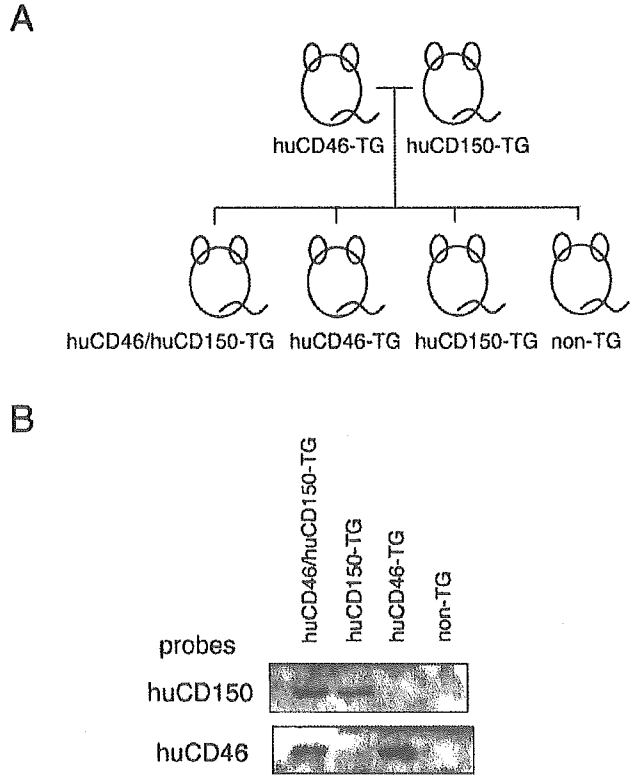

FIGURE 2. Scheme for generation of double TG mice and confirmation of genotypes. *A*, Heterogenic human CD46 and human CD150 TG mice were mated, and Mandelian inheritance of the offspring of huCD46/huCD150, huCD46, huCD150 is diagrammatically represented. *B*, Southern blots of gene transfer profiles of double TG mice.

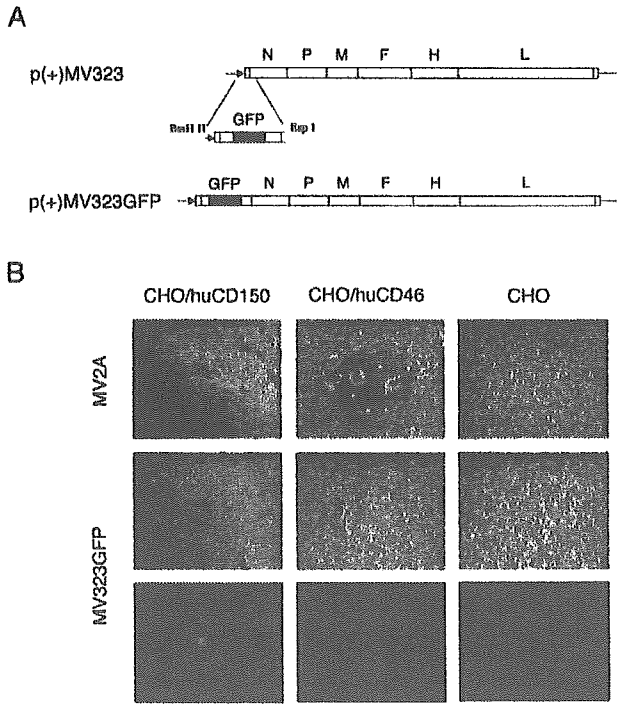

FIGURE 3. Generation of EGFP-labeled wild-type MV (MV323GFP). *A*, Scheme for GFP-labeled MV323 (MV323GFP) construction shows the p(+)MV323 plasmid (*upper*) coding wild-type MV IC strain antigenome and a construct of EGFP. The EGFP construct was placed upstream of the N gene of IC. The construct of p(+)MV323GFP that expresses GFP and viral proteins is also shown (*lower*). *B*, Receptor usage of MV323GFP. Rescued virus MV323GFP from p(+)MV323GFP was inoculated on CHO/huCD150, CHO/huCD46, and intact CHO cells, and 2 days later receptor tropism of MV323GFP was confirmed by syncytium formation and GFP fluorescence. MV2A (ED strain) rescued from p(+)MV2A was used as a control that can use both receptors. Magnification, ×100.

high MOI inoculation (Fig. 4*B*). Thus, the CD11c-positive splenocytes that are representative of DCs are less susceptible to wild-type MV compared with other cell types.

Wild-type MV fails to infect the TG mice in vivo

Next we examined in vivo MV infection. Six- to 10-wk-old double TG, huCD150 TG, huCD46 TG, or non-TG mice were injected i.p., i.v., intranasally, or s.c. with MV323GFP at a dose of 5×10^4 to 1×10^6 $TCID_{50}$. None of the TG mice showed any sign of illness. Two to 4 days after inoculation, the sections of brain, spleen, thymus, lymph nodes, lung, kidney, liver, intestine, and heart from each mouse were examined for GFP-positive cells to monitor infection by MV323GFP. No GFP-positive cells were detected in any tissue. Following i.p. injection of MV323GFP in newborn 2-day-old or 1-wk-old TG or normal mice, no GFP was detected. Intravenously transferred MV323GFP-infected splenocytes to double TG mouse also did not show any GFP-positive lesions (data not shown).

MV fails to effectively replicate in mouse DC

It was reported earlier that huCD150 were minimally expressed in human monocyte-derived DCs and up-regulated by stimulation of TLRs (20). We confirmed the expression and up-regulation of huCD150 in mDCs from huCD46/huCD150 TG mice (Fig. 5*A*). mDCs prepared from the TG mice by conventional method (the purity confirmed by the CD11c marker) expressed both huCD150 and huCD46 with a pattern similar to human monocyte-derived DCs. When mDCs were stimulated with LPS, a ligand of TLR4, expression of huCD150 was up-regulated. Similar huCD150 up-regulation was observed by stimulation with MALP-2, which is a ligand of TLR2. Of note, the level of huCD46 was not influenced in mDC in which TLRs were stimulated.

Because GFP-positive cells reflecting MV323GFP infection were barely detected in CD11c-positive splenocytes (Fig. 4*B*), we examined whether mDCs prepared from TG or non-TG mice were susceptible to MV. The mDCs from each TG mouse line were inoculated with MV323GFP at MOI = 0.25 (Fig. 5*B*). Only a few GFP-positive cells were found in mDCs from the double TG (Fig. 5*B*, left) and huCD150 TG, but not huCD46 TG and non-TG mice (data not shown). The results were compared with human monocyte-derived DCs. At MOI = 0.25 (Fig. 5*B*, *right*), a number of human DCs turned GFP-positive.

We have reported that human DCs were more susceptible to wild-type MV strains than were the laboratory-adapted and vaccine strains (20). The difference was caused by the ability of MV strains to induce type I IFN in DCs (43, 44). Human monocyte-derived DCs were inoculated with MV323, MV2A, or mock viruses at MOI = 1, whereas human IFN-β was actually produced in culture supernatants as measured by ELISA. As expected, human DCs infected with MV323 produced lower levels of IFN-β than did DCs infected with MV2A (Fig. 6*A*). Then, we examined type I IFN levels in culture supernatants of mDCs challenged with wild-type strain MV323. mDCs prepared from each TG mouse line were incubated with the same lots of MV strains at MOI = 1, and mouse IFN-β mRNA levels were measured by quantitative PCR (Fig. 6*B*). When MV323 infected mDCs from huCD46/huCD150

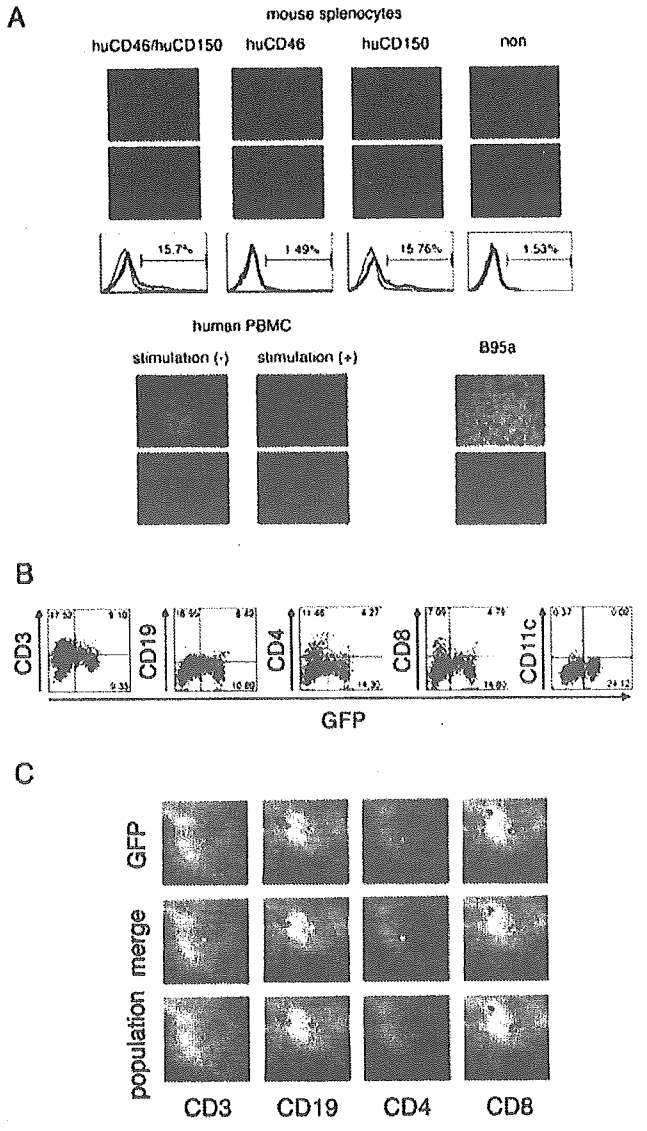

FIGURE 4. In vitro and ex vivo analysis for MV323GFP infection in splenocytes from TG mice. The splenocytes isolated from each TG mouse were stimulated with or without PMA, ionomycin, and, IL-2 and infected with MV323GFP at MOI = 0.25 for 2 days. Cells were observed by fluorescence microscope (*A*), FACS (*B*), and confocal microscope (*C*). *A*, Human PBMC and B95a cells were used as controls. B95a cells were not prestimulated, whereas either stimulated or unstimulated human PBMC and mouse splenocytes were used for infection studies. Magnification, ×40. Infectivity of splenocytes from the indicated mouse line was measured by FACS analysis (*bottom*). Fluorescence intensity reflects the levels of the MV-associated GFP. *B*, Positive cell populations (*inset*) are shown as a percentage. *C*, GFP (infected cells, green) are indicated (*upper*) and the splenocyte populations (CDs, red) are indicated (*lower*). Their merging profiles (yellow) are also shown (*middle*). Magnification, ×200.

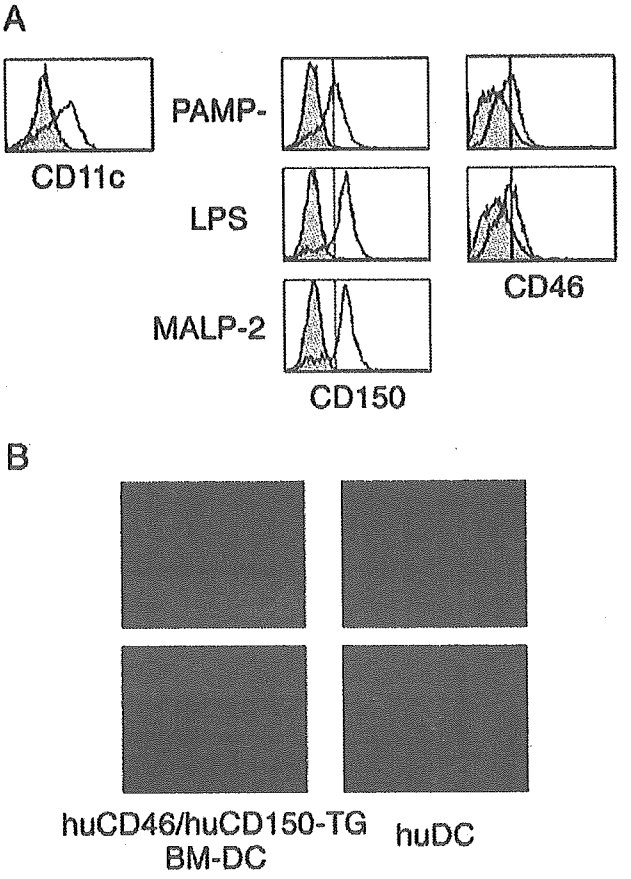

FIGURE 5. Expression profiles of huCD46 and huCD150 and testing MV sensitivity of mDCs. *A*, huCD46 and huCD150 on CD11c-positive mDCs. The expression of CD11c, huCD46, and huCD150 in mDCs prepared from huCD46/huCD150 TG mice were examined using FACS. TLR4 agonist (LPS) and TLR2 agonist (MALP-2) were used as mDC maturation inducers. Control mDCs (PAMP−) were cultured without any TLR stimulator. *B*, Efficacy of MV infection in human monocyte-derived DCs and mDC prepared from the double TG mice. mDCs from each mouse line and human monocyte-derived DCs were infected with MV323GFP at MOI = 0.25. Two days later, cells were observed under fluorescence microscope. Magnification, ×40.

TG and huCD150 TG, the mRNA levels of type-I IFN (IFN-α and IFN-β) were markedly increased in the mDCs (Fig. 6, *B* and *C*). MV2A induced IFN-α and IFN-β in the mDCs from double TG, huCD150 TG, and huCD46 TG (Fig. 6, *B* and *C*). Induction of IFN-α was less compared with IFN-β. In mouse blood cells, however, neither IFN-β nor IFN-α message was virtually detected: only a trace amount of IFN-γ message was measurable (data not shown). Local, rather than systemic, induction of type I IFN-β would occur by mDCs in response to wild-type MV. The levels of total IFN-β production depend on the combined actions of MV strain types and IFN inducibility of mDCs. Because IL-12 p40 and IL-10 were induced from human DCs as a consequence of MV infection, we also measured the mRNA level of murine IL-12 p40 and murine IL-10 of mDCs (Fig. 6, *D* and *E*). Results showed that the mRNAs of these cytokines were also induced by MV infection via the MV receptors. MV infection was confirmed by determination of the mRNA level of the H protein gene of MV (Fig. 6*F*). Although the same lots of virus stocks were used for infection, MV323 replicated better in mDCs from huCD150 TG than huCD46/huCD150 TG mouse lines. MV2A replication was high in mDCs from huCD150 TG and low in mDCs from the double TG and huCD46 TG mouse lines. The coexpressed huCD46 would probably inhibit viral replication in mouse mDCs.

DC have critical role in the establishment of MV infection in vivo

Differential susceptibility to MV of mDCs prepared from huCD46/huCD150 TG mouse vs human DCs earlier described, allowed us to speculate that DCs play a critical role in the development of systemic MV infection in vivo. Because type I IFN induced by

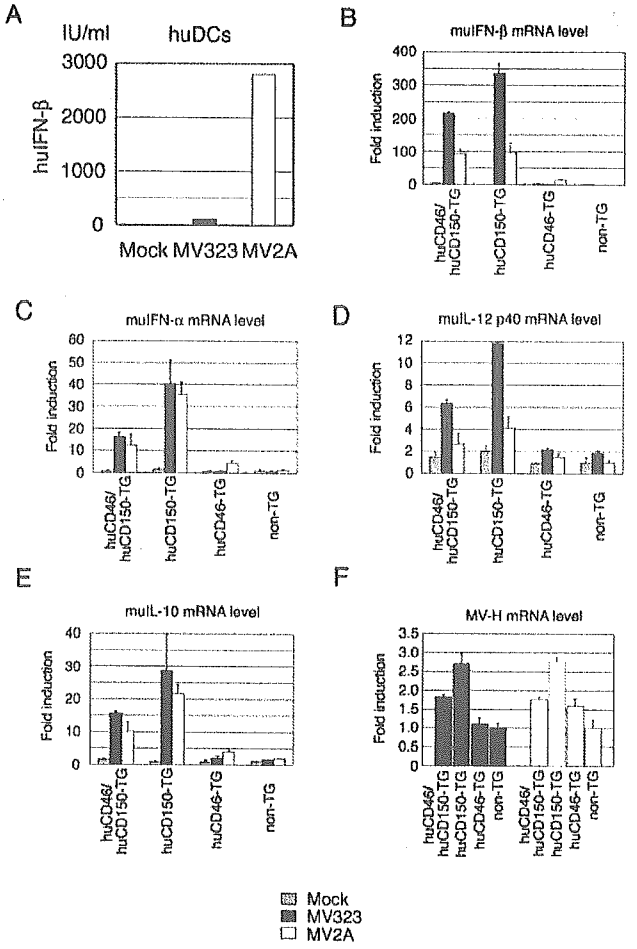

FIGURE 6. MV-induced molecules and cytokines in mouse bone-marrow-derived mDCs. Cytokine induction and MV replication profiles in mDCs prepared from TG mouse lines. *A*, Human monocyte-derived DCs were infected with MV323 or MV2A at MOI = 1. At 24 h after infection the levels of human IFN-β in the supernatants were measured using ELISA. *B*, mDCs were prepared from each indicated TG mouse line and infected with MV323 or MV2A at MOI = 1. At 24 h after infection, the mRNA levels of mouse IFN-β affected by infection with MV323 or MV2A in mDCs were measured by quantitative PCR. Fold induction against mock infection in non-TG is shown. *C–F*, mDCs prepared as in *A* and *B* were similarly infected with MV323 or MV2A at MOI = 1. At 24 h after infection, the mRNA levels of mouse (mu) IFN-α (*C*), IL-12 p40 (*D*), IL-10 (*E*), and MV-H (*F*) were measured by quantitative PCR. Relative fold induction against mock infection in non-TG is shown. The experiments were performed at least three times and represented results are shown.

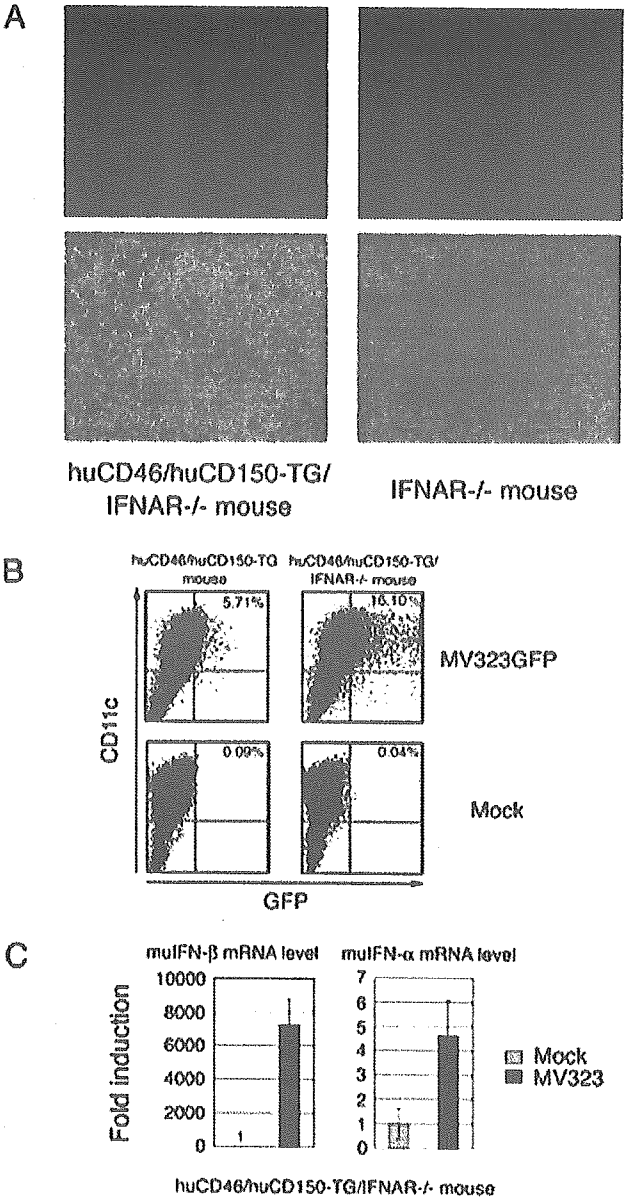

FIGURE 7. mDCs from huCD46/huCD150 TG/IFNAR$^{-/-}$ mice are susceptible against MV infection. *A*, mDCs susceptible to MV. mDCs prepared from huCD46/huCD150 TG/IFNAR$^{-/-}$ mice or IFNAR$^{-/-}$ mice (control) were inoculated with MV323GFP at MOI = 0.25. At 24 h later, cells were observed under fluorescence microscope. Magnification, ×40. *B*, mDCs from huCD46/huCD150 TG and huCD46/huCD150 TG/IFNAR$^{-/-}$ mouse prepared as in *A* were infected with MV323GFP at MOI = 0.25. At 24 h later, cells were stained with PE-labeled mouse CD11c and measured by FACS analysis. *C*, mDCs prepared as in *A* were infected with mock or MV323GFP for 24 h at MOI = 1. The mRNA levels of mouse IFN-β and IFN-α were measured by quantitative PCR. Relative fold induction against mock infection is shown.

wild-type MV acted on huCD46/huCD150-positive mDCs in a positive feedback manner, we generated huCD150/huCD46/IFNAR1$^{-/-}$ triple mutant mice, by mating the double TG mice with the IFN $\alpha\beta$ receptor 1 (IFNAR1) knockout mouse. First, we examined whether mDCs from huCD150/huCD46/IFNAR1$^{-/-}$ mutant mice were susceptible to wild-type MV infection in vitro. When the mDCs were inoculated with MV323GFP at MOI = 0.25, many GFP-positive mDCs were detected by fluorescence microscopy (Fig. 7*A*) and FACS analysis (Fig. 7*B*). Moreover, robust type I IFN production was detected in mDCs (Fig. 7*C*). The mDCs were incubated with MV323 at MOI = 1 for 24 h, and then the mRNA levels of mouse IFN-β and IFN-α were measured using quantitative PCR. As viral replication increased, IFN-β production also increased (Fig. 7, *B* and *C*). IFN-α production was largely suppressed because of the lack of the secondary signal via IFNAR1 (Fig. 7*B*, *right*). Because mDCs in triple mutant mice became susceptible to wild-type MV, we next analyzed the results of in vivo MV infection. For this, the triple mutant mice were injected i.p. with MV323GFP at a dose of 5×10^4 $TCID_{50}$. Two days later, some of the tissues were collected and their sections were directly observed by inverted fluorescence microscope (Fig. 8*A*). GFP-positive cells were detected in mesenteric lymph nodes, inguinal lymph nodes, parathymic lymph nodes, and spleen. Yet,

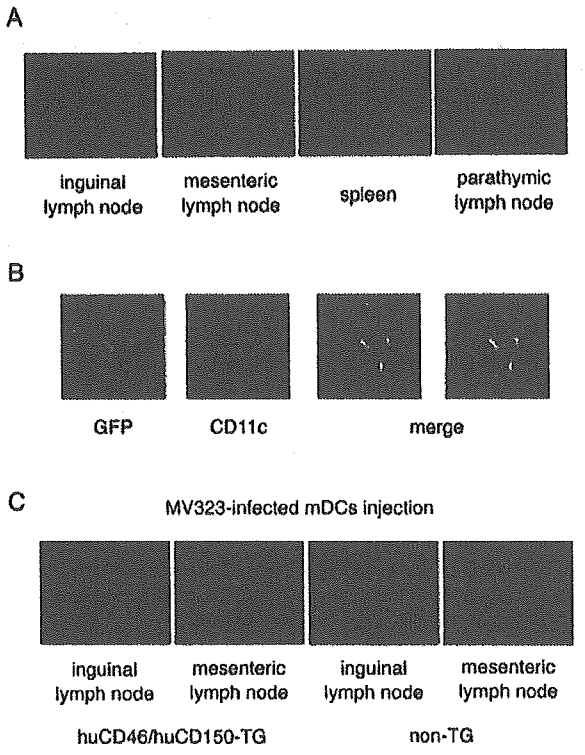

FIGURE 8. mDCs participate in systemic viral spreading. *A*, MV infection visualized in the triple mutant mice by in vivo inoculation of MV323GFP. Representative photographic images of GFP-positive organs in huCD46/huCD150 TG/IFNAR$^{-/-}$ mice are shown. At 48 h after i.p. injection of 10^5 $TCID_{50}$ of MV323GFP, the mice were dissected and tissues were directly examined under an inverted fluorescence microscope. Magnification, ×100. *B*, CD11c-positive cells reserve MV323GFP. Mesenteric lymph nodes were fixed with 4% paraformaldehyde and the sections were stained with PE-conjugated anti-mouse CD11c mAb (red). EGFP (green) and CD11c (red) were observed by confocal microscopy. Merging profiles are shown to the *right*. Magnification, ×400. *C*, MV infection visualized by transfer of MV-infected mDCs into IFNAR1$^{+/+}$ TG mice. mDCs prepared from huCD46/huCD150 TG/IFNAR$^{-/-}$ mice were infected with MV323GFP at MOI = 1. At 24 h later, MV323GFP-infected mDCs (1×10^6) were i.v. injected into huCD46/huCD150 TG, huCD46 TG, huCD150 TG, and non-TG mice (IFNAR1 is normal in all TG mice). Two days later, each mouse was dissected and tissues were directly examined under an inverted fluorescence microscope. Magnification, ×100. The results were confirmed by two additional experiments.

no positive cells were found in brain, thymus, lung, heart, liver, kidney or intestine.

We prepared sections from mesenteric lymph nodes, and stained with PE-conjugated anti-mouse CD11c mAb. As shown in Fig. 8*B*, GFP-positive and CD11c-positive cells merged in the lymph nodes. Thus, we speculated that MV-infected mDCs delivered viruses to local draining lymph nodes. To confirm this hypothesis, we i.v. transferred MV323GFP-infected mDCs (1×10^6 cells, MOI = 1) from huCD150/huCD46/IFNAR1$^{-/-}$ mice to huCD46/huCD150, huCD150, huCD46, or non-TG mice (IFNAR1 is normal in each mouse strain). Two days later, the affected mice were dissected and specified tissues were directly observed by inverted fluorescence microscope (Fig. 8*C*). In mesenteric and inguinal lymph nodes from huCD46/huCD150 TG and huCD150 TG mice, GFP-positive lesions were detected. No such lesions were observed in affected huCD46 TG and non-TG mice. Based on these results, we infer that in this model MV-infected mDCs facilitate establishing systemic MV infection. IFNAR1$^{-/-}$ mDCs allowing viral replication inside the cells, trigger successive viral delivering to lymph nodes.

Discussion

We generated TG mice that express MV receptors huCD46 and huCD150. The expression profiles and the distribution of these receptors in these mice were similar to those in humans. Mouse splenocytes from the double TG mice became susceptible to wild-type MV strains in vitro. Nevertheless, despite the fact that the TG mice expressed both huCD46/huCD150, they were highly resisted MV infection in vivo irrespective of the route of MV administration. huCD150 but not huCD46 was up-regulated in response to TLR stimulators and conferred high susceptibility to wild-type MV on mDCs. In vivo, normal CD11c-positive DCs were usually insensitive to wild-type MV infection, whereas these DCs turned susceptible to MV if their amplifiable IFN-inducing ability in response to MV infection was spoiled. Systemic MV infection was observed in the huCD46/huCD150 double TG mice with no IFNAR1 by direct MV injection or in the double TG mice with normal IFNAR1 by transfer of MV-infected mDC to them. Thus, in vivo MV permissiveness was accomplished when the IFNAR1 gene was simultaneously disrupted in DCs.

A crucial question remains elusive that this TG mice model with natural expression profile of huCD46 and huCD150 serve as a model for the investigation of human wild-type MV infection. Different from human, IFNAR1$^{-/-}$ mice induce basal production of IFN-β in response to IFN regulatory factor (IRF)-3 activation, but secondary amplifiable response is severely impaired (45, 46). Opposing to the results on human DC, mouse mDC more efficiently induce type I IFN in response to infection by wild-type MV than to infection by attenuated MV strains (Fig. 6). Wild-type MV strains barely induce amplified type I IFN production in human DCs, whereas attenuated strains robustly induce it (M. Taniguchi, N. Begum, and T. Seya, unpublished observation). Thus, pathogenicity of wild-type MV strains may not be reflected in our mouse model. MV strains, either wild-type or laboratory-adapted, appear to replicate well in human DCs if they induce only minute degrees of type I IFN (20). We would say that our mouse model in part reflects MV infection of humans whether we choose MV strains with lesser ability to induce IFN-β or choose to activate the secondary amplified type I IFN pathway. Further study is needed to confirm these issues.

Which of CD11c-positive DCs, mDCs, or pDCs play a major role in MV-mediated IFN induction is a critical question. Both mDC and pDC are CD11c-positive and possess the pathways that induce IFN type I in response to RNA viruses (47). Our infection study on mouse mDC is: 1) MV more prominently induces IFN-β than IFN-α in IC-infected double TG mice (Fig. 7, *C* and *D*); 2) double TG mice without IFNAR1 induced an extremely lower level of IFN-α as compared with the TG mice with normal IFNAR1 (Figs. 7*C* and 8*B*). This is true in human in which wild-type MV strains efficiently replicate in DCs and barely induce type I IFN (20, 43). Recent reports after the submission of our study clearly revealed that the MyD88-IRF-7 pathway in pDC is the main route of IFN-α induction in mice in several virus species (48, 49). Perhaps, mDC induces IFN-β via Toll-IL-1R homology domain-containing adapter molecule 1-mediated or RNA helicase RIG-I-mediated IRF-3 activation pathways, which is independent of MyD88-dependent IRF-7 activation pathway. We could test this issue regarding MV if the mouse IFN-α and IFN–β are separately determined by ELISA. At least blood cells barely induce the messages of type I IFN in response to MV infection (data not shown), and the role of pDC in our mouse MV infection model remain to

be settled. Further MV infection studies using mice disrupting either of the IFN-inducing pathways will be required to clarify the source of type I IFN in MV-infected TG mice.

Wild-type MV-H protein acts as a ligand for TLR2 (2) and up-regulates CD150 in human monocytes (50). Although in vitro analysis speculated that DC matures into a stage vulnerable to wild-type MV through TLR2 signaling (51), whether wild-type MV actually infects mDC in vivo remains undetermined. Our mouse model offers the possibility that the matured DC is the primary target of wild-type MV strains. Testing the role of TLR2 by MV-H protein stimulation in immature DC maturation is feasible in our model. Dysfunction of Ag-presenting capacity of DC through MV infection may be associated with modulation of TLR signaling by MV.

Previous reports suggested that MV efficiently infected human DC and suppressed DC-mediated allostimulatory lymphocyte proliferation (MLR) (52–54). Both wild-type and vaccine strains induce defective MLR response irrespective of the induction levels of type I IFNs. Testing MLR in intact vs MV-infected mDCs prepared from these TG mice will give us a hint to resolve DC-based immune suppression. The model may enable us to test the role of huCD150 and/or huCD46 on lymphocytes in association with infected mDCs.

A comparison of the double TG mice with huCD150 TG mice in their ability to produce differential cytokine levels suggests that huCD150 TG mice more efficiently induce all four cytokines tested than do huCD46/huCD150 TG mice (Fig. 6, *B–E*). The replication level of MV-H was high in huCD150 TG mice and relatively low in huCD46/huCD150 TG mice (Fig. 6*F*). The results suggest that CD46 expressed on mDCs acts as a negative regulator for wild-type MV replication. According to previous reports, mouse macrophages expressing human CD46 significantly resist MV infection (55). These cells produced high levels of NO and IFN-$\alpha\beta$ upon infection by MV (55, 56), suggesting that huCD46 enhances IFN-$\alpha\beta$ production in mouse macrophages. Thus, it is possible that simultaneous expression of huCD46 and huCD150 in mDC down-regulates MV replication. Alternatively, huCD46 in lymphocytes deliver negative or positive signal in lymphoproliferation depending on the type of tail sequences as reported previously (25, 26). There are two major isoforms of huCD46 with differential cytoplasmic tails (CYTs), CYT1 and CYT2 (25, 57). NO is induced in macrophages in a CYT1>CYT2 fashion (55, 58). In mouse lymphocytes from TG mice with either CYT1 or CYT2, differential functions of these isoforms were clearly observed (26). Relationship between the huCD46 isoforms and IFN-β production will be an issue to be tested in our model.

In human myeloid DCs, TLR3 and TLR8 participate in type I IFN production (59). Mouse mDCs lack TLR8 (60, 61). RIG-I participates in sensing viral infection inside the cells to activate a signaling pathway for induction of type I IFN (62). Yet, TLR3 may sense viral infection, as in West Nile virus entry into the brain (63). Human monocytes may express CD150 in the adenoid tissue to catch up inspired MV (50). Therefore, interpretation of differential responses between mouse mDC and human immature DC in wild *vs* vaccine strains need to take these recent findings into consideration. It is important to further analyze the natural intranasal infection of MV and its relationship to the properties of wild-type MV-infected CD11c-positive mDCs.

Our findings can be summarized as follows: 1) mouse CD11c-positive mDCs in huCD46/huCD150 double TG mice are barely susceptible to wild-type MV until IFNAR1 is depleted; 2) in the double TG mice with no IFNAR1, only CD11c-positive mDCs are vulnerable to MV at an early phase of infection in vivo; 3) robust IFN type I induction due to IFNAR1 by mDCs confers natural resistance to wild-type MV on mice; and 4) the huCD46/huCD150 double TG mouse when injected with MV-infected mDCs i.v. permits systemic infection by MV. Therefore, in this mouse model mDC carries MV to draining lymph nodes. Although the results were deduced from an artificial model case, the TG mice with or without IFNAR1 would be applicable for the study of MV-mediated initial IFN-β induction in mDC. With these mice, virus-mediated initial IFN-β inducing response and its related pathways involving IRF-3 and IRF-7 in mDCs may be critically analyzed. This is the first report on the generation of wild-type MV-sensitive mice.

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Disclosures

The authors have no financial conflict of interest.

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Fish soluble Toll-like receptor (TLR)5 amplifies human TLR5 response via physical binding to flagellin

Tadayuki Tsujita[a,b,c,1], Akihiro Ishii[a,c,1], Hironobu Tsukada[a,b], Misako Matsumoto[a,b,c], Fang-Sik Che[b], Tsukasa Seya[a,b,c,*]

[a] *Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari-ku, Osaka 537-8511, Japan*
[b] *Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 631-0101, Japan*
[c] *Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8637, Japan*

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Abstract

Fish has a soluble form of TLR5 ortholog (TLR5S), which does not exist in mammals. We identified TLR5S from rainbow trout and named rtTLR5S, which was about 38% homologous to the extracellular domains of human (hu) and mouse TLR5. Adjuvancy of rtTLR5S to flagellin response by human TLR5 (huTLR5) was tested in this study. A chimera constructed of rtTLR5S and the intracellular TIR of huTLR5 expressed on HeLa cells signaled the presence of various species of bacterial flagellin resulting in NK-κB activation. huTLR5S, when co-expressed with rtTLR5S in HeLa cells, augmented response to flagellin resulting in robust huTLR5-mediated NF-κB activation. Physical binding of flagellin to rtTLR5S was detected under the conditions where huTLR5 induced rtTLR5S-amplified NF-κB activation. Signal amplification by rtTLR5S was specific to huTLR5: no other huTLRs tested were responded to rtTLR5S. These results suggest that the soluble TLR5 serves as an adjuvant augmenting flagellin-TLR5-mediated NF-κB activation even in human.

Keywords: Toll-like receptors; Adjuvant; Bacterial flagellin

1. Introduction

Toll-like receptor (TLR) family proteins consist of an extracellular domain containing leucine-rich repeats (LRRs) and a C-terminal flanking region, and a cytoplasmic signaling domain, called the Toll/IL-1 receptor homology domain (TIR) [1]. Human (hu) and mouse TLR5 recognize bacterial flagellin and deliver signal to activate a transcription factor NF-κB [2]. TLR5, together with TLR4, also activates interferon-regulatory factor 3 (IRF-3) in response to flagellin [3]. According to the genome projects of *Fugu rubripes* [4], TLRs are distributed in fish with similar structural variations to TLRs of human and mouse [5]. However, three major differences are found in the fish TLR system: (1) there is a soluble form of TLR5 ortholog in the fish but not in human; (2) there is no TLR4 ortholog in the fish; and (3) novel members of TLRs named TLR21, TLR22 [5] and TLR14 [6] exist specific to fish. Fugu (fg) TLR members TLR2, 3, 5, 7, 8 and 9 structurally correspond to those of mammals [5]. Although mammalian TLRs recognize a variety of microbial pattern molecules, almost no functional information on the fish TLRs has been reported.

We have investigated the functional properties of the TLR5-like soluble protein in the rainbow trout (rtTLR5S) [7]. Here, We show that rtTLR5S physically bound flagellin and markedly potentiated human TLR5 (huTLR5)-mediated NF-κB activation. This rtTLR5S function was exerted exclusively toward TLR5. Thus, fish soluble TLR5 may serve as an adjuvant amplifying membrane TLR5 signaling even across species.

* Corresponding author. Tel.: +81 11 706 5073; fax: +81 11 706 7866.
E-mail address: seya-tu@med.hokudai.ac.jp (T. Seya).
[1] The first two authors have equally contributed.

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2. Materials and methods

2.1. Cells and reagents

Cells were cultured in the following medium containing 10% heat-inactivated fetal calf serum (FCS; JRH biosciences, Lenexa, KS) and antibiotics [100 unit/ml penicillin and 100 Δg/ml streptomycin (Invitrogen, San Diego, CA)]. The human cervical carcinoma cell line HeLa (Japanese Cell Resource Bank, Osaka, Japan) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 0.1 mM non-essential amino acids solution and 1 mM sodium pyruvate. HEK293FT cells (RIKEN, Wako, Japan) were cultured in DMEM. In some experiments, we used an alternative HeLa substrain established in our laboratory [8].

E. coli DH5α was purchased from Invitrogen. *Vibrio anguillarum* was prepared in National Research Institute of Aquaculture, Fisheries Research Agency, Mie, Japan. Flagellin of *P. avenae* was purified in Nara Institute of Science and Technology [9]. Recombinant flagellin A and C were purified from the cDNAs of *V. anguillarum*. Practical procedure for purification was described in a previous report [10]. LPS was purchased from BD Biosciences (San Jose, CA). poly(I:C) was from Amersham Biosciences (Buchinghamshire, UK). MALP-2 was synthesized in Biologica (Nagoya, Japan) [11]. Peptidoglycan (PGN) purified from *Staphylococcus aureus* and Polymyxin B was from Sigma–Aldrich (St. Louis, MO). All nucleotide primers were synthesized by Hokkaido System Sciences (Sapporo, Japan).

2.2. Molecular cloning of a soluble form of TLR5 in the rainbow trout (rtTLR5S)

We obtained an EST clone that contains leucine-rich repeat (AF281346) from rainbow trout exposed to *V. anguillarum*. Full-length rtTLR5S cDNA was cloned using SMART RACE cDNA Amplification Kit (BD Bioscienses) [7].

PCR was performed with liver cDNA templates and rtTLR5S gene-specific internal primers paired with either Primer 1 (P1)- or P2-specific end primers (BD Biosciences). The PCR amplicons were cloned to assess sequence accuracy. Finally, full-length rtTLR5S cDNA was obtained from the liver cDNA library by RT-PCR using primers F2 and R2 (Table 1). The cDNA encoding rtTLR5S without signal sequences was placed between the *Hind*III and *Sal*I sites of pFLAG-CMV plasmid.

2.3. Computer analysis

Alignment and motif analysis of cDNAs were performed with GENETYX, Clustal W on a Macintosh G4. The gene sequences of rainbow trout (rt) β-actin (AF157514) were obtained from the NCBI.

BLAST search analyses were carried out in the Fugu BLAST server (http://fugu.hgmp.mrc.ac.uk/blast/) using tblastn program. To predict the exon/intron boundaries, we used GeneMark.hmm (http://opal.biology.gatech.edu/GeneMark/) [12] and GeneScan (http://genes.mit.edu/GENSCAN.html) programs [13]. Obtained results were further improved manually comparing mammalian TLRs. The domain structures were predicted using SMART program. We additionally used TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) program to predict the TM regions in the rtTLR5S as described previously [14]. This program also supported the absence of a TM domain in rtTLT5S. The alignments of TLR protein sequences were performed using clustalW in WWW server of DDBJ (http://hypernig.nig.ac.jp).

Table 1
Primers (5′–3′) used in this study

rtTLR5S	
F1	ATTTCCTCTCCTCTCCTGACCCAG
F1–2	TCGCTTCCACTGTGACGGTGGCCTG
F2	CAGTGCTTCAAGTTATCTGCGAGG
R1	TGCCATCTGGGAGAGCCCTCAGCGA
R2	TGTATCATTCACTGGTCATTCAGC
P1	CTAATACGACTCACTATAGGGCAAGCAG-TGGTAACAACGCAGAGT
P2	AAGCAGTGGTAACAACGCAGAGT
F3	GATTGAGCCAGGTGCGTTTGTGGG
R3	GAGGCCCTCTAAGGATGTGATCT
F4	GGAGTGTGTTTGGACGTATTTGAAAA
R4	TCCACATCGTTTCCAGTCAAAACC
R5	GCTGTAATTCAACAGGCTGACACC
β-Actin forward	ATTCGCCGGAGATGACGCGCCTCG
β-Actin reverse	CCTTCTGCATCCGGTCAGCGATGC

2.4. Reporter gene assay

A chimera cDNA was constructed by fusing cDNA encoding Flag-tagged extracellular domain of rtTLR5S (amino acids 29–597) to that encoding the C-terminal flanking region, transmembrane and cytoplasmic domains of huTLR5 (amino acids 574–858) [7]. The construct was cloned into the pFLAG-CMV expression vector [15]. Full-length huTLR5 was obtained from human monocyte cDNA by RT-PCR as described [2]. The cDNA of huTLR5 was placed in the pFLAG-CMV plasmid. The promoter region of human E-selectin (ELAM) (−241 to −54) was ligated between *Kpn*I and *Hind*III sites of pGV-E2 (Toyo Inc., Tokyo, Japan) [15]. This plasmid (designated pELAM-luc) was used as a reporter for NF-κB activation. phRL-TK vector was purchased from Promega.

The subline of HeLa we used was TLR2-, TLR3-, TLR4- and TLR5-negative on cell surface (data not shown). HeLa cells were plated in 24-well plates (1×10^5 cell per well), and 24 h later, they were transiently transfected by LipofectAMINE Plus reagent (Invitorogen) with pFLAG-CMV for expression of the chimera, huTLR5, rtTLR5S (100 ng) or vector alone for control, together with a pELAM-luc

reporter gene (100 ng) [16]. The total amount of transfected DNA (400 ng) was adjusted by adding empty vector. One nanogram of phRL-TK was used as an internal control. After 36 h, cells were treated with flagellin A (1 μg/ml), flagellin C (1 μg/ml), LPS (1 μg/ml), poly(I:C) (50 μg/ml) or MALP-2 (1 μM) at 37 °C for 5 h. These are ligands for TLR5, TLR4, TLR3 and TLR2, respectively. To analyze the rtTLR5S dose dependency, HeLa cells were transfected with pFLAG-CMV/huTLR5 (100 ng), various amounts (0–50 ng) of pFLAG-CMV/rtTLR5S, pELAM-luc (100 ng) and phRL-TK (1 ng). Twenty-four hours later, cells were transferred to a 96-well plate and then stimulated with 0–1 μg of flagellin A, and allowed to stand for 5 h. For the luciferase assay, the cells were lysed with lysis buffer (Promega). Luciferase activity was measured with the reagents and protocols from Dual luciferase reporter gene assay kit (Promega) using a luminometer (model: BLR-201, Aloka, Tokyo, Japan) [16]. The specific activity was calculated as light intensity with a *Renilla* luciferase internal control. Values were expressed as mean relative stimulation with standard deviation (S.D.) from triplicate values of a minimum of three separate experiments.

To confirm the specificity of rtTLR5S adjuvant effect, HeLa cells were transfected with the plasmid containing huTLR cDNA (200 ng), the ELAM-luc plasmid (100 ng) and phRL-TK (2 ng), together with rtTLR5S or control vector (0.5 μg each) using Polyfect transfection Reagent (QIAGEN). After 36 h, cells were treated with PBS (non-stimulant), flagellin A (1 μg/ml), MALP-2, Pam3 (100 nM), or LPS (10 ng/ml) for 5 h. The luciferase assay was performed as described above, and luciferase activity was normalized by non-stimulant control. For this study, we used a HeLa cell subline with lower reactivity to luciferase reporter than the conventional strain [8].

2.5. Flagellin-rtTLR5S binding assay

HEK293FT cells (10^8 cells) were transfected with the plasmids (20 μg) for expression of FLAG-tagged huTLR2 (control) or rtTLR5S and cultured in DMEM/10% FCS. Typically, 48 h later, cell lysates (1% NP-40 extract) and supernatants were harvested. These samples were incubated with GST-tagged flagellin (5 μg) for 60 min at 4 °C. GST-tagged flagellin A in the samples was immunoprecipitated with anti-GST Ab (10 μg) and protein G-Sepharose (20 μg). The precipitates were washed twice in PBS/0.02% NP-40, pH 7.4 and analyzed by SDS-PAGE followed by immunoblotting using anti-FLAG Ab. The two-step recognition allowed us to detect flagellin A-bound rtTLR5S.

3. Results

3.1. A soluble form of rainbow trout Toll-like receptor 5 ortholog

The primary a.a sequence of rtTLR5S (AB062504) was 664 a.a, consisting of 1992 bp ORF and 1223 bp 3′-UTR [7,17]. BLAST search analyses suggested that this protein was most similar to mouse (mo) TLR5 in mammals (Fig. 1A). Motif search, *K–D* plot (Fig. 1B) and Crustal W comparison suggested that this fish LRR protein contained no transmembrane portion or TIR. SMART analysis defined 11 LRRs in this protein. rtTLR5S had high homology to the soluble fgTLR5 (58%) (Fig. 1A). rtTLR5S was 37 and 38% homologous to the LRR domains of huTLR5 and moTLR5, respectively (Fig. 1A). rtTLR5S showed <25% homologous to those of other human or mouse TLRs. Nine of the 11 cysteines in the LRR domains of mo/huTLR5 were conserved in rtTLR5S (Fig. 1A).

3.1.1. Recognition of flagellin by a chimera rtTLR5-TIR of humanTLR5

Since flagellin is a ligand for hu/moTLR5 [2], we next tested whether rtTLR5S recognizes flagellins from a variety of bacteria. For this trial, we made a chimera receptor cDNA consisting of cDNAs of rtTLR5S and the transmembrane and TIR domains of huTLR5. NF-κB reporter assay was performed in HeLa cells. We successfully expressed this molecule on HeLa cells, which was confirmed using anti-His-tag Ab. If rtTLR5S has an ability to recognize flagellin, this chimera receptor signals the presence of flagellin by the ectodomain and activates NF-κB via the human TIR in the HeLa cells. A representative result using the chimera-expressing HeLa cells is shown in Fig. 2A. This chimera molecule activated the NF-κB reporter gene in HeLa cells to a similar degree to huTLR5, when their expression levels were almost the same (Fig. 2A). Flagellin A and C had identical rtTLR5-stimulation potency. Flagellin of other plant bacteria, *P. avenae* similarly stimulated the chimera (Fig. 2B). Hence, rtTLR5S interacts with flagellins via its LRR domains.

To test the specificity, we stimulated this chimera with MALP-2 (a ligand of TLR2), poly(I:C) (a ligand of TLR3), LPS (a ligand of TLR4), CpG DNA (a ligand for huTLR9) and flagellin C (a ligand of TLR5) (Fig. 2C). The appropriate doses of these ligands were determined with human TLRs as in part described previously [8,11,18]. The chimera molecule as well as human TLR5 exclusively recognized a wide variety of flagellins to activate NF-κB reporter gene. We next tested the specificity of the partner of rtTLR5S in HeLa cells. Cells were stimulated with various TLR ligands after being transfected with cDNAs of huTLR2, huTLR4 (together with MD-2 and CD14) [18] or huTLR5 with or without rtTLR5S (Fig. 3). The NF-κB reporter activities were induced by MALP-2 and Pam3 in huTLR2-expressing cells and by LPS in huTLR4-expressing cells. The activity, however, was not affected by simultaneous expression of rtTLR5S. The rtTLR5S-mediated reporter amplification was observed only in the cells expressing huTLR5 with flagellin (Fig. 3). Thus, the action of rtTLR5S is specific toward huTLR5 and flagellin.

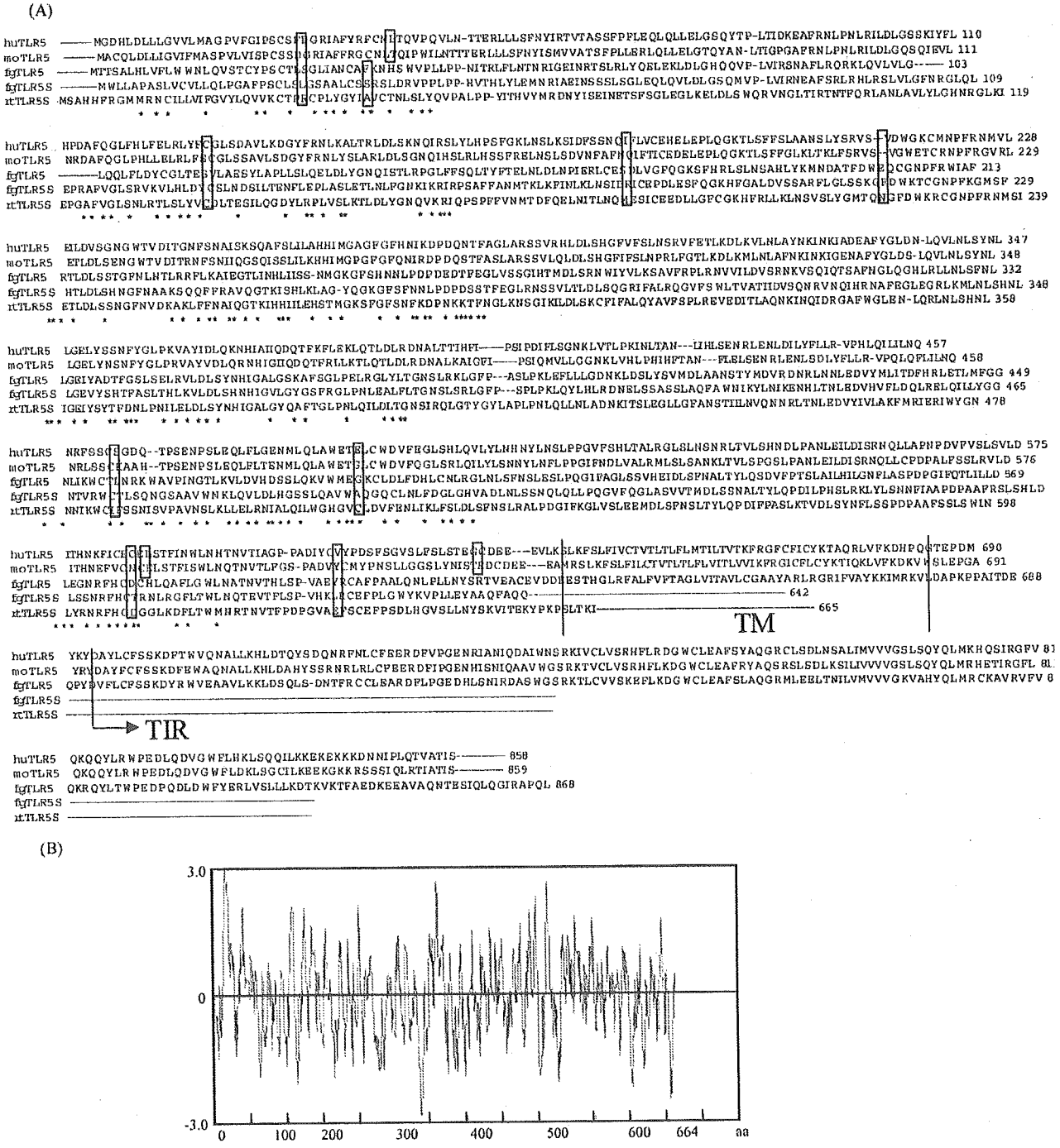

Fig. 1. Predicted amino acid sequence of rtTLR5S and the alignment of rtTLR5S and TLR5 of other species. Panel A: Comparison of rtTLR5S with the extracellular LRR domains of hu/mo/fgTLR5. The cysteines conserved across mammals and fishes are boxed. The transmembrane and TIR domains are indicated. Asteriks indicate the conserved amino acids. huTLR5, human TLR5; moTLR5, mouse TLR5; rtTLR, rtTLR5S; fgTLR5, fugu TLR5. Each domain was predicted using SMART program. Some transmembrane regions were predicted by TMHMM program. Panel B: *K–D* plot analysis of rtTLR5S.

3.2. rtTLR5S augments huTLR5-mediated NF-κB activation in transfectants

Physiological properties of rtTLR5S were examined using a transfectant model. HeLa cells expressing huTLR5 only or those expressing huTLR5 plus rtTLR5S were stimulated with flagellin A. NF-κB reporter was significantly increased in the cells if the rtTLR5S was co-transfected (Fig. 4A). Similar results were obtained with HEK293 cells which naturally express huTLR5 but not other TLRs (data not shown).

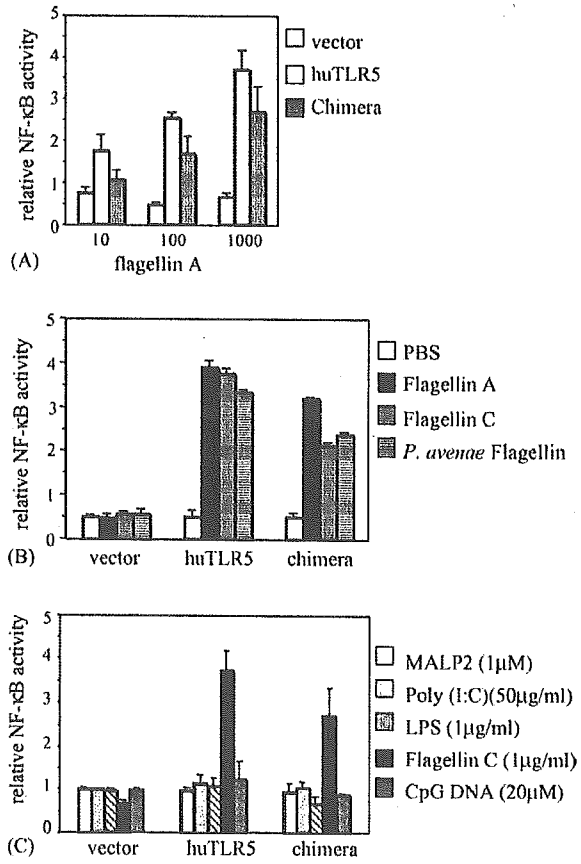

Fig. 2. Flagellin activates NF-κB in HeLa cells expressing the rtTLR5S chimera receptor. Panel A: NF-κB activation via the chimera receptor in response to flagellin. Luciferase reporter assay for measurement of NF-κB activation in HeLa chimera and huTLR5 transformants. HeLa cells transfected with ELAM-luciferase reporter plasmid were cotransfected with empty vector (vector control is shown on the left), huTLR5 (center) or the chimera (right). After 36 h, cells were stimulated with flagellin A with indicated concentrations for 5 h. The degree of NF-κB activation was determined by luciferase assay in cell lysates. Panel B: The chimera receptor responds to flagellins with various origins. Cells expressing huTLR5 or the chimera were stimulated with flagellin A (1 μg/ml), flagellin C (1 μg/ml) or *P. avenae* flagellin for 6 h. NF-κB activation was determined as in (A). Panel C: Specific recognition of flagellin by rtTLR5S. Cells expressing huTLR5 or the chimera were stimulated with the indicated TLR ligands. NF-κB activation was measured as in (A).

rtTLR5S acted as an enhancer of membrane TLR5-dependent flagellin response. Fish soluble TLR5 conforms a positive feedback loop for flagellin response leading to robust NF-κB activation in membrane TLR5-positive cells irrespective of the cell species.

To physically confirm this functional mode of flagellin, we checked direct interaction of GST-tagged flagellin with FLAG-tagged rtTLR5S by immunoprecipitation. HEK293FT cells were transfected with pFLAG-CMV/rtTLR5S and proteins from cells and supernatants were probed with anti-FLAG Ab by blotting analysis. Alternatively, the proteins were immunoprecipitated with anti-GST Ab and analyzed by immunoblotting using anti-

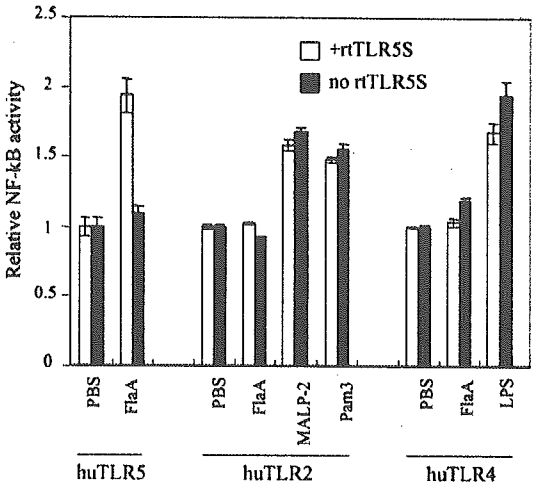

Fig. 3. Specific enhancement by rtTLR5S of flagellin/TLR5-mediated NF-κB activation. HeLa cells were transfected with the indicated plasmids for huTLR expression together with the ELAM-luc plasmid for luciferase assay. Cells were simultaneously transfected with the rtTLR5S plasmid (open bars) or vector only (closed bars). Forty-eight hours later, cells were then stimulated with the TLR ligands (1 μg/ml flagellin A (FlaA), 100 nM MALP-2, 100 nM Pam3) for 5 h. Reporter assay was performed as in the text.

FLAG Ab. In either cell lysate or supernatant, we identified a band of rtTLR5S (Fig. 4B). The results were confirmed in an additional experiment (Fig. 4C), suggesting that rtTLR5S amplifies host TLR5 response by assembling flagellin.

4. Discussion

Unique properties of the soluble TLR5 in fish are shown in this study. A soluble form of TLR5 from rainbow trout physically interacts with flagellin and this complex elicites high magnitudes of membrane TLR5 response. Not only fish TLR5 but also human TLR5 induces robust NF-κB activation in response to this complex. In vivo studies suggest that flagellin exerts endotoxin-like properties in fish [7]. Soluble forms of TLR5 appear to be distributed in a variety of fish species. The function of soluble TLR5 may cause the high susceptibility of fish to flagellin. It is notable that human lacks soluble TLR5 but huTLR5 still reserves capacity to augment flagellin response in concert with soluble TLR5. rtTLR5S failed to cooperate with other human TLRs (Fig. 3), the adjuvancy of soluble TLR5 being specific to TLR5. Soluble TLR5 may serve as a good adjuvant for flagellin-mediated activation of the human immune system.

In human, TLR5 activates NF-κB in a MyD88-dependent fashion. TLR5 also activates type I IFN if TLR4 functions with TLR5 [3]. Flagellin has been reported to act as a Th2 inducer [19] and an effective adjuvant for cancer immunotherapy [20]. The potential of adjuvancy of flagellin would be augmented if soluble TLR5 simultaneously administered. We have tried to produce a large amount of human soluble TLR5 using a variety of protein expression system

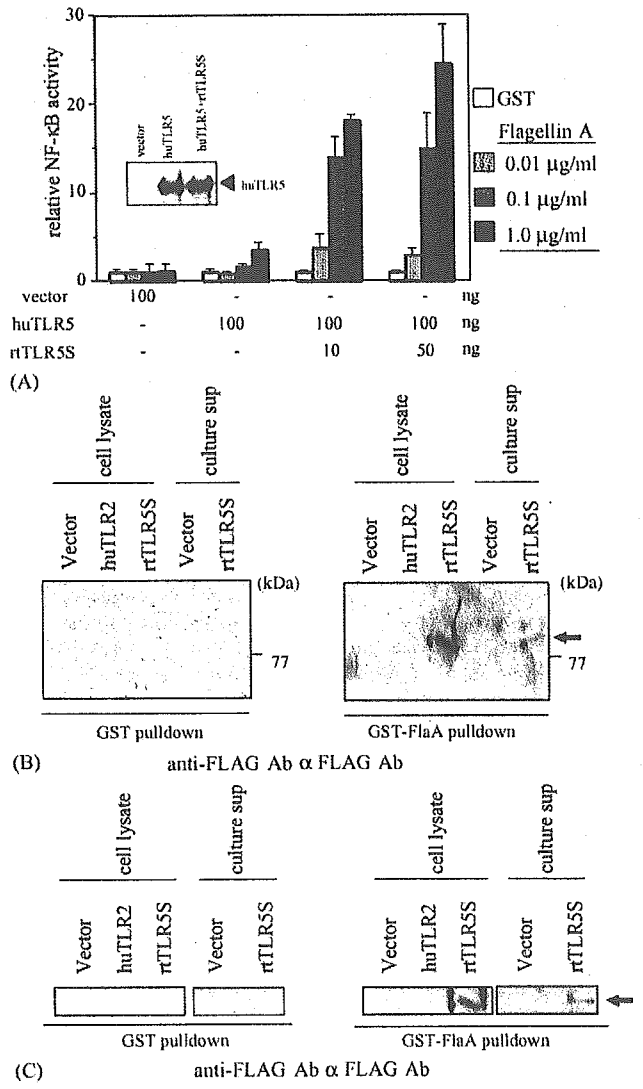

Fig. 4. Interaction of flagellin to rtTLR5 S and huTLR5. Panel A: HeLa cells were transfected with vectors indicated combinations and stimulated with flagellin A (indicated doses) or control GST protein (1 μg/ml). At timed intervals (typically 5 h), NF-κB reporter assay was performed as in Fig. 3. Expression of huTLR5 was confirmed by SDS–PAGE followed by immunoblotting using anti-FLAG Ab (inset panel). Cotransfection of rtTLR5S with huTLR5 barely affected the huTLR5 expression level (inset panel). Notice, the rtTLR5S band could not be detected in the cell lysate or supernatant (see the text). Panels B and C: 293FT cells were transfected with the indicated plasmids. At timed intervals, cell lysates and supernatants were harvested. GST-tagged flagellin was immunoprecipitated with anti-GST Ab. The precipitates were analyzed by SDS–PAGE followed by immunoblotting using anti-FLAG Ab that could probe rtTLR5S. Arrow indicates the TLR5S bound to flagellin A (FlaA).

in vain (data not shown). Creation of a recombinant protein with low antigenicity with reference to rtTLR5S would be more appropriate for this purpose. This is feasible since the alignment analysis (Fig. 1) suggests that we can pick up the portion important for flagellin recognition within the conserved region.

TLR5 gene expression was observed in mouse macrophages, epithelial and endothelial cells but not in dendritic cells [1,21]. TLR5 mRNA expression has been described to be mainly restricted to myelomonocytic cells [21]. The expression levels of TLR5 are low compared to other TLR members (data not shown). Interestingly, TLR5 gene expression was down-regulated by both virus infection and IFN stimulation [22]. In fish, soluble TLR5 is differentially up-regulated in the liver to positively respond to TLR5-mediated inflammatory responses to bacterial flagellin [7]. Flagellin-mediated adjuvancy may be exerted in different route and fashion from other TLR agonists that act on dendritic cells.

Our interest is that the flagellin-recognition system is conserved across the human and fish, and in the fish lineage, this system developed into a more sophisticated one. Bacterial flagellin can be caught up in their body fluids by the soluble form of TLR5. The advantage of this recognition system for flagellin is comparable to the system of the LPS recognition by TLR4. A soluble LBP and CD14 facilitate LPS recognition by TLR4 on the immune cell surface [23,24]. In mice, soluble form of TLR4 may function as a regulatory factor for LPS recognition [25]. In human, an artificial soluble form of TLR4 and MD-2 form a complex that attenuates LPS-induced NF-κB activation [26]. Thus, soluble TLR4 acts as a negative regulator in TLR4 signaling in mammals [25,26]. Unexpectedly, rtTLR5S acted as an enhancer for flagellin-mediated NF-κB activation in huTLR5-expressing cells (Fig. 4A). If we exogenously administer rtTLR5S or other soluble forms of TLR5 to a local lesion, innate immune response to flagellin adjuvant will be augmented to raise patients' immune potential. High antibody (Ab) production and tumor-regressing activities will be expected in human patients. The amplified adjuvant properties by rtTLR5S are worth testing for developing a new therapeutic potential.

Recent report suggested that in lamprey leucine-rich repeat-containing proteins make a variation for lymphocyte receptors through gene conversion [27]. Since no humoral factors structurally similar to Ab have been found in lamprey, a functional substitute for Ab may cover humoral immunity in lamprey. Soluble TLR-like LRR proteins are candidates for substitutes of Ab. In this view, the soluble TLR5 may reflect a prototype of humoral immunity being conserved in fish. rtTLR5S recognizes wide variety of flagellins which induce acute phase host protective response. This process may be indispensable as a primary defense in lower vertebrates living in the water. Although soluble TLR5 may participate in host defense against microbes in human as well as in fish [28], it is still unknown whether soluble TLR5 can amplify fish TLRs other than TLR5.

We herein demonstrated that rtTLR5S physically forms a complex with flagellin, and that rtTLR5S successfully amplifies flagellin signal in concert with the membrane form huTLR5 in human cells. The amplifiable flagellin-recognition system was evolved in fish as a sophisticated molecular cascade along with the NF-κB system [6,29],

and this amplifiable system is essentially conserved even in human if a soluble form TLR5 is supplemented. If this is the case, it would be possible to reconstitute a flagellin form with more appropriate function by producing a recombinant flagellin [28] where antigen peptides are incorporated to facilitate vaccination. This trial is in progress in our laboratory.

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Antibodies against human Toll-like receptors (TLRs): TLR distribution and localization in human dendritic cells

Tsukasa Seya[1,2], Kenji Funami[1], Mitsue Taniguchi[2], Misako Matsumoto[1,2]

[1]*Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan*
[2]*Department of Immunology, Osaka Medical Center for Cancer, Osaka, Japan*

We have produced monoclonal antibodies (mAbs) against human Toll-like receptors (TLRs). These mAbs recognize the natural conformation of the extracellular domain leucine-rich repeats and, thereby, are suitable for immunoprecipitation, flow cytometric analysis and immunostaining. Using these mAbs, we determined the distribution of TLRs in a variety of human cell populations. Human TLRs that recognize bacterial components, particularly TLR-1, TLR-2, TLR-4 and TLR-6, reside on the cell surface and are expressed in myeloid and monocyte-derived dendritic cells (DCs) but not in plasmacytoid DCs (pDCs). Human TLR-3 resides in putative endosomes in myeloid DCs. Thus, the human myeloid DC subset harbors a unique and distinctive TLR repertoire. These mAbs will be useful to test the localization and distribution of human TLRs in a variety of cells and organs. Functional studies of human TLRs will also be feasible since certain of them are function-blocking mAbs.

Keywords: Monoclonal antibodies, human TLRs, TLR distribution, TLR localization, human dendritic cells

Current knowledge of human TLRs and adapters

Many microbial molecules have unique signatures that activate the host immune system and are called pattern molecules. Host animals generally do not possess such signatures: therefore, the pattern molecules are microbe-specific. Microbial patterns with a high potential for host immune activation have been designated as adjuvants. Adjuvants potentially induce antibody (Ab) production, as well as cytotoxic T cells (CTL) and NK/NKT cell activation through a maturation of host dendritic cells.[1,2] Myeloid dendritic cells (mDCs), a representative of immune antigen-presenting cells, and plasmacytoid dendritic cells (pDCs), formerly identified as type I IFN-producing cells, are the targets for most of the adjuvants.

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Correspondence to: Tsukasa Seya, Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan.
Tel: +81 11 706 5073; Fax: +81 11 706 7866;
E-mail: seya-tu@med.hokudai.ac.jp

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Recently, the Toll-like receptors (TLRs) were found to serve as receptors for a variety of adjuvants.[3] TLRs recognize microbial patterns by the extracellular leucine-rich repeat-containing domain and deliver a signal through the cytoplasmic domain, named the Toll–IL-1 receptor homology domain, TIR. Since each TLR recruits distinct sets of adapter molecules that, in turn, recruit specific downstream signaling molecules,[2,3] each adjuvant confers a unique immune response on the DC subsets. So far, ten TLRs and four adapters have been identified, and the selection of TLRs and adapters appears to determine the specific TLR signaling pathway leading to the activation of transcription factors such as NF-κB, c-Jun (AP-1), IRF-3, IRF-5 or IRF-7.[3–6] In mDCs, MyD88 is a pivotal adapter that recruits IRAK/TRAF6 and/or IRF-5, leading to the activation of NF-κB so as to induce the cytokines, TNF-α, IL-6, IL-8 and IL-12, whereas TICAM-1 (TRIF), another functional adapter, mainly mediates activation of IRF-3 and the IFN-β promoter.[3,7] In pDCs, interestingly, MyD88 serves as a major adapter to activate not only NF-κB but also IRF-7, which in turn acts to produce IFN-α.[5,6] The results here are consistent with the expression profile of IRF-7, which is inducible in mDCs but constitutive in pDCs.

Adjuvants usually activate TLRs to induce mature dendritic cells to enter a professional stage of antigen-presenting cells. Each TLR appears to confer differential phenotypes on matured DCs. To understand the molecular localization and dynamics of human DC TLRs in conjunction with the stage of DC maturation and the mechanism whereby TLRs take up their ligands in the compartment where they reside, we have produced mAbs against human TLRs that specifically recognize each individual TLR member.[8-12] Our results, taken together with other functional reports on TLRs, suggest the following: (i) each human TLR has its own distribution profile; (ii) TLRs in humans DCs respond to given adjuvants in distinct ways; and (iii) human TLRs possess functions similar to their mouse counterparts.[12,13] We have put forward the hypothesis that adjuvants selectively activate adapter sets via TLRs, leading to the induction of effecters, such as CTL, Ab and/or NK.[2,13] These results provide insight into the mechanism of the adjuvant-based stimulation of immune potential, which may be useful for testing DC-mediated immunotherapy in patients with cancer and/or infectious disease.[2,13]

Methods for the preparation of Abs against human TLRs and adapters

The cDNAs of all human TLRs were cloned by PCR, labeled with a tag and placed in mammalian expression vectors (pCMV and pEFBos). We transferred the plasmid into the mouse B cell line BaF3 and used TLR-expressing BaF3 cells as the immunogen.[8] Mice were subcutaneously injected with the immunogen conjugated with Freund complete adjuvant (FCA). Our method for mAb production allowed the production of mAbs against human TLR-1, TLR-2, TLR-3 and TLR-6. We also produced mAbs against TLR-7 and TLR-8, which are undergoing further characterization. mAbs against human TLR-4 (HTA125) were provided by Dr Miyake.[14] We have not yet provided mAbs against human TLR-5 or TLR-10. An Ab against fish TLR-5 did not cross-react with human TLR-5.[16]

Rabbit polyclonal Abs against human MyD88, Mal/TIRAP, TRIF/TICAM-1 and TRAM/TICAM-2 were produced in our laboratory by a published method.[17] Briefly, rabbits were immunized with rabbit kidney (RK)13 cells overexpressing human adapters. All four adapters, MyD88, Mal/TIRAP, TRIF/TICAM-1 and TRAM/TICAM-2 are present in mDCs , judging from the results of immunoblotting. The endogenous expression level of TICAM-1 was very low compared to the levels of other three adapters.[18] The practical method for production of these Abs and the results on the properties of the adapters will be presented elsewhere.

Properties of the mAbs against human TLRs

The properties of the anti-TLR mAbs we produced are summarized in Table 1. Endogenous as well as overexpressed TLRs were immunoprecipitated with the relevant mAbs using various blood cells and cell lines.[8-11] The estimated molecular masses of these TLRs were larger than those expected from the cDNA sequences (Table 1). Post-translational modification, mainly glycosylation, may have caused this discrepancy. By flow cytometric analysis, these mAbs recognize the surface-expressed TLRs.[8-11] Intracellular TLRs are detectable if the cells are permeabilized.[10,19] By confocal analysis, endogenous TLRs can be stained even in human DCs, although the color intensity is faint.[11,19] On intracellular TLR-3 analysis,[18,19] monocyte-derived DCs and peripheral myeloid DCs were stained as expected. Function-blocking analysis was performed in monocytes (TLR-1, TLR-2, TLR-4, and TLR-6) or fibroblasts (TLR-3). Except for the anti-TLR-1 mAb, all mAbs partially or almost completely blocked ligand recognition followed by failure of signal input.[8-11] The results are summarized in Figure 1.

Table 1. Human Toll-like receptors and mAbs

huTLR	Amino acids	MW	mAb	Adapters	Ligands	Modes	Chromosome
TLR-1	786	85 kDa	TLR1.136	M-1/M-2	Tri-acyl BLP	M-type	4p14
TLR-2	784	82 kDa	TLR2.45	M-1/M-2	PGN, BLP	M-type	4q32
TLR-3	904	110 kDa	TLR3.7	T-1 (M-1)	dsRNA	T/M-type	4q35
TLR-4	839	95 kDa	HTA125	M-1/M-2 T-1/T-2	LPS, Taxol RSV-F	M/T-type	9q32
TLR-6	796	89 kDa	TLR6.127	M-1/M-2	Di-acyl BLP	M-type	4p14

M-1, MyD88; M-2, Mal/TIRAP; T-1, TICAM-1; T-2, TICAM-2; M-type, MyD88-dependent NF-κB activation pathway; T-type, IRF-3/IRF-7-mediated type I IFN inducing pathway. TLR-1 and TLR-6 are members of the TLR-2 subfamily and in conjunction with TLR-2 recognize more precise BLP patterns to activate the TLR-2 pathway. BLP, bacterial lipoprotein; PGN, peptidoglycan; RSV-F, F protein of respiratory syncytial virus.

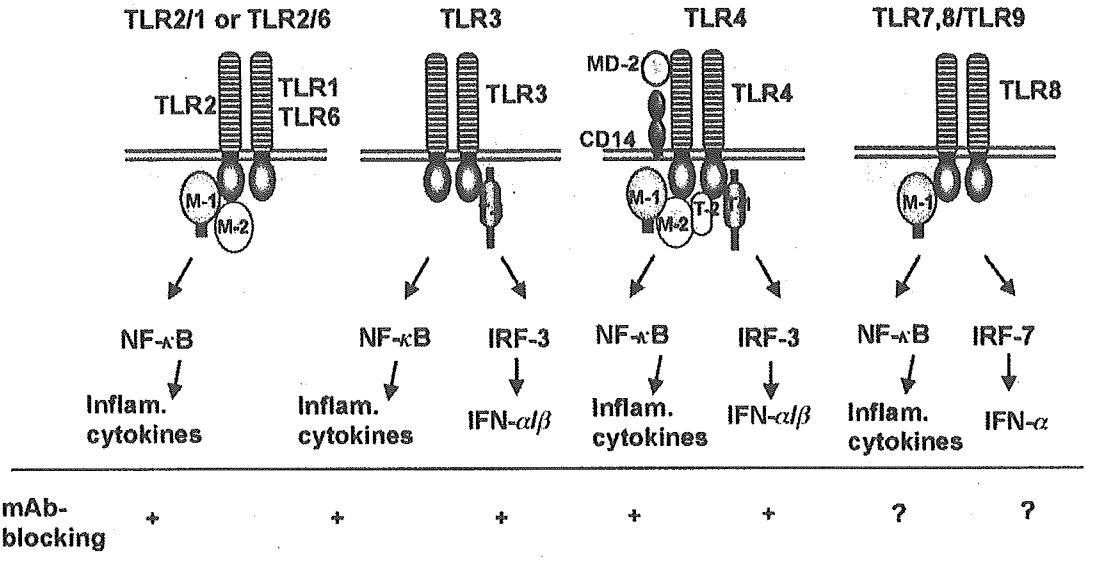

Fig. 1. Human TLRs and their functions: mAbs blocking the TLR function. Human TLRs and the selection profiles of adapters are shown. The outputs inhibited by our mAbs are indicated below the figure. '+' means that Ab-blocking is feasible; '?' means not yet tested. TLR-1, TLR-2, TLR-4 and TLR-6 are localized on the surface of mDCs, while none of these TLRs are on pDCs. These TLRs essentially recognize microbial products. Four other TLRs recognize nucleic acid derivatives: TLR-3 and TLR-8 are localized to intracellular compartments of mDCs, while TLR-7 and TLR-9 are localized to those of pDCs.[27,28,30,34] Confirmation is in progress using these mAbs.

Distribution of TLRs in dendritic cells

Human monocytes and neutrophils were used to test the expression profiles of TLRs. Monocytes and neutrophils express TLR-1, TLR-2, TLR-4 and TLR-6 with higher surface levels than other cell types.[11,12] Flow cytometric analyses using anti-TLR mAbs revealed differential expression profiles for two DC subsets – mDCs and pDCs.[10] We isolated mDCs using BDCA1-beads and pDCs using BDCA4-beads from fresh human blood. Although there is an individual-to-individual variation in the levels of TLRs on these DC subsets, similar consensus expression profiles were obtained from > 4 donors. Human mDC express TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 on the cell surface and TLR-3 inside the cell (Table 2). In contrast, human pDCs express these TLRs at less than the detection limit, if at all, even though these TLR mRNAs were detected.[20] The results are not always consistent with the summary deduced from the results of RT-PCR.[20] TLR-6 is a typical example: a strong mRNA signal was detected but no TLR-6 protein was observed in human pDCs (Fig. 2). The surface expression levels of TLR-1 and TLR-6 were low even in human mDCs compared to that of TLR-2.[11] In conclusion, human mDCs possess all TLRs that recognize bacterial products, while pDCs do not express these TLR species. The TLR-1 and TLR-6 proteins, which act as partners of TLR-2 for the recognition of tri- or di-acylated bacterial lipoproteins,[21,22] were not expressed on the pDC surface.[11,19] Notably, the distribution profiles of nucleotide-recognition TLRs were found to be completely different between the mDCs and pDCs, as previously described.[20] mDCs are reported to express TLR-3 and TLR-8 whereas pDCs express TLR-7 and TLR-9, which was confirmed by our findings using mAbs (unpublished observation). The intracellular localization of these TLRs has also been mentioned in several reports.[19,23,24]

Table 2. Toll-like receptor expression in human dendritic cell subsets

	Freshly isolated			*In vitro* differentiated cells*	
	Monocyte	mDC (BDCA1+)	pDC (BDCA4+)	DCs	Macrophages
TLR-1	++	+	–	+	++
TLR-2	++	++	–	++	++
TLR-3	–	++	–	++	+
TLR-4	++	+	–	+	+
TLR-6	++	+	–	+	+

TLR-3 is a nucleotide recognizing TLR and resides in intracellular compartments.
*differentiated from peripheral blood monocytes

Possible ligand recognition system of TLRs

The mouse has counterparts for all the human TLRs except TLR-8 according to genomic analysis.[3] The functional

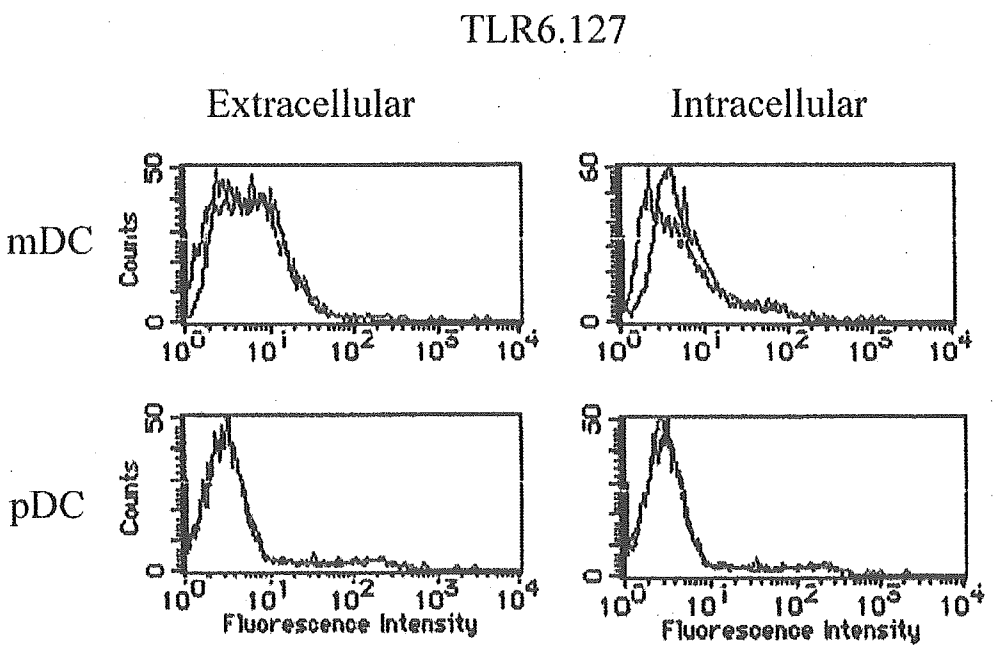

Fig. 2. mDCs but not pDCs express minute amounts of TLR-6 protein on the cell surface. Human mDCs and pDCs were isolated using the indicated bead method and their solubilized preparations were produced as described previously.[11] The results of flow cytomtry are shown in this figure. Although the levels of TLR-6 were minimal on the surface of mDCs, in other samples the values of mean fluorescence intensities for TLR-6 were significantly positive.

properties of these mouse TLRs have been determined in gene-disrupted mice.[3,25] Human TLRs are hypothesized to be similar in functional features to their mouse TLR counterparts.[1,3] TLRs are type I, membrane-associated receptors. TLRs with nucleotide-recognition properties reside in cytoplasmic compartments.[19,26] Although we have not yet defined the subcellular localization of each TLR, the TLR-containing organelle must express the extracellular pattern-recognition domains of the relevant TLRs inside the organelles. Nucleotides, including RNA and DNA, irrespective of host or microbial origin, reside in the cytoplasm or nuclei. Thus, TLRs and their ligands are separately distributed.

TLR-3 has been investigated regarding the relationship between its dsRNA recognition properties and subcellular localization since the specific mAb against human TLR-3 is available. dsRNA is a typical product of RNA virus replication that occurs in the cytoplasm and its receptor TLR-3 resides in putative endosomes in human mDCs.[19] One possible interpretation of this distinct ligand-receptor localization is that virus-infected cells are taken up into mDCs by phagocytosis, then lysed endosomaly, thereby dsRNA is released and binds their cognate TLRs situated in (or being recruited to) the endosomes.[27] The case may be similar in other nucleotide-recognition TLRs (TLR-7, TLR-8 and TLR-9). Alternatively, though difficult to occur, the ligands for these TLRs are delivered from the cytoplasm or nuclei to the endosomes where the TLRs accumulate. This would be possible if mDCs *per se* are infected with viruses. In this situation, however, intracellular RNA receptors – RIG-I, MDA5 and PKR – are pivotal factors with regard to cytoplasmic RNA recognition.[28,29] If the organelles harboring TLRs are identified, one could test the second possibility and the molecules responsible for the intra-organelle TLR-3 response.

TLR localization and ligand recognition

The addition of poly-I:C or CpG DNA provokes activation of mDCs and pDCs, respectively.[19,26,27,30] Type I IFNs are produced in response to these ligands. In fibroblasts and epithelial cells, TLR-3 is expressed on the cell surface,[8,10] and responds to the ligand poly-I:C on the surface. However, in mDCs, TLR-3 is expressed in certain cytoplasmic compartments,[10,19] suggesting that an unidentified poly-I:C-taking up receptor exists on the surface of mDCs and carries the ligand to the intracompartmental TLR-3. This poly-I:C (and probably dsRNA)-binding receptor remains unidentified. The same is true for CpG DNA, the receptor TLR-9 being localized in the endoplasmic reticulum (ER).[26,30] TLR-9 recognizes CpG DNA in the endosome and the MyD88–IRF-7-mediated IFN-α induction depends on the retention time CpG DNA resides there.[30] It is possible that a molecule possessing the targeting sequence for the ER or endosome plays a role in carrying CpG DNA to TLR-9 in organelles.

TLRs in association with their functions in DCs

DCs are central to T/B-cell activation. DCs facilitate the production of Abs by inducing the differentiation of B lymphocytes. T lymphocytes are differentiated by matured DCs into T helper I (Th1), Th2 and cytotoxic T cells (CTLs). DCs take up antigens, alter their functions in response to pattern molecule adjuvant and migrate to draining lymph-nodes. When antigen and adjuvancy coincide to stimulate DCs maturation, antigenic peptide presentation is augmented on major histocompatibility complex (MHC).[31] The up-regulation of co-stimulatory and MHC molecules, expression of chemokine receptors, secretion of cytokines and chemokines and presentation of antigens are all accelerated by adjuvants in DCs.[1] TLRs on DCs are involved in these pivotal events in the activation of lymphocytes, *i.e.* acquired immunity. Regulatory T-cell (Treg) function is suppressed by IL-6, which is produced by mDCs in response to TLR activation.[31] Memory and cross-priming are probably important features of DC-mediated lymphocyte activation, and these DC functions may be related to TLR signaling.[27] Thus, the simultaneous stimulation of DCs by microbial patterns and antigens induces robust immune activation. It is notable that DC subsets have distinct receptor-expression profiles which enable them to respond to specific types of pathogens. In addition, DCs express pattern-recognition receptors other than the TLRs, most of which act as microbe-uptake receptors[32,33] and cytoplasmic pattern-recognition receptors.[28,29] Lectins, Ig superfamily and complement receptors are representative of the phagocytosis receptors which DCs express. RIG-I, MDA5, PKR and DNA kinases are examples for the latter receptors. Functional assignment of the TLRs and these phagocytic/cytoplasmic receptors in DCs will be important to delineate specifically the DC pattern recognition system. The lack of a means to probe endogenous TLRs by immunochemical methods has, until now, precluded analysis of the molecular interassociation between the TLRs and the other pattern-recognition receptors. We favor the hypothesis that both TLR signaling and other receptor-mediated internalization mechanisms function together to induce mature DCs in an appropriately responsive fashion.[13,33,34] Further investigation is needed to clarify this issue.

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The Kinase Complex Responsible for IRF-3–Mediated IFN-β Production in Myeloid Dendritic Cells (mDC)

Miwa Sasai[1,2], Misako Matsumoto[1] and Tsukasa Seya[1,*]

[1]Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638; and [2]Department of Molecular Immunogy, Nara Institute of Science and Technology, Ikoma, Nara 631-0101

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Type I interferons (IFN) IFN-α and -β play a central role in the induction of antiviral immunity, which involves up-regulation or activation of a large number of IFN-inducible genes in host immune competent cells. Initial events in the antiviral response may occur in myeloid dendritic cells (mDCs), and the proteins expressed provoke early responses to cope with concomitant infection in the host. The participation of transcription factors IRF-3/7, AP1 and NF-κB in IFN-β promoter activation in mDCs is well established. An initial trigger of this event is a viral dsRNA that is recognized by proteins with an RNA-binding motif. Toll-like receptor (TLR) 3 on membranes and RIG-Iin the cytoplasm are molecules with dsRNA-recognition ability. Our main aim in the present review is to describe how IRF-3 and/or NF-κB are activated through the initial recognition of dsRNA by these pattern-recognition receptors. By analogy to the trimolecular complex of IKKγ, IKKα and IKKβ, thus far, IRF-3–activated kinases have been reported to be kinase complexes with trimolecular assembly. Two kinases, TBK1 and IKKε, are thought to be linked to regulatory subunit TANK or NAP1 with no kinase activity like IKKγ. The TLR3 and RIG-I pathways converge upstream of IRF-3, possibly at NAP1, the regulatory subunit of IRF-3-activating kinase. Thus, a novel function of the regulatory subunit has emerged. These proteins are involved in the TLR3 and RIG-I pathways, and act as adapters bridging on the dsRNA-recognition unit and IRF-3–activating kinases in addition to their kinase-regulatory function. Here, we summarize the properties of regulatory subunits NAP1 and TANK, and the mode of activation of NF-κB and IRF-3 in conjunction with the unique properties of the TLR3 function.

Key words: Toll-like receptor, type I interferon, viral infection.

Abbreviations: dsRNA, double-stranded RNA; FADD, Fas-associated protein with the death domain; IFNAR, interferon-α/β receptor; IKK, IκB kinase; IRF, interferon-regulatory factor: mDC, myeloid dendritic cell; NAP, NAK-associated protein; pDC, plasmacytoid dendritic cell; RIP, receptor-interacting protein; STAT, signal transducers and activation of transcription; TBK, TANK-binding kinase; TICAM, Toll/IL-1 receptor homology domain–containing molecule; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor–associated factor; VAK, virus-activated kinase.

TLR is a type I transmembrane protein consisting of extracellular leucine-rich repeats (LRRs) and an intracellular Toll-IL-1 receptor homology domain (TIR). Humans possess a family of TLRs consisting of ten TLR proteins. Homologous or heterologous combinations of them recognize a variety of microbial pattern molecules (*1*, *2*). These TLRs are expressed in myeloid cells, particularly in mDCs. The recognition of multifarious microbial patterns by primary pattern–recognition receptors in a manner unique to TLRs is the first defense for host immunity against various pathogens in these cells. Many microbial pattern molecules have been reported, most of which serve as agonists for TLRs.

Poly(I:C), a representative dsRNA, has been reported to be a potent type I IFN inducer (*3*), but its mechanism remained unknown until the discovery of the function of TLR3. Human and mouse TLR3 acts as receptors for poly(I:C) in mDCs and some epithelial cells (*4*, *5*). The unique IFN-inducing property of TLR3 is in part characterized by its specific selection of TLR adapter TICAM-1 (also called TRIF) (*6*). Of the four adapters that have the Toll-IL-1β homology (TIR) domain, TICAM-1 binds directly to TLR3 and indirectly to TLR4 (*7*). Subsequently, TICAM-1 allows the selection for the pathway to activation of IRF-3 followed by IFN-β promoter activation (*8*). In myeloid dendritic cells (mDCs), this pathway is pivotal for the induction of type I IFN. Another adapter, MyD88, induces IFN-α only in plasmacytoid DC (pDCs) by activating IRF-7 through a different molecular interaction (*9*, *10*). Two additional adapters, Mal/TIRAP and TICAM-2 (TRAM), essentially function as bridging adapters, as delineated in Fig. 1 (*11*).

A trimolecular complex consisting of IKKγ, IKKα and IKKβ has been well characterized, but a complex of IRF-3-activated kinases has not yet been clearly demonstrated. However, recent reports suggested that the putative trimolecular kinase complex TANK, IKKε and TBK1 participates in activation of not only NF-κB but

*To whom correspondence should be addressed. Tel: +81 11 706 5073, Fax: +81 11 706 7866; E-mail: seya-tu@med.hokudai.ac.jp

