Suppression of NF-κB and AP-1 activation in monocytic cells persistently infected with measles virus

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Available online 28 December 2006

Abstract

A major cause of the high morbidity and mortality associated with measles infection is attributed to virus-mediated immunosuppression. In this report, we present evidence for a novel strategy of immunosuppression by the measles virus. We observed a marked suppression of lipopolysaccharide (LPS)-induced IL-8, RANTES, TNF-α and IL-6 production and NF-κB activation in human monocytic cell lines persistently infected with measles virus. This effect was not observed in human epithelial cell lines persistently infected with measles virus. There were no significant differences in expression levels of Toll-like receptors (TLRs) and their associated molecules, or other intracellular signaling molecules of the NF-κB signaling pathway in measles-virus-infected monocytic cells compared to uninfected cells. Infected monocytic cells exhibited decreased LPS-induced DNA binding of NF-κB and phosphorylation of JNK, namely activation of transcription factors NF-κB and AP-1. NF-κB was constitutively activated in human epithelial cells persistently infected with measles virus, and LPS treatment resulted in further activation. The cell-type-specific suppression of NF-κB activation represents a potential strategy of escape from the host immune system by measles virus via induced immunological silencing in infected cells.

Keywords: Measles virus; NF-κB; AP-1; Immunosuppression; Monocyte; Toll-like receptor; Signal transduction

Introduction

Measles is a highly contagious disease characterized by a prodrome of fever, cough, coryza and conjunctivitis followed by Koplik’s spots and a generalized maculopapular rash. The complications of measles, such as alveobronchiolitis and otitis media, are generally believed to be caused by secondary infections (Griffin, 2001; Oxman, 2002). Immunosuppression by measles virus is considered to be a major cause of the high morbidity and mortality rates of acute measles and the resultant secondary complications. Encephalitides and alveobronchiolitis are the major causes of death from measles virus infection.

Natural infection with the measles virus (MeV) is initiated when the virus comes in contact with epithelial cells in the respiratory tract, oropharynx or conjunctivae. MeV grows rapidly in the epithelial cells of the respiratory tract, then translocates into regional lymph nodes, at which point viremia and the general symptoms of measles infection are manifest. MeV is carried throughout the entire body by infected blood cells such as monocytes and T-cells in the blood stream and lymphatic fluids (Griffin, 2001). MeV has acquired several mechanisms of immunosuppression, which enable the virus to escape immune surveillance and inhibit attempts by the host immune system to remove virus and virus-infected cells.

A number of putative molecular mechanisms of viral escape from host immune defenses have been reported. During MeV infection, lymphocytes are unresponsive to mitogens and undergo cell cycle arrest at the G0/G1 transition (Engelking et al., 1999; Naniche et al., 1999; Wang et al., 2003; Yanagi et al., 1992). We previously reported that growth arrest also occurred in epithelial cells infected with MeV due to upregulation of interferon regulatory factor 1 (IRF-1). However, this likely represents a host strategy of the defense system for suppressing viral propagation rather than a viral strategy for escape from host defense (Yokota et al., 2004). Viral H and F glycoproteins expressed on the surface of infected cells directly inhibit T-cell activation and proliferation through disruption of the Akt signaling pathway (Avota et al., 2001). Cell-free viral nucleoprotein
(NP) released from MeV-infected cells inhibits antibody production by B cells though engagement of their Fcγ II receptor (FcγRII) (Ravanel et al., 1997). MeV infection leads dendritic cells to undergo abnormal maturation (Hahn et al., 2004, 2005; Schneider-Schaulies et al., 2003; Servet-Delprat et al., 2003). MeV infection impairs CD40 signal transduction in T-cells and results in altered cytokine production and T-cell proliferation (Servet-Delprat et al., 2000). Karp et al. (1996) reported that MeV infection inhibits lipopolysaccharide (LPS)- and Staphylococcus aureus Cowan I cell-induced interleukin (IL)-12 production in human primary monocytes. MeV infection also alters production of interferons (IFNs) and IFN-mediated signal transduction. Naniche et al. (2000) reported that wild MeV strains suppress type I IFN production in human peripheral blood mononuclear cells (PBMC), while Vero cell-adapted MeV strains (laboratory strains) strongly induced type I IFN. Schlender et al. (2005) reported that MeV inhibit Toll-like receptor 7 (TLR7) and TLR9-mediated type I IFN production in plasmacytoid dendritic cells. MeV-mediated suppression of IFN signal transduction system is shown to be mediated in part by the viral accessory protein V (Palosaari et al., 2003; Takeuchi et al., 2003). We previously reported that IFN signaling is suppressed to a similar extent in cells infected with wild and laboratory strains of MeV (Yokota et al., 2003) (Okabayashi et al., submitted for publication).

In this study, we present evidence for a novel strategy of suppression of the host immune response by MeV, namely suppression of NF-κB and AP-1 activation in monocyctic cells. NF-κB is a transcriptional activator that plays a central role in the host immune system, in both innate and adaptive immunity, inflammation and cell proliferation and survival (Hoffmann and Baltimore, 2006; Karin and Greten, 2005). Activation of NF-κB results in upregulation of a large number of genes, such as those that encode proinflammatory cytokines, chemokines, IFNs, cytokine receptors, adhesion molecules, transcription factors, anti-apoptotic proteins and anti-microbial proteins. Thus, through downregulation of NF-κB, MeV-infected monocyctic cells may escape rejection by becoming immunologically silent.

Results

Suppression of proinflammatory cytokine and chemokine production in human monocyctic cell lines infected with MeV

We established several cell lines persistently infected with MeV strain Hälle according to previously described methods (Fujii et al., 1988, 1990). We assessed at least three independent persistently infected lines for each cell line used in the experiments of this study in order to exclude the possibility of clonal selection during infection. Persistently infected monocyctic cell lines (THP-1 and U937) and epithelial cell lines (SiHa and OSC70) produced constitutively infectious virus at a similar level [approximately 10^7 plaque forming unit (pfu/ml)].

THP-1 and U937 monocyctic cells persistently infected with MeV produced a similar amount of a chemokine IL-8 as uninfected cells (Fig. 1). LPS treatment upregulated IL-8 production in uninfected THP-1 and U937 cells following differentiation by treatment with active vitamin D3. In contrast, LPS-induced IL-8 production was suppressed in MeV-infected THP-1 and U937 cells following active vitamin D3 treatment (Figs. 1A and D). Suppression of IL-8 production was observed not only with LPS (a TLR4 agonist) but also when cells were stimulated with peptidoglycan (a TLR2 agonist) (Fig. 1B). On the other hand, U937 cells persistently infected with mumps virus constitutively produced a high level of IL-8 in the absence of active vitamin D3 treatment and LPS stimulation (Fig. 1C). SiHa and OSC70 epithelial cells persistently infected with MeV constitutively produced markedly elevated levels of IL-8 compared to uninfected cells. While MeV-persistently-infected SiHa and OSC70 cells highly produced IL-8, they showed still more IL-8 induction by LPS treatment (Figs. 1E and F).

We also examined production of other proinflammatory cytokines. Production of regulated upon activation normally T-cell expressed and secreted (RANTES), tumor necrosis factor-α (TNF-α) and IL-6 induced by LPS were also suppressed in MeV-infected monocyctic cell lines (Fig. 2). Induction of RANTES (Figs. 2A and D) and TNF-α (Fig. 2C) by LPS stimulation was suppressed in monocyctic cell lines persistently infected with MeV. U937 cells did not produce detectable amounts of TNF-α. IL-6 production of THP-1 and U937 was very low but significantly suppressed in MeV-infected cells compared to uninfected cells (Figs. 2B and E).

We performed acute MeV infection experiment. We used high infection dose at MOI 5 in the acute infection in order that cells were fully infected with virus during the early time points. During acute MeV infection, IL-8 production was induced in THP-1 and U937 cells, compared to cells that did not receive virus (Figs. 3 and 4A). The results suggested that suppression of MeV-infected IL-8 production in MeV-infected monocyctic cells correlated with the establishment of the stationary infection state, namely persistent infection. On the other hand, LPS-induced upregulation of IL-8 was suppressed during acute MeV infection as similar to MeV-persistently-infected cell lines. Similar results were obtained for other MeV strains such as Edmonston and a clinical isolate SMU (data not shown). In contrast, infection with mumps virus also induced IL-8 production in THP-1 and U937 during acute infection, and LPS-induced upregulation of IL-8 during acute mumps virus infection was similar to that seen in uninfected cells (Fig. 3A). We also performed acute infection experiment using human monocyte/macrophage fraction derived from PBMC as normal and primary cells. Human primary monocyte/macrophage strongly induced IL-8 by the stimulation with LPS. Whereas MeV (both strain Hälle and a wild strain AK1) infection weakly induced IL-8, LPS stimulation did not induce so high IL-8 production compared to uninfected cells (Fig. 5). Fig. 5 shows a representative result. Similar results were obtained by using monocyte/macrophage fraction donated from three different donors (data not shown). We also observed IL-6 and TNF-α induction by LPS was suppressed in the acutely infected primary monocyte/macrophage, as similar to IL-8 (data not shown).
Both MeV-induced and LPS-induced IL-8 upregulation was observed in the epithelial cell lines T24 and OSC70 during acute MeV infection (Figs. 4B and C). The viral mRNA levels were comparable among the cells tested, THP-1, U937, T24 and OSC70. These results suggested that cell-type-specific suppression of LPS-induced IL-8 production occurred as a result of both acute and persistent MeV infection in monocytic cells.

Effect of antiviral agent ribavirin on LPS-induced IL-8 production in monocytic cell lines persistently infected with MeV

To confirm that the suppression of LPS-induced IL-8 production was due to MeV infection, and not to selection of an unresponsive clone during isolation of the persistently infected cell lines, we examined elimination of virus in MeV-infected cells by ribavirin, an antiviral agent. Ribavirin is effective for MeV in vitro (Kirsit et al., 1983). The MeV-infected THP-1 cells produce infectious virus at approximately 10^3 pfu/ml. Ribavirin treatment reduced virus production under detectable level by the plaque formation assay. Treatment with ribavirin restored LPS-induced IL-8 production in THP-1 cells persistently infected with MeV to levels similar to uninfected THP-1 cells (Fig. 6). This result indicated that the suppression of LPS-induced IL-8 production was due to the infection by MeV.

Suppression of NF-κB activity in human monocytic cell lines persistently infected with MeV

Expression of IL-8 is mainly regulated at the levels of transcription by NF-κB (Mukaida et al., 1994). We examined NF-κB activity in LPS-treated and -untreated cells persistently infected with MeV using an enzyme-linked DNA/protein interaction assay (ELDIA) (Fig. 7). NF-κB activity was similar levels in both non-infected and MeV-infected THP-1 and U937 cells (Figs. 7A and B). NF-κB was constitutively activated in epithelial cells persistently infected with MeV (Fig. 7C). Moreover, further activation of NF-κB was induced by LPS treatment in the MeV-infected epithelial cells to levels similar to that seen in uninfected cells. Transcriptional activity of NF-κB was examined by luciferase reporter assay (Fig. 8). Luciferase reporter under control of NF-κB was upregulated by treatment with LPS in uninfected cells. In contrast, the basal levels of luciferase reporter was significantly lower than that in uninfected cells and the luciferase reporter was not upregulated.
even by LPS treatment in THP-1 cell lines persistently infected with MeV. These results indicated that NF-κB activation induced by both viral infection and LPS-stimulation is suppressed in monocytic cells persistently infected with MeV.

Expression of Toll-like receptors (TLRs) and other signal transduction molecules involved in NF-κB activation in monocytic cell lines persistently infected with MeV

We examined expression levels of TLRs, MD2 and CD14, which are components of the receptor complexes for microbial factors. TLR4, MD2 and CD14 are required for recognition of LPS and LPS-mediated signal transduction (Fitzgerald et al., 2004). There were no significant differences in the levels of all TLRs tested and MD2 mRNA in uninfected THP-1 and MeV-infected THP-1 cells (Fig. 9). The mRNA level of CD14 was significantly elevated by MeV infection. In contrast, the levels of TLR3, 7 and 8 and CD14 mRNA decreased in MeV-infected U937 cells. We also examined the cell surface expression of TLR2, TLR4 and CD14 in THP-1 (Fig. 10) and U937 (data not shown) by flow cytometry. The levels of cell surface expression well correlate with the mRNA levels. The differences in the response to MeV infection between U937 and THP-1 cells suggested that the changes in expression levels of these proteins following MeV infection were not related to the suppression of NF-κB in these cells.

We examined protein levels of intracellular signal transduction molecules involved in NF-κB pathway by Western blotting analysis (Fig. 11). The protein levels of NF-κB p50 subunit, MyD88, TRAF6, TAK1 and TAB2 were not significantly altered by persistent MeV infection in U937 and THP-1 cells. The levels of p65/RelA, c-Rel and IkB increased in THP-1 cells persistently infected with MeV, but we did not observe a similar increase in the levels of these proteins in U937 cells persistently infected with MeV. We were unable to detect IRAK in THP-1 cells, most
likely because it was below the level of detection by Western blotting. The results suggested that the suppression of NF-κB activation by MeV was not due to alternations in the expression levels of the signaling molecules involved in NF-κB activation.

**Suppression of JNK activation and AP-1 activity in human monocytic cell lines persistently infected with MeV**

We examined LPS-induced JNK phosphorylation in cells persistently infected with MeV. TLR- and IL-1-receptor-activated signaling pathways activate not only NF-κB, but also MAP kinase-related signaling molecules, such as JNK (Lee et al., 2000; Ninomiya-Tsuji et al., 1999). We found that LPS-induced JNK phosphorylation was suppressed in monocytic cell lines persistently infected with MeV (Fig. 12A). We also examined the activity of transcription factor AP-1, which is downstream of JNK, by luciferase reporter gene assay. Similar to NF-κB (Fig. 8), AP-1 was not activated by LPS treatment in THP-1 cells persistently infected with MeV, while it was dramatically activated by LPS in uninfected THP-1 cells (Fig. 12B). These results indicated that MeV inhibited both LPS-induced NF-κB and JNK activation. The mode of inhibition by MeV infection seems to involve a common signaling pathway for NF-κB and JNK.

**Discussion**

In this study, we found that activation of NF-κB and AP-1 was suppressed in MeV-infected monocytic cells. Generally, NF-κB is rapidly activated following viral infection. Helin et al.
proinflammatory cytokines, such as IL-8, RANTES, IL-6 and AP-1 suppression influenced on the production of various infection. The suppression of NF-κB and AP-1 was likely to require steady state of 

κB in the nuclear fraction were determined by ELDIA. The specific binding of NF-κB to immobilized oligo DNA was confirmed using mutated oligo DNA and inhibition of binding using soluble oligo DNA (data not shown). The data represent the mean±SD of triplicate wells in one experiment.

Karp et al. (1996) reported that human primary monocytes infected acutely with MeV show suppressed IL-12 and macrophage inflammatory protein-1β (MIP-1β) induction by LPS or S. aureus Cowan 1 cells. But induction of TNF-α, IL-6 and MIP1β was not significantly altered in the MeV-infected monocytes compared with uninfected cells. The reason of this contradictory result is still unknown.

During viral infection, components of the virus are recognized by pattern recognition molecules in host cells. Recognition activates signaling pathways that result in, among other things, activation of NF-κB. Pattern recognition molecules toward virus identified to date are TLRs and cytosolic proteins containing caspase-recruiting domain (CARD), such as retinoic acid inducible gene-1 (RIG-1) and melanoma differentiation-associated gene-5 (mda5) (Hiscott et al., 2006; Johnson and Gale, 2006). MeV was expected to be recognized by various pattern recognition molecules. Viral single-stranded RNA can be recognized by TLR7 and TLR8. Viral double strand RNA can be recognized by RIG-1, mda5 and TLR3 (Bowie and Haga, 2005; Hiscott et al., 2006; Johnson and Gale, 2006).

Furthermore, not only NF-κB and production of type I IFNs. In the current study, we found that MeV infection suppressed the activation of NF-κB in monocytic cells. MeV suppressed both virus-induced and LPS-induced NF-κB activation. We previously observed that MeV-infected U937 cells produced markedly higher levels of type I IFNs (Yokota et al., unpublished results). Thus, MeV specifically suppressed NF-κB activation and not IFN production in monocytic cells.

Yokota et al. (2001) reported that MeV activates NF-κB in the human lung epithelial cell line A549. Consistent with this, we observed that viral infection activated NF-κB in the epithelial cells lines SiHa, T24 and OSC70. In monocytic cell lines THP-1 and U937, suppression of LPS-induced NF-κB activation occurred during the early phase of acute infection of MeV, while suppression of virus-induced activation occurred during steady state infection, namely the persistent infection phase. The complete suppression of NF-κB and AP-1 was likely to require steady state of infection. The suppression of NF-κB activity suggested to be commonly possessed by MeV, including laboratory strains and wild strains. Mumps virus, which is also belonging to Paramyxoviridae, did not share such activity. The NF-κB and AP-1 suppression influenced on the production of various proinflammatory cytokines, such as IL-8, RANTES, IL-6 and TNF-α, induced by stimulants. Such suppression of proinflammatory cytokine induction was observed in persistently infected monocyctic cell lines and acutely infected primary monocytes/macrophages and monocytic cell lines. Karp et al. (1996) reported that human primary monocytes infected acutely with MeV show suppressed IL-12 and macrophage inflammatory protein-1β (MIP-1β) induction by LPS or S. aureus Cowan 1 cells. But induction of TNF-α, IL-6 and MIP1β was not significantly altered in the MeV-infected monocytes compared with uninfected cells. The reason of this contradictory result is still unknown.

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Fig. 7. Effect of MeV infection on LPS-induced NF-κB activation in monocytic and epithelial cell lines. (A) THP-1 (light gray bar) and MeV-persistently-infected THP-1 (dark gray bar) cells; (B) U937 and MeV-persistently-infected U937 cells; and (C) OSC70 and MeV-persistently-infected OSC70 cells. THP-1, MeV-infected THP-1, U937 and MeV-infected U937 cells were treated with active vitamin D3 for 16 h, then treated with 100 ng/ml E. coli LPS. OSC70 and MeV-infected OSC70 cells were treated with 100 ng/ml E. coli LPS. The cells were harvested at the indicated times, and nuclear fractions were prepared. The levels of activated NF-κB in the nuclear fraction were determined by ELDIA.

The specific binding of NF-κB to immobilized oligo DNA was confirmed using mutated oligo DNA and inhibition of binding using soluble oligo DNA (data not shown). The data represent the mean±SD of triplicate wells in one experiment.

Fig. 8. Effect of MeV infection on LPS-induced NF-κB activation determined by a reporter gene assay. THP-1 cells and THP-1 cells persistently infected with MeV were transfected with reporter plasmid containing luciferase reporter gene linked to tandem repeat of NF-κB binding motif (pNF-κB-Luc) and pRL-TK as an internal reference of transfection efficacy. The transiently expressed transfectants were subsequently incubated in normal condition for 24 h, and then with active vitamin D3 for 12 h. The cells were treated with E. coli LPS for 12 h, or not treated. The resulting cells were lysed, and the luciferase activities in the cell lysates were measured. All experiments were performed in triplicate. The results are expressed as fold induction (±standard deviation), which is relative to the value obtained from uninfected THP-1 cells without LPS treatment.
TAK1, which is the common route to NF-κB and JNK activation (Lee et al., 2000; Ninomiya-Tsuji et al., 1999). The detail molecular mechanism for suppression of these transcription factors is being investigated.

Numerous effects on host cells have been proposed as immunosuppressive strategies of MeV for evading the host immune system as described in the Introduction section. We propose that suppression of NF-κB activity represents another putative immunosuppressive strategy of MeV that is utilized specifically in monocytic cells, and not in epithelial cells, where NF-κB is strongly activated by MeV infection (Helin et al., 2001). For example, we showed that chemokine, such as IL-8 and RANTES, induction was suppressed in MeV-infected monocytic cells. The suppression of chemokines should impair leukocyte, especially neutrophil, activation and recruitment of the activated leukocytes to the foci of infection. Many viruses have various strategies for suppression of chemokine production and function (Glass et al., 2003; Mahalingam et al., 2001).

The determinants of the cell-type specificity of immunosuppression by MeV have yet to be elucidated. In MeV infection in humans, the primary target is the airway epithelium. MeV proliferates in airway epithelial cells and then goes on to infect secondary targets, such as dendritic cells, lymphocytes and monocytes. MeV spreads throughout the body by means of infected monocytes and lymphocytes via the blood and lymphatic fluids (Griffin, 2001; Oxman, 2002). Suppression of NF-κB activation, which means immunologically silent, in MeV-infected monocytic cells may be a strategy utilized by the virus to escape rejection and allow the spread of infection via the blood stream throughout the entire body.

**Materials and methods**

**Cells and viruses**

The human monocytic cell lines THP-1 and U937, the human cervical squamous carcinoma cell line SiHa and the human uroepithelial cell line T24 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human oral squamous carcinoma cell line OSC70 was previously described (Yonekura et al., 2003). All cells were routinely cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum. Human monocyte/macrophage fraction were prepared from PBMC derived from healthy adults as described previously (Yokota et al., 2006). MeV strains, Halle, Edmonston, SMU and AK1, were described previously (Yokota et al., 2004). Mumps virus strain Torii was used in this study. In acute infection experiments, virus was infected at a multiplicity of infection (MOI) of 5. THP-1, U937, SiHa, T24 and OSC70 cell lines persistently infected with MeV were established according to the method previously described (Fujii et al., 1988, 1990). Virus titers were determined using a plaque forming assay using Vero cells as the indicator cell.

**TLR agonist treatment**

THP-1 and U937 were pre-treated with $1 \times 10^{-8} \text{M 1α,25-dihydroxyvitamin D}_3$ (active vitamin D$_3$) for 16 h before LPS treatment. Suspension cells were inoculated at $5 \times 10^5$ cells/ml. Adherent cells were grown to more than 90% confluent. The cells were treated with 100 ng/ml (unless otherwise mentioned) LPS derived from *Escherichia coli* O111:B4 (Sigma-Aldrich, St. Louis, MO). In some cases, peptidoglycan was used instead of LPS. *S. aureus* peptidoglycan was purchased from Fluka (Steinheim, Switzerland).

**Ribavirin treatment**

Cells were treated with 30 μg/ml ribavirin (Sigma-Aldrich) for 8 days. Every 2 days, culture medium was changed. Viral
titers in the culture supernatant of ribavirin-treated cells were below the detection limit following ribavirin treatment. Treated cells were subsequently treated with active vitamin D₃ and LPS, as described above.

Western blotting

Rabbit antibodies against NF-κB p50 and TAK1 were purchased from Upstate Biotechnologies (Lake Placid, NY). Rabbit antibodies against NF-κB p65 (RelA) (Ab-1) and c-Rel (Ab-1) were purchased from Oncogene Research Products (Cambridge, MA). Rabbit antibodies against MyD88 (HFL-296), TAB2 (H-300) and JNK1 (FL) and mouse monoclonal antibodies against IRAK (F-4), TRAF6 (D-10) and phospho-JNK (G-7) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-IκBα antibody was purchased from Cell Signaling (Beverly, MA).

Fig. 10. Cell surface expressions of TLR2, TLR4 and CD14 in MeV-infected and -uninfected THP-1 cells determined by flow cytometry. Cells were pre-treated with active vitamin D₃, stained with phycoerythrin-labeled antibody against TLR2, TLR4 or CD14 and analyzed by flow cytometry. Hatched lines indicate the levels of staining using isotype-matched unrelated control antibodies.

Fig. 11. Protein levels of intracellular signaling molecules involved in NF-κB activation in MeV-infected and -uninfected monocytic THP-1 and U937 cells. Cells were pre-treated with active vitamin D₃, and the protein expression of p50, p65, c-Rel, IκBα, MyD88, IRAK, TRAF6, TAK1 and TAB2 were determined by Western blot analysis. Actin was carried out as a control experiment. MeV HA was determined as a control for MeV infection.

Fig. 12. Effect of MeV infection on LPS-induced JNK phosphorylation and AP-1 activity determined by a reporter gene assay in THP-1 cells. (A) THP-1 cells and THP-1 cells persistently infected with MeV were treated with active vitamin D₃ for 16 h, and then treated with 100 ng/ml E. coli LPS. The cells were harvested at the indicated time points after LPS treatment and lysed. Total protein levels of JNK1 and phosphorylation state of JNK were determined by Western blot using anti-JNK1 antibody and anti-phosphorylated JNK antibody, respectively. (B) THP-1 cells and THP-1 cells persistently infected with MeV were transfected with reporter plasmid containing luciferase reporter gene linked to tandem repeat of AP-1 binding motif (pAP-1-Luc) and pRL-TK as an internal reference of transfection efficacy. The transiently expressed transfectants were subsequently incubated in normal condition for 24 h, and then with active vitamin D₃ for 12 h. The cells were treated with E. coli LPS for 12 h, or not treated. The luciferase activities in the resulting cell lysates were measured. All experiments were performed in triplicate wells in one experiment. The results are expressed as fold induction (±standard deviation), which is relative to the value obtained from uninfected THP-1 cells without LPS treatment.
Preparation of total cell lysates, SDS–polyacrylamide gel electrophoresis and Western blotting were carried out as previously described (Yokota et al., 2003). Alkaline phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin antibodies (BioSource International, Camarillo, CA) and bromochloroindolylphosphate/Nitro blue tetrazolium were used as secondary antibodies and enzyme substrate for Western blotting, respectively.

**Reverse transcription real-time polymerase chain reaction (RT-PCR)**

Total RNA was isolated from cells using an RNeasy Mini kit (Qiagen, Hiden, Germany). The cDNA was prepared from total RNA by a Reverse Transcription System kit (Promega, Madison, WI) using random primer. Real-time PCR was performed using a TaqMan Gene Expression Assays kit (Applied Biosystems, Foster City, CA) with 7300 Fast Real-Time PCR system (Applied Biosystems). TLR2, 3, 4, 7 and 8, MD2 and CD14 mRNAs were determined. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as a control for normalization. The specificity of the reaction products was confirmed by performing real-time PCR directly using RNA preparation. MeV hemagglutinin (HA) mRNA and mumps virus V protein mRNA were detected using OneStep RT-PCR kit (Qiagen) described previously (Yokota et al., 2003; Kubota et al., 2001).

**Flow cytometry**

Flow cytometry was performed using FACSCalibur (Beckton-Dickinson, San Jose, CA) as previously described (Shimizu et al., 2004). Phycoerythrin-labeled mouse monoclonal antibodies against TLR2 (clone TL2.1), TLR4 (clone HTA125) and CD14 (clone 61D3) and their isotype controls (phycoerythrin-labeled mouse monoclonal IgG1 and IgG2a antibodies) were purchased from eBioscience (San Diego, CA).

**Enzyme-linked immunosorbent assay (ELISA)**

IL-8, RANTES, TNF-α and IL-6 in culture supernatants was quantified using a respective ELISA Development kit (Genzyme-Technie, Minneapolis, MN).

**Enzyme-linked DNA/protein interaction assay (ELDIA)**

Nuclear extracts of cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). DNA binding of activated NF-κB was determined by ELDIA (Benotmane et al., 1997; Yokota et al., 2004) using a BD Mercury Transfactor NFκB p50 Kit (BD Bioscience). This system measures proteins bound to immobilized double-stranded oligonucleotides using specific antibodies. Binding specificity to a particular DNA motif was confirmed by inhibition experiments using unlabeled double-stranded competitor oligonucleotides at a concentration of 200 ng/well. The following oligonucleotides were used as competitor (wild) and as control (mutant) for NF-κB p50: wild, 5′-GCCATGGGGG-GATCCCCGGGC-3′; mutant, 5′-GCCATGGGCGCATCCC-CGGGC-3′.

**Reporter gene assay using luciferases**

Activity of transcription factors was determined by a dual luciferase reporter assay by transiently transfecting reporter plasmids containing either the binding motif of NF-κB or AP-1. The reporter plasmids, pNF-κB-Luc and pAP-1-Luc (PathDetect in Vivo Signal Transduction Pathway cis-Reporter System), were purchased from Stratagene (La Jolla, CA). pNF-κB-Luc and pAP-1-Luc harbor tandem repeats of the enhancer elements (TGGGACTTCTCCG)5 and (TGACTAA)7, respectively, upstream of the firefly luciferase gene. Reporter plasmids (1 μg) and a reference plasmid (0.1 μg of pRL-TK vector, harboring the HSV thymidine kinase promoter just upstream of Renilla luciferase, Promega, Madison, WI) were mixed and transfected into THP-1 cells or THP-1 cells persistently infected with MeV using Superfect reagent (Qiagen) according to the manufacturer’s instruction manual. After cultivation for 24 h, the cells were treated with active vitamin D3 for 12 h. The transfected cells were treated or not treated with 100 ng/ml LPS for 12 h. The cells were lysed, and the firefly luciferase and Renilla luciferase activities were measured. The experiments were performed in triplicate. Reporter activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity.

**Acknowledgments**

This research was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and the grant provided by the Ichiro Kanehara Foundation.

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