhibited particular transcription factor leading to cytokine gene expression.

Materials and Methods

Drugs

Compound **1** and FK506 were prepared in our Research Laboratories. Structure of **1** is shown in Fig. 1. When we performed *in vitro* test, **1** and FK506 were dissolved in acetonitrile and further diluted in medium and added to the culture.

Reagents and Antibodies

Mouse IL-2 and IL-12 ELISA kits were purchased from BioSource International, Inc. (Camarillo, CA). Hamster

* Corresponding author: kiyotaka-fujine@po.fujisawa.co.jp

anti-mouse CD40 mAb, FITC-conjugated rat anti-mouse CD4 mAb, PE-conjugated hamster anti-mouse CD154 mAb, mouse anti-human CD3 mAb and mouse anti-human CD28 mAb were purchased from BD PharMingen (San Diego, CA). Microbeads conjugated rat anti-mouse MHC Class II (I-A^b) mAb and microbeads conjugated hamster anti-mouse CD11c mAb were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Cell Preparation, Cell Proliferation Assay and Cytokine Production Assay

T cells were concentrated from splenocytes of female C57BL/6 mice (Charles River Japan) with nylon wool column. Antigen presenting cells (APCs) containing B cells, macrophages and dendritic cells (DCs) were obtained from splenocytes with magnetic activated cell sorting (MACS) technique using microbeads labeled anti-MHC class II mAb. DCs were purified from splenocytes with MACS technique using microbeads labeled anti-CD11c mAb.

Cells were cultured in 0.1 ml RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Moregate, Bulimba, Australia), 50 mM 2-mercaptoethanol (Nakarai Chemical, Kyoto, Japan), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Rockville, MD) at 37°C in a humidified atmosphere of 5% CO_2. Splenocytes (1×10^5 cells/well) were stimulated by soluble anti-mouse CD3 mAb (1 μg/ml) in 96-well U-bottomed microtiter plates. The amount of IL-2 and IL-12 in supernatant at 24 hours after initiating culture was quantitated using ELISA kit. Cell proliferation was quantitated by MTT (3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay. Following incubation for 3 days, 10 μg of MTT was added to each well and cells were incubated for another 4 hours. Reduced MTT in lymphocytes was extracted by 2-propanol and absorbance at 550 nm was measured.

T cells (5×10^4 cells/well) were stimulated with anti-CD3 mAb immobilized to solid phase in 96-well flat-bottomed microtiter plates, as APC independent T cell proliferation. After 12-hours incubation, CD154 expression on cell surface was detected with flowcytometry using FITC labeled anti-CD4 mAb and PE labeled anti-CD154 mAb. The amount of IL-2 in supernatant at 24 hours after initiating culture was quantitated using ELISA kit. Following incubation for 2 days, Cell proliferation was quantitated by MTT assay.

$CD11c^+$ splenocytes were stimulated with LPS and anti-CD40 mAb and cell proliferation was quantitated by MTT assay.

To elucidate whether **1** would suppress dominantly either T cell or APC, washing out drug and cell reconstitution was performed as follows. Briefly, T cells, APCs or mixture of T cells and APCs were incubated in the presence or absence of drugs at 37°C for 1 hour. To each of them, APCs, T cells and medium were added, following washing cells with medium. After 3-day incubation, cell proliferation was quantitated by MTT assay.

AP-1, Nuclear Factor of Activated T Cells (NF-AT) and Nuclear Factor-kappa B (NF-κB) Activation Assay

Human Mv1Lu cells were transiently co-transfected by electroporation with pAP-1-Luc (Stratagene, La Jolla, CA) and phRL-TK (Promega, San Luis Obispo, CA), plasmids containing AP-1 binding site fused to firefly luciferase and containing thymidine kinase promoter fused to renilla luciferase as internal control, respectively. Cells were resuspended in fresh EMEM (Sigma) medium supplemented with 10% fetal bovine serum (Moregate), MEM non-essential amino acids (Invitrogen), MEM sodium pyrvate (Invitrogen), and plated on serial dilution of **1** in the presence or the absence of 10 ng/ml of TGF-β1 in 96-well U-bottomed plates (100 μl per well).

Similarly, Human Jurkat T cells were taransiently co-transfected by electroporation with phRL-TK and pNF-AT-TA-Luc (BD Biosciences Clontech, Palo Alto, CA), plasmids containing NF-kB binding site fused to a luciferase. Cells were cultured in serial dilution of **1** in the presence and the absence of anti-human CD3 mAb (1 μg/ml) and anti-human CD28 mAb (1 μg/ml).

Human endotherial cell line, HEC293 were taransiently co-transfected by lipofectamine (Life Technologies, Taby, Sweden) with phRL-TK and pNF-κB-Luc (BD Biosciences Clontech), plasmids containing NF-kB binding site fused to a luciferase. Cells were cultured in serial dilution of **1** in the presence and the absence of human TNF-α (5 ng/ml).

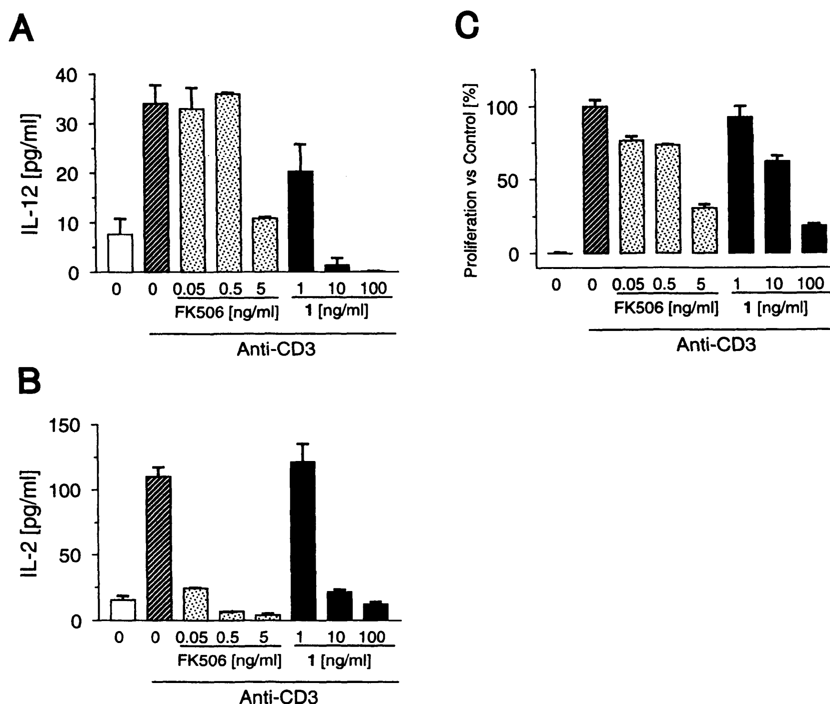
The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 18 hours. After removal of supernatant, cell lysis buffer was added to each well, and cells were lysed for 30 minutes. Luminescence was immediately measured using Wallac 1420 ARVOsx multi label counter (EG&G Berthold, Wallac Sverige, Upplands Vasby, Sweden).

Results

Compound 1 Inhibits IL-2 and IL-12 Production by Splenocyte Stimulated by Soluble Anti-CD3 mAb

We have previously shown that a novel compound, **1**,

Fig. 2. Inhibitory effects of **1** against IL-12 (A) and IL-2 (B) production and ploliferation (C) by splenocytes stimulated with soluble anti-CD3 antibody.



isolated from the cultured broth of *Pseudomonas fluorescens*, inhibited splenocytes proliferation stimulated with anti-CD3 mAb. Then we determined whether **1** inhibited cytokine produced by splenocytes. Splenocytes were stimulated with soluble anti-CD3 mAb for 24 hours in the presence of **1** or FK506, and the amount of IL-2 and IL-12 in supernatant of culture were measured by ELISA. Compound **1** markedly decreased IL-2 and IL-12 production. As shown in Fig. 2, 10 ng/ml of **1** strikingly inhibited IL-2 and IL-12 production in spite of insufficient inhibition of cell proliferation. In contrast, FK506 specifically inhibited IL-2 production comparing to IL-12 production. These results suggest that **1** potentially suppress both T cell and APC activation.

Compound 1 Dominantly Acts against Not T Cell but APC

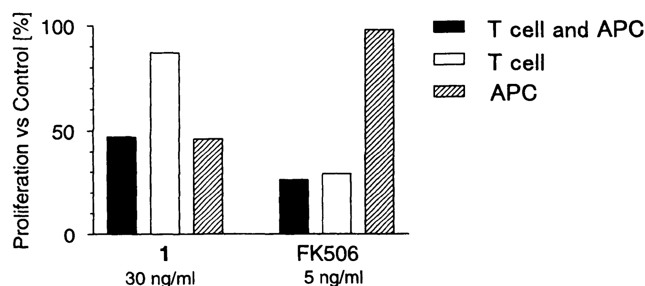
To determine whether **1** acts against T cell or APC, drug exposure to the cells and washing out and cell reconstitution were carried out. First, T cells were obtained with nylon wool column and APCs (expressing MHC class II) were obtained using MACS technique from splenocytes. Compound **1** was exposed to T cell or APC for 1 hour and washed out. Each cell fraction exposed with **1** was reconstituted with APC or T cell respectively and then incubated in the presence of soluble anti-CD3 mAb for 3 days. Treatment of APCs with **1** inhibited proliferation of reconstituted cell as same as treatment of mixture of APCs and T cells, but exposure to T cells showed no inhibition (Fig. 3). In the case of FK506, treatment of T cells suppressed proliferation of reconstituted cell. It is suggested that dominant effect of **1** against APC lead to inhibition of splenocyte activation initiated by T cell receptor triggering.

Compound 1 Inhibits Not Purified T Cell Activation but Purified Dendritic Cell

We subsequently analyzed the effect of FK506 or **1** on purified T-cell activation in response to immobilized anti-CD3 mAb stimuli. As shown in Fig. 4, FK506, T-cell specific inhibitor attenuated cell proliferation, IL-2 production and CD154 expression on the cell surface. In contrast, all of those events was not affected by 100 ng/ml **1** at which IL-2 and IL-12 production by splenocyte was completely inhibited (Figs. 2, 4). Therefore, **1** showed no

effect on activation of purified T cells, in spite of their inhibitory effect on splenocyte activation. These results suggest that **1** exerts their action depending on the presence of splenic non-T cell or **1** acts directly against APC. Indeed, **1** inhibited proliferation of CD11c$^+$ DCs stimulated with LPS and anti-CD40 mAb in dose-dependent manner, whereas FK506 did not (Fig. 5). Thus, we confirm that **1** dominantly act against not T cell but APC.

Fig. 3. The effect of **1** on proliferation of reconstituted T cell and APC stimulated with soluble anti-CD3 mAb.



T cell, APC or mixture of T cell and APC were exposed to drugs for 1 hour and washed. Each T cell or APC was reconstituted with naïve APC or naïve T cell and then was stimulated with soluble anti-CD3 mAb.

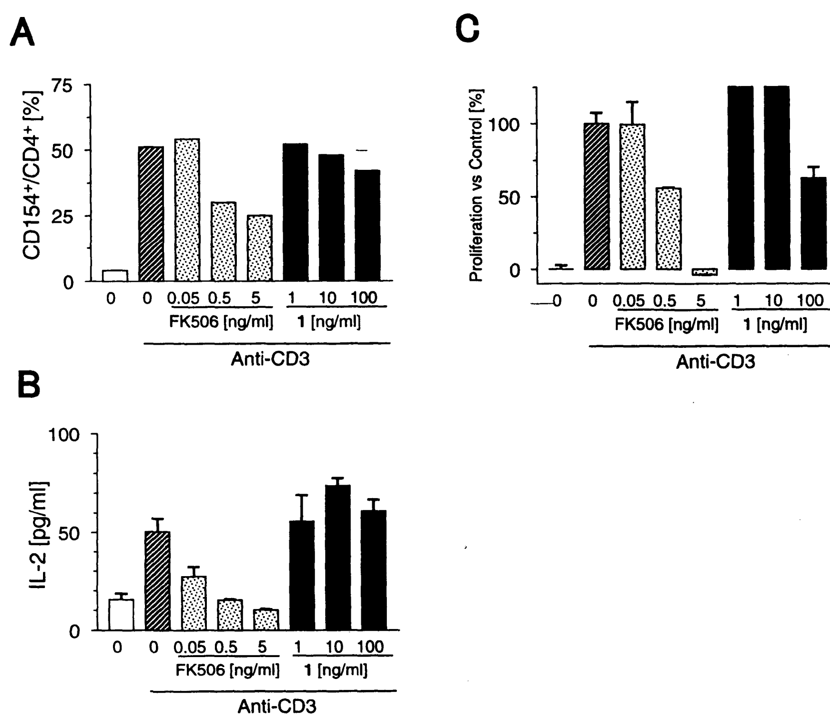
Compound 1 Inhibits AP-1 Promoter Activity in Reporter Gene Assay

As described above, **1** inhibited IL-2 and IL-12 production by splenocyte. It has been reported that binding of transcription factor, such as nuclear factor of activated T cells (NF-AT), activating protein-1 (AP-1) and nuclear factor kappa B (NF-κB) regulates cytokine gene expression. To investigate whether **1** inhibits promoter activity of transcription factor, reporter gene assay was performed. As described in Material and Method, reporter genes constructed by ligation of NF-AT, AP-1 or NF-κB binding site to luciferase were transformed into Mv1LU, Jurkat or HEC293, respectively. Transformant was cultured in the presence or absence of **1**. As shown in Fig. 6, **1** inhibited luciferase reporter gene expression driven by AP-1, whereas neither by NF-AT nor NF-κB.

Discussion

As reported in preceding paper, **1** was found *in vitro* screening system insensitive to FK506. Compound **1**

Fig. 4. The inability of **1** to inhibit CD154 expression (A), IL-2 production (B) and proliferation (C) by T cells stimulated with immobilized anti-CD3 antibody.

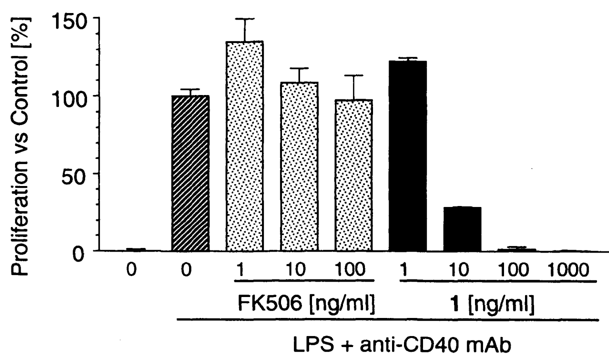


inhibited not only splenic proliferation stimulated by anti-CD3 mAb but also those stimulated LPS *in vitro*[4]. These results gave us interest for investigation of mode of action of **1**.

As shown in Fig. 2, **1** inhibited both IL-2 and IL-12 production by splenocyte. IL-2 and IL-12 are cytokine produced by T cell and APC, respectively. So, we speculated **1** targeted both T cell and APC in contrast to specific T cell inhibitor, FK506. Indeed, inhibition of IL-12 production was not observed with FK506.

However, drug exposure and cell reconstitution revealed that **1** had less inhibitory effect on T cell. Pretreatment of T cell with FK506 reduced proliferation with complementation of APC. Conversely, APC pretreated with **1** reduced proliferation with complementation of T cell (Fig. 3). In addition, **1** inhibited neither IL-2 production nor proliferation by purified T cell stimulated with immobilized anti-CD3 mAb, which is APC independent event (Fig. 4). APC-oriented effect of **1** was confirmed by analysis of proliferation of DC (Fig. 5). These results confirmed that **1** acted dominantly against not T cell but APC. Based on these data, we speculate that **1** directly act against APC and that APC exposed by **1** is not able to transduce sufficient activating signal to T cell, which results in failure to produce IL-2 in the system with intact splenocyte.

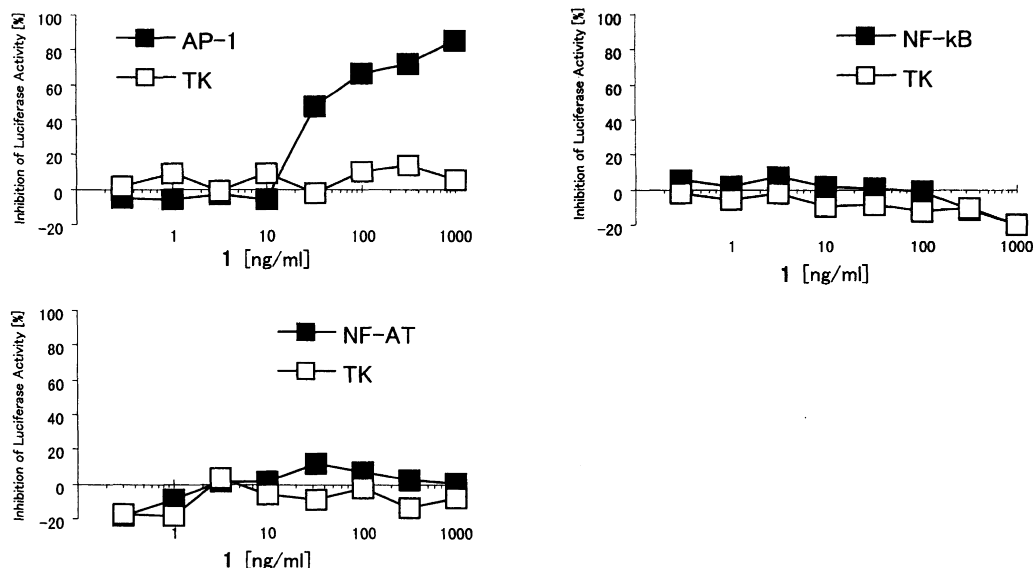
Fig. 5. Inhibitory effects of **1** against dendritic cells proliferation stimulated with LPS and anti-CD40 mAb.



Transcription factors, NF-AT, NF-κB and AP-1 regulate various gene expressions associated with lymphocyte activation[5~8]. FK506 is known to inhibit CN activity by formation of complex with FK binding protein (FKBP) and prevent from transport of NF-AT into nucleus, leading to inability to produce IL-2[9,10]. Interestingly, **1** reduced AP-1 transcription activity in spite of null effect on NF-AT and NF-κB in reporter gene assay (Fig. 6). This AP-1 inhibition of **1** is consistent with its inhibition of IL-12 production. IL-12 is a heterodimeric cytokine composed of p35 and p40, produced by macrophage and DC. Binding of AP-1 to DNA is critical for IL-12 p40 promoter activation[11].

It has also been reported that LPS-stimulated and CD40-mediated signals induce AP-1 transcriptional activity in B cell, macrophage and DC[12~15]. Therefore, it is reasonable to inhibit activation of DC by AP-1 inhibitor, **1** (Fig. 4). As described in preceding paper, **1** was discovered during course of screening program using LPS-stimulated splenocyte proliferation[4]. This activation is insensitive to

Fig. 6. Compounnd **1** inhibited AP-1 signal in luciferase reporter gene assay.



calcinuerin inhibitor. This screening system may be appropriate for search for AP-1 inhibitor.

NF-AT and AP-1 complex regulate IL-2 as well as IL-12 gene expression[16,17]. In our experiments, however, AP-1 inhibitor **1** did not reduce IL-2 production by purified T cell (Figs. 4 and 6). FK506 inhibited NF-AT transcription in reporter gene assay (data not shown) and attenuated IL-2 production by purified T cell (Fig. 4). This may implicate that AP-1 inhibition alone was sufficient for IL-12 inhibition, but insufficinet for IL-2 inhibition.

The present results provide new insight of mode of action of **1**, novel immunosuppressant. Although acute rejection mediated by T cell has been controlled by CN inhibitor in clinical, it is insufficient for suppression of subsequent immune response mediated by antibody produced by B cell[18]. It has also been reported that immature DC can cause immune tolerance[19]. In view of APC-oriented effect of **1**, this novel drug may contribute to establish new therapeutic method in transplantation. We will evaluate combinatorial effect with FK506 and **1** that have different target cells *in vitro* and *in vivo* in accompanying paper[20].

Acknowledgment

We wish to thank Dr. HIDEKAZU MIZUHARA for advice in the preparation of this manuscript, and Ms. MICHIKO USHIODA, Ms. CHIYOKO ISHIHARA and Ms. SATOKO FURUKAWA for assistance throughout this work.

References

1) RUEGGER, A.; M. KUHN, H. LICHTI, H. R. LOOSLI, R. HUGUENIN, C. QUIQUEREZ & A. VON WARTBURG: Cyclosporin A, a Peptide Metabolite from Trichoderma polysporum (Link ex Pers.) Rifai, with a remarkable immunosuppressive activity. Helv. Chim. Acta. 59: 1075~1092, 1976
2) KINO, T.; H. HATANAKA, S. MIYATA, N. INAMURA, M. NISHIYAMA, T. YAJIMA, T. GOTO, M. OKUHARA, M. KOHSAKA & H. AOKI: FK-506, a novel immunosuppressant isolated from a *Streptomyces.* II. Immunosuppressive effect of FK-506 *in vitro.* J. Antibiotics 40: 1256~1265, 1987
3) TAKEMOTO, S. K.: Maintenance immunosuppression. Clin. Transpl.: 223~236, 2001
4) FUJINE, K.; M. TANAKA, K. OHSUMI, M. HASHIMOTO, S. TAKASE, H. UEDA, M. HINO & T. FUJII: FR252921, a novel immunosuppressive agent isolated from *Pseudomonas fluorescens* No. 408813. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities of FR252921, FR25922 and FR256523. J. Antibiotics 56: 55~61, 2003
5) GRANELLI-PIPERNO, A. & P. NOLAN: Nuclear transcription factors that bind to elements of the IL-2 promoter. Induction requirements in primary human T cells. J. Immunol. 147: 2734~2739, 1991
6) ZHU, C.; K. GAGNIDZE, J. H. GEMBERLING & S. E. PLEVY: Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. J. Biol. Chem. 276: 18519~18528, 2001
7) NEWELL, C. L.; A. B. DEISSEROTH & G. LOPEZ-BERESTEIN: Interaction of nuclear proteins with an AP-1/CRE-like promoter sequence in the human TNF-alpha gene. J. Leukoc. Biol. 56: 27~35, 1994
8) OHLSSON, B. G.; M. C. ENGLUND, A. L. KARLSSON, E. KNUTSEN, C. ERIXON, H. SKRIBECK, Y. LIU, G. BONDJERS & O. WIKLUND: Oxidized low density lipoprotein inhibits lipopolysaccharide-induced binding of nuclear factor-kappaB to DNA and the subsequent expression of tumor necrosis factor-alpha and interleukin-1beta in macrophages. J. Clin. Invest. 98: 78~89, 1996
9) SCHREIBER, S. L.: Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 251: 283~287, 1991
10) CLIPSTONE, N. A. & G. R. CRABTREE: Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 357: 695~697, 1992
11) ZHU, C.; K. GAGNIDZE, J. H. GEMBERLING & S. E. PLEVY: Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. J. Biol. Chem. 276: 18519~18528, 2001
12) FUJIHARA, M.; M. MUROI, Y. MUROI, N. ITO & T. SUZUKI: Mechanism of lipopolysaccharide-triggered junB activation in a mouse macrophage-like cell line (J774). J. Biol. Chem. 268: 14898~14905, 1993
13) PHUCHAREON, J. & T. TOKUHISA: Deregulated c-Fos/AP-1 accelerates cell cycle progression of B lymphocytes stimulated with lipopolysaccharide. Immunobiology 193: 391~399, 1995
14) PHUCHAREON, J. & T. TOKUHISA: Deregulated c-Fos/AP-1 modulates expression of the cyclin and the cdk gene in splenic B cells stimulated with lipopolysaccharide. Cancer Lett. 92: 203~208, 1995
15) MANN, J.; F. OAKLEY, P. W. JOHNSON & D. A. MANN: CD40 induces interleukin-6 gene transcription in dendritic cells: regulation by TRAF2, AP-1, NF-kappa B, and CBF1. J. Biol. Chem. 277: 17125~17138, 2002
16) JAIN, J.; P. G. MCCAFFREY, Z. MINER, T. K. KERPPOLA, J. N. LAMBERT, G. L. VERDINE, T. CURRAN & A. RAO: The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature 365: 352~355, 1993
17) JAIN, J.; P. G. MCCAFFREY, V. E. VALGE-ARCHER & A. RAO: Nuclear factor of activated T cells contains Fos and Jun. Nature 356: 801~804, 1992
18) BAID, S.; S. L. SAIDMAN, N. TOLKOFF-RUBIN, W. W. WILLIAMS, F. L. DELMONICO, A. B. COSIMI & M. PASCUAL: Managing the highly sensitized transplant recipient and B cell tolerance. Curr. Opin. Immunol. 13: 577~581, 2001
19) SHORTMAN, K. & Y. J. LIU: Mouse and human dendritic cell subtypes. Nat. Rev. Immunol. 2: 151~161, 2002
20) FUJINE, K.; H. UEDA, M. HINO & T. FUJII: FR252921, a novel immunosuppressive agent isolated from *Pseudomonas fluorescens* No. 408813. III. *In vivo* activities. J. Antibiotics 56: 68~71, 2003