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# A new NF-κB inhibitor attenuates a $T_H1$ type immune response in a murine model

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**Abstract Nuclear factor kappa B (NF-κB) plays a wide variety of pathophysiological roles and modulation of its pathway can be a good novel drug target. Here, we found that our recently synthesized NF-κB inhibitor attenuated an ovalbumin-specific delayed-type hypersensitivity response in vivo and suppressed production of IL-12 by dendritic cells and $T_H1$ cytokines by T cells in vitro. These findings suggest that the activation of NF-κB mounts $T_H1$ type immune responses, and that this new NF-κB inhibitor has a therapeutic potential in this context.**


*Keywords:* Nuclear factor-kappa B; Delayed type hypersensitivity; Inhibitor; Dendritic cell; T cell; $T_H1$

## 1. Introduction

Nuclear factor kappa B (NF-κB) family of transcription factors exist as homodimers or heterodimers of five distinct proteins (p50, p52, p65, RelB, and cRel) [1]. NF-κB proteins are present in the latent cytoplasm bound to inhibitors of NF-κB (IκBs). After activation by inducers, IκB proteins are phosphorylated, ubiquitylated, and degraded by the proteasome. The degradation of IκB allows NF-κB proteins to translocate to the nucleus and to bind their cognate DNA binding sites, which regulates the transcription of a large number of genes, including antimicrobial peptides, cytokines, chemokines, and stress-responses proteins [2]. Several studies have shown that the activation of NF-κB plays roles in a number of inflammatory conditions such as asthma, rheumatoid arthritis, heart disease, and dermatitis [3,4]. Based on these findings, inhibition of NF-κB transcriptional activation may represent an attractive target in the development of novel anti-inflammatory drugs. Many pharmaceutical companies have tried to develop selective inhibitors of NF-κB, including targeting the DNA-binding activity of individual NF-κB proteins using decoy oligonucleotides [4–6]. Recently, synthetic decoy oligonucleotides containing the NF-κB *cis* element have prevented a certain number of diseases as myocardial infarction and rheumatoid arthritis [5,7,8]. However, there has been no satisfactory NF-κB inhibitory compound for in vivo use at present.

Antigen-specific immune responses are involved in the pathogenesis of a variety of diseases. To establish this response, the interaction between T cells and antigen presenting cells, as dendritic cells, is essential for clonal stimulation and differentiation from naïve $CD4^+$ cells into helper T cells. There are two different fates of helper T cells, $T_H1$ cells expressing IL-2 and IFN-κ involved in cellular immunity, and Th2 cells releasing IL-4, IL-5, IL-6, IL-10 and IL-13, mediating humoral immunity [9]. In this step, IL-12 produced by dendritic cells is dominant in directing the development of $T_H1$ cells [10], and it is reported that NF-κB functions in T cell activation, $T_H1$ differentiation, and dendritic cell maturation [3,4,11–14]. These in vitro studies suggest that NF-κB plays an important role in antigen-specific $T_H1$ type immune responses in vivo.

Here, we report that a newly synthesized NF-κB inhibitor functions specifically to NF-κB activity in vitro and suppressed antigen-specific delayed-type hypersensitivity (DTH) response as a $T_H1$ type immune response model.

## 2. Materials and methods

### 2.1. Materials

C57BL/6 female mice were used as mentioned otherwise and all animal experiments were approved by the Committee on Animal Research of Kyoto University. DSKB-2, a new NF-κB inhibitor, was synthesized in our laboratory [19]. For in vivo experiments, this compound was applied intraperitoneally (1 mg/kg) to mice twice a day until the end of experiments, or topically on the ear skin at a dose of 20 μl of 0.1 mg/ml acetone solution. DSKB-2 was applied to mice 5 min after ultraviolet (UV) irradiation or 1 h before ovalbumin (OVA) sensitization.

### 2.2. Cell preparation, culture, and luciferase assay

Cells were prepared from the spleen essentially as previously described [15]. Splenic $CD11c^+$dendritic cells and $CD4^+$ T cells were purified with beads-conjugated antibodies to CD11c and CD4 using an Auto MACS (Miltenyi Biotec, Gladbach, Germany). The purified cells were shown to be more than 80% positive for $CD11c^+$and 95% positive for $CD4^+$ cells confirmed by flow cytometry EPICS XL instrument (Beckman Coulter, Fullerton, CA). The cells were cultured in flat-bottomed 96-well plates at 37 °C as described [16]. For T cell proliferation, $CD4^+$ cells ($2 \times 10^5$/well) were activated in plates precoated

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*Abbreviations:* NF-κB, nuclear factor kappa B; UV, ultraviolet; DTH, delayed-type hypersensitivity; OVA, ovalbumin

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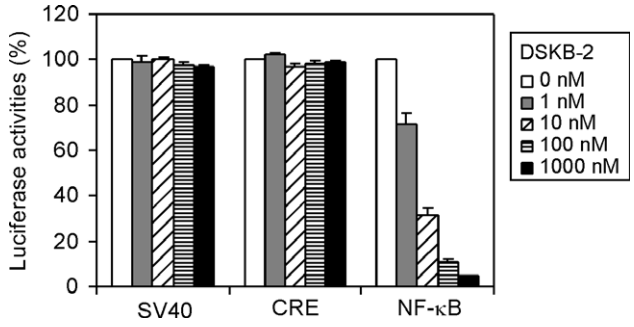


Fig. 1. Effect of DSKB2 on SV-40, CRE, and NF-κB. A549 cells transfected with constitutive SV40 driven luciferase gene exhibited the luciferase activity. And A549 cells transfected with CRE driven luciferase gene exhibited the luciferase activity in the presence of cAMP. Both of these activities were not affected by the exposure of DSKB2 at indicated doses. On the other hand, when A549 cells with NF-κB driven luciferase gene were stimulated with IL-1 for 4 h, DSKB2 inhibited the luciferase activity in a dose dependent manner. Data shown are representatives of three independent experiments, showing means ± S.E.M. in triplicated wells.

with the anti-CD3 antibody (10 μg/ml, PharMingen) for 72 h in triplicate. For mixed lymphocyte reaction, dendritic cells ($6 \times 10^4$/well) isolated from the spleen of BALB/c mice were irradiated and cultured for 72 h in triplicate with allogeneic $CD4^+$ cells ($2 \times 10^5$/well) from the spleen of C57BL/6 mice. The proliferation of $CD4^+$ cells was determined as incorporation of [$^3$H]thymidine for the last 16 h as described [16]. The amounts of IL-2, IFN-κ and IL-12 at 72 h of culture were measured by EIA kits (Biotrak) as per the manufacturer's protocols.

For luciferase assay, luciferase plasmid containing the NF-κB, constitutively active SV40, or cyclic AMP responsive element (CRE) consensus sequence was co-transfected with pSV2neo (Clontech, USA) into A549 cells using lipofectamine (Lifetech Oriental K.K., Tokyo), and selected by adding G418 sulfate (1 mg/ml, Lifetech Oriental K.K.).

### 2.3. Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

The nuclear extracts from the spleens of OVA-sensitized mice were isolated according to the method of Schreiber et al. [17] with a slight modification. Briefly, the spleens from four mice that received vehicle or NF-κB inhibitor for indicated period were minced and homogenized in cold buffer I (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) with a tissue homogenizer. The homogenates were allowed to swell on ice for 10 min, mixed with 10% NP-40, and vortexed for 10 s. The homogenates were centrifuged for 10 min. After removal of the supernatant, the nuclear pellets were resuspended in buffer II (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). The nuclear extracts were centrifuged and the supernatants were used. The DNA probe consisted of the NF-κB consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or AP-1 consensus sequence (5′-CGCTTGATGACTCAGCCGGAA-3′). The oligonucleotide 5′-labeled with rhodamine and unlabeled oligonucleotides were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). The nuclear extracts (5 μg/ml) were incubated with rhodamine-labeled NF-κB probe in the binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 30 mM KCl, and 0.2% (w/v) Tween 20) at room temperature for 15 min. The nuclear protein and oligonucleotides complexes were separated from free probes on native 5% polyacrylamide gel (BioRad, Richmond, CA) in 0.25× TBE (Tris–borate–EDTA) buffer. The gel was scanned using a fluorescent image analyzer (FMBIO II; Hitachi, Yokohama, Japan).

### 2.4. UV radiation

Mice were anesthetized and the dorsal ears of mice were exposed to a bank of fluorescent sunlamps (FL.20SE.30; Toshiba Medical Supply, Tokyo, Japan) with an emission spectrum from 270 to 375 nm, peaking at 305 nm from above at a dose of 3 kJ/m$^2$ [18]. The irradiance of UVB was measured by a radiometer (UVR-305/365D (ll); Toshiba Medical Supply).

### 2.5. DTH response

For an OVA-induced DTH, mice were sensitized by intraperitoneal injection of 100 μg of OVA (Sigma) in complete Freund's adjuvant. Seven days later, mice were injected subcutaneously with 30 μg of OVA in incomplete Freund's adjuvant into a hind footpad. The change in footpad thickness was measured 48 h after the challenge by subtracting the thickness of the contralateral footpad injected with incomplete Freund's adjuvant alone from that of the footpad injected with both OVA and adjuvant.

### 2.6. Statistics

Data are presented as means ± S.E.M. and were analyzed by Student's unpaired two-tailed $t$ test. A $P$ value of $<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Specificity of a new NF-κB inhibitor, DSKB-2, in vitro

DSKB-2, a new NF-κB inhibitor, is an indan derivative with inhibition of NF-κB transcriptional activation in A549 cells stimulated with TNF-α or IL-1β (TNF-β: $IC_{50} = 89$ nM; IL-1β; $IC_{50} = 51$ nM) and in Jurkat cells ($IC_{50} = 38$ nM, $TC_{50} = >10\,000$ nM) [19,20]. Initially, we assessed this compound's specificity to NF-κB in vitro. When A549 cells transfected with NF-κB driven luciferase gene were stimulated with IL-1 (1 ng/ml) for 4 h, DSKB-2 inhibited the luciferase activity in a dose dependent manner. On the other hand, when A549 cells transfected with constitutively active SV40 or CRE driven luciferase genes, which was independent of the activation of NF-κB, were stimulated with or without cAMP (1 mM), the luciferase activities were not affected by the exposure of DSKB-2 (Fig. 1). These data suggested that DSKB-2 inhibited the activation of NF-κB specifically in vitro.

### 3.2. In vivo impact of a new NF-κB inhibitor on UV-induced skin inflammation

Since the in vitro effect of NF-κB has been evaluated as above, we then evaluated the in vivo effect of this compound. It is known that a single dorsal exposure of mouse skin to UV radiation causes marked cutaneous inflammation and that the extent of inflammation can be examined by measuring the ear swelling responses. It was previously reported that ear swelling became detectable within 24 h after irradiation and progressed over the next several days, and this ear swelling was significantly reduced by blocking NF-κB gene transactivation with NF-κB decoy oligonucleotides [18]. Therefore, using UV-induced skin inflammation assay, we initially confirmed an in vivo effect of DSKB-2. Mice treated with intraperitoneal injections or skin painting of DSKB-2 showed significantly reduced ear swelling responses to UV irradiation at a dose of 3 kJ/m$^2$ at each time point tested after 72 h compared with control mice with PBS treatment (Fig. 2). These present results suggest that the DSKB-2 functions efficiently in vivo.

### 3.3. Impaired DTH response by the new NF-κB inhibitor

Using DTH as a $T_H1$ immune response model, we investigated the effect of NF-κB in an acquired immune response to an exogenous antigen. We sensitized mice with an intraperitoneal injection of OVA mixed with complete Freund's adjuvant. Seven days later, we challenged the mice with a subcutaneous injection of OVA mixed with incomplete Freund's adjuvant into a hind footpad. The extent of footpad swelling was significantly reduced by intraperitoneal injection

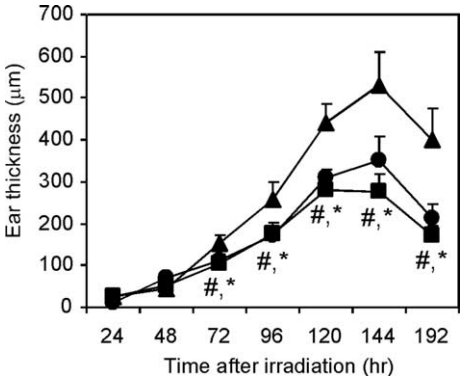


Fig. 2. Inhibition of UV-induced ear skin swelling by application of NF-κB inhibitor. C57BL/6 mice treated with an intraperitoneal injection of NF-κB inhibitor DSKB-2 (closed circles), PBS alone (closed triangles), or topical application of DSKB-2 (closed square). These mice were exposed to UV radiation at a dose of 3 kJ/m$^2$ and examined for ear swelling responses at the indicated time points. Data shown are representative of three independent experiments, showing means ± S.E.M. ($n=4$) of ear thickness change compared before irradiation. Statistically significant differences ($P<0.05$) compared with the UV plus PBS group (# vs. intraperitoneal injection, and *vs. topical application group).

with DSKB-2 (Fig. 3). Moreover, we extracted nuclear protein from the spleen at each indicated time after OVA sensitization and examined NF-κB activity using EMSA. This experiment showed that NF-κB activity is induced both one and five days after OVA sensitization and this induction is attenuated by the DSKB-2 (Fig. 4A). Since dendritic cell maturation occurs ~1 day after sensitization, and T cells proliferate and differentiate 3–6 days after sensitization, the above data suggest that NF-κB activity is induced at two different phases, early phase (day 1) during dendritic cell maturation, and later phase (day 5) during T cell activation and differentiation into $T_H1$. Moreover, we confirmed the specificity of NF-κB/DNA binding reaction using the nuclear extracts from the spleen of mice 5 days after OVA. The nuclear extracts were incubated with rhodamine labeled oligonucleotide containing a consensus NF-κB binding site in the presence or absence of a 100-fold molar excess of unlabeled oligonucleotide containing AP-1 and NF-

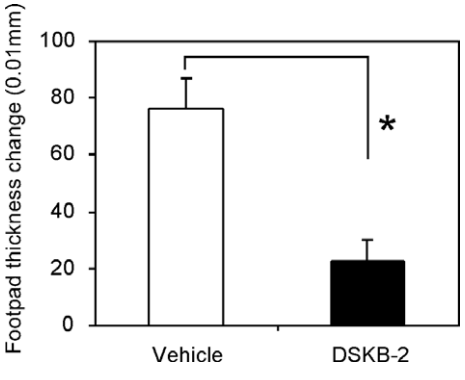


Fig. 3. Impaired OVA-specific DTH response by NF-κB inhibitor. Mice were sensitized by intraperitoneal injection of OVA and complete Freund's adjuvant. Footpad swelling induced by OVA challenge in sensitized mice ($n=6$ per group) was measured. Footpad swelling treated with DSKB-2 is significantly impaired compared to vehicle treated mice ($*P<0.05$).

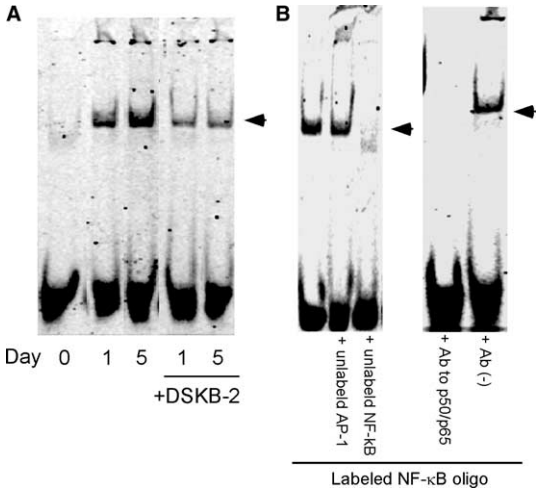


Fig. 4. Effect of a new NF-κB inhibitor on NF-κB activity in the spleen from OVA-sensitized mice. Mice were treated with or without DSKB-2 and immunized with OVA. Nuclear proteins were extracted from the spleens on day 0, 1, and 5 after immunization. The nuclear extracts were incubated with rhodamine-labeled NF-κB probe and its activity of each sample was analyzed by EMSA as indicated (A). Using the nuclear extracts from the spleen of mice 5 days after OVA immunization, the specificity of NF-κB/DNA binding reaction was examined in the presence or absence of a 100-fold molar excess of unlabeled oligonucleotide containing AP-1 and NF-κB (B, left panel) or antibodies to p50 and p65 (B, right panel). Arrowheads indicate the NF-κB band.

κB (Fig. 4B, left panel), where the NF-κB band is disappeared by unlabeled oligonucleotide containing NF-κB, but not by unlabeled oligonucleotide containing AP-1. Moreover, the NF-κB band is supershifted by the presence of antibodies to p50 and p65 which constitute NF-κB (Fig. 4B, right panel). These results clarified that the band observed does indeed represent binding of NF-κB.

### 3.4. NF-κB inhibitor attenuates dendritic cell activation and T cell proliferation in vitro

The above results suggested that NF-κB regulated dendritic cell and/or T cell function. To examine this issue, we first examined the effect of DSKB-2 in dendritic cell-independent T cell proliferation stimulated by anti-CD3 antibody and found that DSKB-2 attenuated T cell proliferation in a dose dependent manner (Fig. 5A). Consistently, DSKB-2 inhibited the production of $T_H1$ cytokines, IL-2 and IFN-γ (Fig. 5B and C). On the other hand, DSKB-2 did not suppress the $T_H2$ cytokine, IL-4, production in this assay (data not shown). Since the above T cell proliferation assay is independent of dendritic cell function, we then performed a mixed lymphocyte reaction assay where splenic C57BL/6 CD4$^+$ T cells were stimulated by irradiated allogeneic splenic BALB/c dendritic cells. This dendritic cell dependent CD4$^+$ T cell proliferation was significantly attenuated by DSKB-2 in a dose dependent manner (Fig. 6A). Moreover, the NF-κB inhibitor suppressed both the production of $T_H1$ cytokine (IL-2 and IFN-γ) by $T_H1$ cells, and the production of IL-12, which is dominant in directing the development of $T_H1$ cells and is produced by dendritic cells (Fig. 6B–D). Therefore, these above results suggest that NF-κB drives $T_H1$ type immune responses by acting not only on dendritic cells but also on T cells.

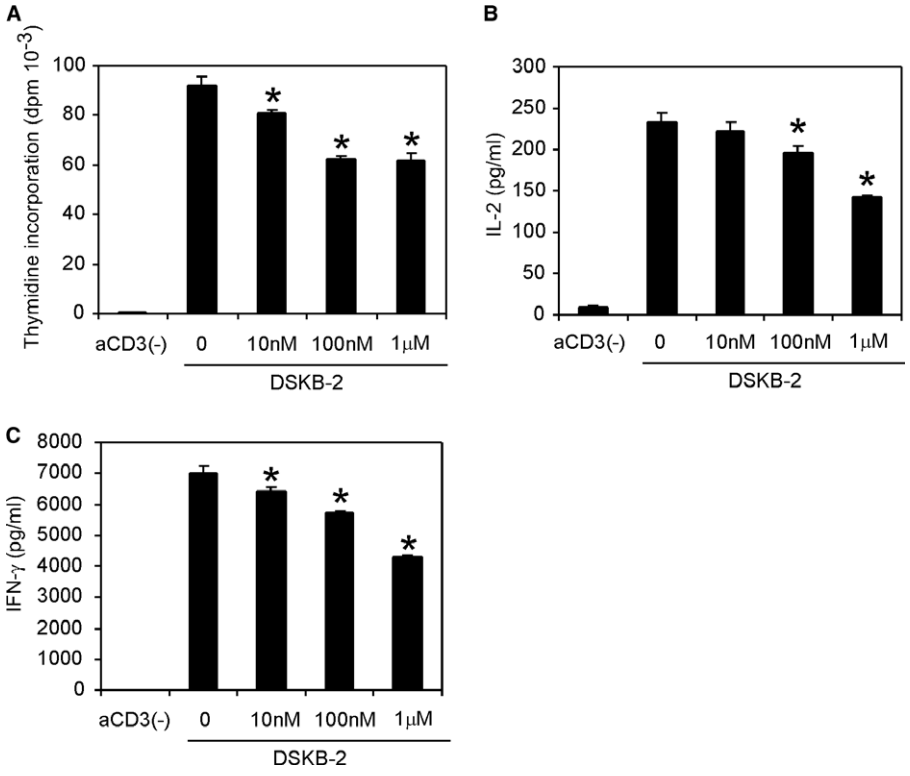


Fig. 5. Effect of NF-κB inhibitor on dendritic cell-independent T cell proliferation and cytokine production stimulated with anti-CD3 antibody. (A–C) $CD4^+$ cells purified from the spleen of C57BL/6 mice were stimulated by stabilized anti-CD3 antibody in the absence (0 nM) or presence of the NF-κB inhibitor (DSKB-2) for 72 h in triplicates. T cells without anti-CD3 stimulation are indicated as aCD3(−). The doses of DSKB-2 are as shown on the graph. T cell proliferation (A), the production of IL-2 (B), and IFN-γ (C) were determined. $*P < 0.05$ vs. vehicle treated (0 nM) group. Representative data from more than three independent experiments are shown.

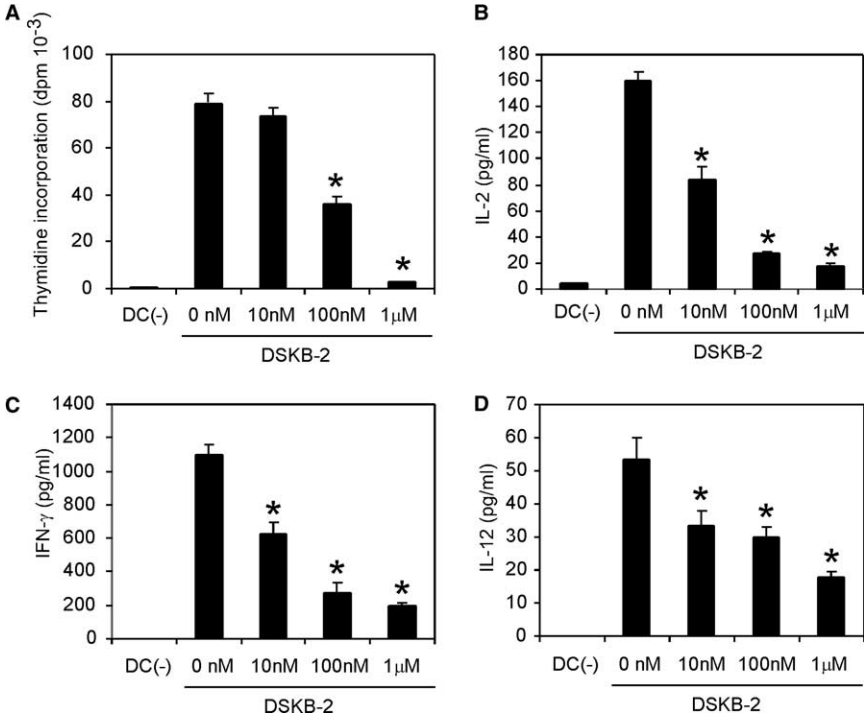


Fig. 6. Effect of NF-κB inhibitor on T cell proliferation and cytokine production stimulated with allogeneic dendritic cells. $CD4^+$ cells from the spleen of C57BL/6 mice were stimulated with or without (DC(−)) irradiated dendritic cells from the spleen of BALB/c mice in the presence or absence of the DSKB-2. T cell proliferation (A), production of IL-2 (B), IFN-γ (C), and IL-12 (D) were determined. $*P < 0.05$ vs. vehicle (0 nM) treated group. Representative data from more than three independent experiments are shown.

## 4. Discussion

In the present study, we showed that a newly synthesized NF-κB inhibitor specifically acted on NF-κB activity in vitro and attenuated antigen-specific $T_H1$ type DTH response in vivo. Consistently, this compound suppressed not only T cell proliferation and $T_H1$differentiation, but also dendritic cell function in vitro.

There are a number of in vitro studies about the role of NF-κB on dendritic cells and T cells. It has recently been reported that NF-κB blocked dendritic cells with adenovirus transferring IκBα and impaired the allogeneic immune reaction, which suggested that the antigen presentation by dendritic cells in vivo is NF-κB dependent [21]. However, the precise roles of NF-κB on antigen-specific acquired immune response in vivo have still not been well investigated. In our study, we first demonstrated that our new NF-κB inhibitor attenuated an antigen-specific acquired DTH response, and that NF-κB activity is induced not only during dendritic cells maturation phase, but also during T cells proliferation and differentiation phase, both of which were suppressed by the NF-κB inhibitor. Interestingly, this inhibitor attenuated both dendritic cell-independent and dependent T cell proliferation, but the extent of suppression by this compound is much more dramatic in a dendritic-dependent T cell proliferation assay. Moreover, we found that this compound suppressed both the production of $T_H1$ cytokines by $T_H1$ cells and the production of IL-12 produced by dendritic cells in vitro. These above results suggest that NF-κB pathway is essential to establish an antigen-specific acquired DTH response, by functioning both during dendritic cells maturation and T cell proliferation and differentiation phase.

Every effort has been made to develop selective inhibitors of NF-κB in vivo, however, no satisfactory NF-κB inhibitory compound for in vivo use has been developed yet. We demonstrated that our new NF-κB inhibitor is specific to NF-κB activity in vitro and suppressed DTH response in vivo in accordance with the local inhibition of NF-κB activity. Inflammatory response activates multiple transcription factors, including NF-κB, AP-1, and AP-2 [22,23]. They are by no means mutually exclusive; rather, they operate in an interdependent manner. Therefore, although we could show the compound specificity to NF-κB activity in vitro, it is difficult to show the in vivo specificity of this compound. However, the ear-swelling response induced by skin irritant chemical painting with phorbol myristate acetate or dinitrofluorobenzene was not affected by the compound (data not shown), which excluded the possibility that this compound had acted simply as a non-specific anti-inflammatory agent.

Since antigen-specific acquired immune responses are involved in a lot of clinical settings, this compound has potential implications for a wide variety of therapeutic uses, including allergy, autoimmunity, and transplantation. We showed that the new NF-κB inhibitor does not affect the $T_H2$ cytokine production in vitro, but it would be interesting to further examine the effect of this compound on $T_H2$ immune response in vivo. On the other hand, NF-κB is also involved in normal cellular physiology. The global inhibition of NF-κB could result in serious side effects as hepatotoxicity during embryonic development. In this respect, this new NF-κB inhibitor is efficient even by topical use in UV-induced skin inflammation. Therefore, this compound would be a good candidate for the control of local inflammation as contact hypersensitivity, psoriasis, and atopic dermatitis without serious systemic side effects.

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