X-100 for 15 min. For the polyI:C stimulation, 100 ng of polyI:C were transfected into HeLa cell in 24-well plates together with IPS-1 or DDX3 expressing vectors, and 24 h after the transfection, the cells were fixed and stained for confocal microscopic analysis. Permeabilized cells were blocked with PBS containing 1% BSA and were labeled with anti-Flag M2 mAb (Sigma), anti-HA polyclonal Ab (Sigma) or Mitotracker in 1% BSA/PBS for 1 h at room temperature. The cells were then washed with 1% BSA/PBS and treated for 30 min at room temperature with Alexa-conjugated Ab (Molecular Probes). Thereafter, micro-cover glass was mounted onto slide glass using PBS containing 2.3% DABCO and 50% of glycerol. The stained cells were visualized at $\times 60$ magnification under a FLUOVIEW (Olympus, Tokyo, Japan).

Acknowledgements: The authors thank Dr. M. Sasai, Dr. T. Ebihara, Dr. K. Funami, Dr. A. Matsuo, Dr. A. Ishii, Dr. A. Watanabe and Dr. M. Shingai in our laboratory for their critical discussions. This work was supported in part by CREST and Innovation, JST (Japan Science and Technology Corporation), the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT, Sapporo Biocluster "Bio-S," the Knowledge Cluster Initiative of the MEXT, Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan, Mitsubishi Foundation, Mochida Foundation, NorthTec Foundation and Takeda Foundation.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S. *et al.***, Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006. **441**: 101–105.

2 **Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R. A., Diamond, M. S. *et al.***, Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc. Natl. Acad. Sci. USA* 2006. **103**: 8459–8464.

3 **Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K. *et al.***, The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 2004. **5**: 730–737.

4 **Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J. *et al.***, IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 2005. **6**: 981–988.

5 **Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R. and Tschopp, J.**, Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005. **437**: 1167–1172.

6 **Seth, R. B., Sun, L., Ea, C. K. and Chen, Z. J.**, Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 2005. **122**: 669–682.

7 **Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z. and Shu, H. B.**, VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol. Cell* 2005. **19**: 727–740.

8 **Sasai, M., Matsumoto, M. and Seya, T.**, The kinase complex responsible for IRF-3-mediated IFN-beta production in myeloid dendritic cells (mDC). *J. Biochem.* 2006. **139**: 171–175.

9 **Ryzhakov, G. and Randow, F.**, SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. *EMBO J.* 2007. **26**: 3180–3190.

10 **Sasai, M., Oshiumi, H., Matsumoto, M., Inoue, N., Fujita, F., Nakanishi, M. and Seya, T.**, Cutting Edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *J. Immunol.* 2005. **174**: 27–30.

11 **Saito, T., Hirai, R., Loo, Y. M., Owen, D., Johnson, C. L., Sinha, S. C., Akira, S. *et al.***, Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc. Natl. Acad. Sci. USA* 2007. **104**: 582–587.

12 **Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H. *et al.***, 5′-Triphosphate RNA is the ligand for RIG-I. *Science* 2006. **314**: 994–997.

13 **Franca, R., Belfiore, A., Spadari, S. and Maga, G.**, Human DEAD-box ATPase DDX3 shows a relaxed nucleoside substrate specificity. *Proteins* 2007. **67**: 1128–1137.

14 **Yedavalli, V. S., Neuveut, C., Chi, Y. H., Kleiman, L. and Jeang, K. T.**, Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. *Cell* 2004. **119**: 381–392.

15 **Kravchenko, V. V., Mathison, J. C., Schwamborn, K., Mercurio, F. and Ulevitch, R. J.**, IKKi/IKKepsilon plays a key role in integrating signals induced by pro-inflammatory stimuli. *J. Biol. Chem.* 2003. **278**: 26612–26619.

16 **Soulat, D., Bürckstümmer, T., Westermayer, S., Goncalves, A., Bauch, A., Stefanovic, A., Hantschel, O. *et al.***, The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response. *EMBO J.* 2008. **27**: 2135–2146.

17 **Venkataraman, T., Valdes, M., Elsby, R., Kakuta, S., Caceres, G., Saijo, S., Iwakura, Y. *et al.***, Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J. Immunol.* 2007. **178**: 6444–6455.

18 **Schroder, M., Baran, M. and Bowie, A. G.**, Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. *EMBO J.* 2008. **27**: 2147–2157.

19 **Gack, M. U., Shin, Y. C., Joo, C. H., Urano, T., Liang, C., Sun, L., Takeuchi, O. *et al.***, TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 2007. **446**: 916–920.

20 **Oshiumi, H., Matsumoto, M., Hatakeyama, S. and Seya, T.**, Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-beta induction during the early phase of viral infection. *J. Biol. Chem.* 2009. **284**: 807–817.

21 **Arimoto, K., Takahashi, H., Hishiki, T., Konishi, H., Fujita, T. and Shimotohno, K.**, Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. *Proc. Natl. Acad. Sci. USA* 2007. **104**: 7500–7505.

22 **Ariumi, Y., Kuroki, M., Abe, K., Dansako, H., Ikeda, M., Wakita, T. and Kato, N.**, DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. *J. Virol.* 2007. **81**: 13922–13926.

23 **Chuang, R. Y., Weaver, P. L., Liu, Z. and Chang, T. H.,** Requirement of the DEAD-Box protein ded1p for messenger RNA translation. *Science* 1997. **275**: 1468–1471.

24 **Owsianka, A. M. and Patel, A. H.,** Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. *Virology* 1999. **257**: 330–340.

25 **Mamiya, N. and Worman, H. J.,** Hepatitis C virus core protein binds to a DEAD box RNA helicase. *J. Biol. Chem.* 1999. **274**: 15751–15756.

26 **Sasai, M., Shingai, M., Funami, K., Yoneyama, M., Fujita, T., Matsumoto, M. and Seya, T.,** NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. *J. Immunol.* 2006. **177**: 8676–8683.

27 **Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. and Seya, T.,** TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 2003. **4**: 161–167.

28 **Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A. *et al.*,** Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* 2003. **171**: 3154–3162.

Abbreviations: **CARD:** caspase recruitment domain · **DEAD:** Asp-Glu-Ala-Asp · **DDX3:** DEAD/H BOX 3 · **IKKε:** I-kappa-B kinase ε · **IRF-3:** IFN regulatory factor-3 · **IP:** immunoprecipitation · **IPS-1:** IFN-β promoter stimulator-1 · **MDA5:** melanoma differentiation-associated gene 5 · **RIG-I:** retinoic acid inducible gene-I · **RLR:** RIG-I-like receptor · **TBK1:** TANK-binding kinase 1 · **VSV:** vesicular stomatitis virus

Full correspondence: Dr. Tsukasa Seya, Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan
Fax: +81-11-706-7866
e-mail: seya-tu@pop.med.hokudai.ac.jp

See accompanying Commentary:
http://dx.doi.org/10.1002/eji.201040447

Received: 30/11/2009
Revised: 8/1/2010
Accepted: 19/1/2010
Accepted article online: 1/2/2010

Molecular Immunology 47 (2010) 1283–1291

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the Toll-like receptor 3/4 pathway

Miwa Sasai[a,1,2], Megumi Tatematsu[a,2], Hiroyuki Oshiumi[a,2], Kenji Funami[a,3], Misako Matsumoto[a], Shigetsugu Hatakeyama[b], Tsukasa Seya[a,*]

[a] Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan
[b] Department of Biochemistry, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan

ARTICLE INFO

Article history:
Received 30 July 2009
Received in revised form
28 November 2009
Accepted 6 December 2009
Available online 4 January 2010

Keywords:
Toll-like receptor 3
TICAM-1 (TRIF)
TRAF2
TRAF6
Interferon-beta

ABSTRACT

Using yeast two-hybrid screening, we found three TRAF proteins TRAF1, 2 and 6, bound the N-terminal region of the TLR3/4 adaptor TICAM-1 (TRIF). TRAF2, a newly identified TICAM-1-binding protein, bound the PxQxS motif (aa 333–338) of TICAM-1 using mutagenesis by alanine substitutions. TICAM-1 is known to induce the activation of NF-κB and IRF-3, which leads to activation of the interferon (IFN)-β promoter, an activity that is conserved in the N+TIR fragment (aa 1–533). By mutation of the two distinct binding sites for TRAF2 and TRAF6 in N+TIR TICAM-1, the induction of IFN-β was completely abrogated. Although the TRAF2 site single mutation only marginally affected TICAM-1-mediated type I IFN induction, it further impaired the function of the TRAF6 site mutant. Moreover, double point mutations of the TRAF2 and TRAF6 binding motifs in TICAM-1 N+TIR reduced the activation of IRF-3 and NF-κB, the critical transcription factors for IFN-β expression. Furthermore, TRAF2/6 functioned as an E3 ligase to induce K63-mediated ubiquitination on N+TIR which was abrogated in the mutant lacking the TRAF2/6 sites in parallel with IFN-inducing activity. Confocal microscopy analysis indicated that TRAF2 and TRAF6 merged with oligomerized (i.e. activated) TICAM-1 N+TIR. However, TRAF3, which is another TRAF family member essential for TLR3-mediated type-I IFN signaling, still assembled in the mutant lacking the TRAF2/6 sites. Our data suggest that the binding of TRAF2 and TRAF6 to TICAM-1 cooperatively activates the IFN-inducing pathway through ubiquitination of TICAM-1, a modification which occurs unrelated to TRAF3 recruitment in the TICAM-1 signaling complex. TRAF2/6 may participate in TICAM-1-mediated IFN-β induction besides TRAF3.

1. Introduction

Tumor necrosis factor receptor-associated factor (TRAF) family proteins are frequently involved in signaling of Toll-like receptors (TLRs) to evoke immune responses (Chung et al., 2002; Kawai and Akira, 2007). Of the TRAF family members, TRAF6 plays a significant role in signal transduction by both the TNF receptor (TNFR) and interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamilies (Chung et al., 2002; Kawai and Akira, 2007; Ye et al., 2002). CpG-DNA activates the TLR9 signaling pathway via myeloid differentiation marker 88 (MyD88) and TRAF6, leading to activation of the IκB kinase complex and c-jun kinases (Häcker et al., 2000). TRAF6 also interacts with MyD88 to mediate NF-κB activation by TLR2 and TLR4 (Mansell et al., 2004). In the absence of TRAF6 in mouse macrophages, ligands for TLR2, TLR5, TLR7, and TLR9 fail to induce activation of NF-κB and MAPKs or produce inflammatory cytokines. TLR4 ligand-induced cytokine production is also markedly reduced in TRAF6−/− cells, although the activation of NF-κB and MAPKs is still observed. Another adaptor of TLR4, known as Toll/IL-1R homology domain-containing molecule (TICAM)-1 (also named TRIF), may compensate for the function of TRAF6 with other TRAFs. In contrast to the reported findings in HEK293 cells (Sato et al., 2003), TLR3 signaling delivered through TICAM-1 is not affected by TRAF6 deletion in macrophages (Häcker et al., 2000). Based on these results, TRAF6 is thought to be essential for MyD88-dependent signaling, but not required for TICAM-1-dependent signaling (Gohda et al., 2004).

TRAF proteins consist of N-terminal RING and zinc-finger domains and C-terminal TRAF-specific domain, which participates in oligomerization and interacts with their receptors (Chung et al., 2002). The TNFR1-associated death domain protein (TRADD) is critical in TNFR1, TLR3, and TLR4 signaling. TRADD deficiency

* Corresponding author. Tel.: +81 11 706 7866; fax: +81 11 706 7866.
E-mail address: seya-tu@pop.med.hokudai.ac.jp (T. Seya).
[1] Present address: Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06510, USA.
[2] The first three authors equally contributed to this work.
[3] Present address: Research Institute, Chiba Institute of Technology, Chiba, Japan.

0161-5890/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2009.12.002

abrogates TNF-induced apoptosis and also prevents recruitment of the ubiquitin ligase TRAF2 (Ermolaeva et al., 2008). The TLR negative regulator FLN29 interacts with TICAM-1, IPS-1, TRAF3, and TRAF6 (Sanada et al., 2008). Hence, although the specific interactions and mechanisms are unclear, TICAM-1 appears to be involved in TRAF-mediated signaling apart from TRAF6.

According to recent reports (Häcker et al., 2006; Oganesyan et al., 2006), cells lacking TRAF3 are defective in type I IFN responses induced by TLR3 and TLR4. Furthermore, the TLR3/4 adaptor, TICAM-1, associates with TRAF3 to activate the downstream IRF-3/7 kinases TBK1 and IKK-ε (Häcker et al., 2006; Oganesyan et al., 2006), suggesting that TRAF3 serves as a critical link between TLR adaptors and the downstream regulatory kinases important for type I IFN induction. However, the molecular interrelationship between TICAM-1 and TRAF2/6 (Supplementary data, Fig. S1) has not been clearly demonstrated.

The TLR3 adaptor TICAM-1 binds directly and indirectly to the TIR domain of TLR3 and TLR4, respectively (Oshiumi et al., 2003a,b), and participates as a molecular platform in assembling IRF-3/7-activating kinases (Funami et al., 2008). In this study, we attempted to identify the molecules recruited to TICAM-1 by yeast two-hybrid screening and immunoprecipitation assays. Here, we show that the TRAF family proteins directly bind TICAM-1 and demonstrate that TRAF2 and TRAF6 bind different sites of the N-terminal TICAM-1 and accelerate its polyubiquitination. Abrogation of TRAF2 and TRAF6 binding results in strong inhibition of TICAM-1-mediated IFN-β induction, which may be independent of the TRAF3 recruitment to TICAM-1.

2. Materials and methods

2.1. Cells and materials

HEK293 cells (RIKEN, Wako, Japan) were cultured in DMEM 10% fetal calf serum (FCS) as previously described (Sanada et al., 2008). The mouse macrophages cell subline RAW264.7 was maintained in RPMI 1640 containing 10% FCS (Hirano et al., 2002). Anti-FLAG M2 monoclonal Ab and anti-HA polyclonal Ab were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Plasmids with HA-tagged TICAM-1 (TICAM-1 (HA)) (Oshiumi et al., 2003a) and TICAM-1 with a mutated RIP homotypic interaction motif (TICAM-1 RHIM) (Funami et al., 2008) were provided as described. Myc-tagged human TRAF2 and TRAF6 were cloned using human HeLa cell-derived cDNA as a template. C-terminal domains of TRAF2 (TRAF2-C) and TRAF6 (TRAF6-C) were subcloned into a plasmid by a method similar to those reported in mouse counterparts (Ishida et al., 1996). Alanine substitution mutants of TICAM-1 were constructed by a reported method using a site-directed mutagenesis kit (Funami et al., 2004). The p-125 luc reporter containing the human IFN-β promoter region (−125 to +19) was a gift from Dr. T. Taniguchi (The University of Tokyo, Tokyo, Japan). Gal4-IRF-3, Gal4-DBD, and p55 UASG-Luc were used for IRF-3 activation (Yoneyama et al., 1998). NF-κB and AP-1 activation were determined as previously described (Oshiumi et al., 2003a).

2.2. Yeast two-hybrid screening

The yeast two-hybrid assay was performed as described previously (Oshiumi et al., 2003a). Briefly, the yeast strain AH109 (Clontech, Palo Alto, CA, USA) was transformed using bait (pGBKT7) and prey (pGADT7) plasmids. The resulting transformants were streaked onto plates and incubated for 3–5 days. A vector containing the TICAM-1 S1 fragment, which included the entire N-terminal domain, was constructed by inserting a TICAM-1 cDNA partial fragment encoding from aa 1–359 into the pGBKT7 multi-cloning site. Yeast two-hybrid screening was performed using human lung cDNA libraries resulting in the identification of 16 independent clones, six of which were positive after retesting in yeast. Of these clones, three encoded partial cDNAs of TRAF proteins. SD-WLH is a yeast synthetic dextrose medium that lacks Trp, Leu, and His amino acids. SD-WLHA lacks adenine in addition to Trp, Leu, and His. SD-WL lacks Trp and Leu and thus acts as a non-selective plate.

2.3. Immunoprecipitation

HEK293 cells were transfected in 6-well plates with plasmids encoding HA-tagged TICAM-1 (or the 1–533 aa mutant N+TIR) and those encoding either TRAF family proteins or TRAF C-domains as indicated in each figure. Twenty-four hours after transfection, total cell lysate was prepared using lysis buffer (50 mM HEPES [pH 7.5] containing 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 30 mM NaF, 5 mM Na_3Vo_4, 20 mM IAA, and 2 mM PMSF), and proteins were immunoprecipitated with either anti-HA polyclonal (SIGMA) or anti-FLAG M2 monoclonal Ab (SIGMA). The precipitated samples were resolved on SDS-PAGE gels, blotted onto a PVDF membrane, and then stained with anti-HA (HA1.1) monoclonal (SIGMA), anti-HA polyclonal, or anti-FLAG M2 monoclonal Ab.

2.4. Reporter gene assay

HEK293 cells (4×10^4 cells/well) cultured in 24-well plates were transfected with the expression vectors for TICAM-1, TICAM-1 RHIM, or TICAM-1 with mutated TRAF binding domains (AAS, PQA, E252A) or empty vector together with the reporter plasmid (100 ng/well) and an internal control vector, phRL-TK (Promega) (2.5 ng/well) using LepofectAMINE 2000 (Invitrogen) as described previously (Oshiumi et al., 2003a). The total amount of DNA (800 ng/well) was kept constant by adding empty vector. After 24 h, cells were lysed in lysis buffer (Promega), and the *Firefly* and *Renella* luciferase activities were determined using a dual-luciferase reporter assay kit (Promega). The *Firefly* luciferase activity was normalized by *Renella* luciferase activity and was expressed as the fold stimulation relative to the activity in vector-transfected cells. Experiments were performed three times in duplicate (unless otherwise indicated in the figure legend).

For the detection of IRF-3 activation, we used the GFL4-IRF-3 reporter gene assay as described previously (Yoneyama et al., 1998). Briefly, cells were transfected with the p55 UASG-Luc reporter plasmid together with Gal4-IRF-3 or Gal4-DBD. Twenty-four hours after transfection, cells were harvested to measure the expression of luciferase using the dual luciferase assay kit (Promega). Data were expressed as the means ± S.D.

2.5. RT-PCR

RAW264.6 or HEK293 cells were transfected with plasmids encoding the TICAM-1 mutants using FuGene6 (Roche) following the manufacturers' instructions. Twenty-four hours after transfection, total RNA was isolated using the RNeasy kit (Invitrogen). The sequences of the primer pairs and PCR conditions used to amplify mouse IFN-β and β-actin were identical to those previously described (Oshiumi et al., 2003b).

2.6. Confocal microscopy

HeLa cells (1.0×10^5 cells/well) were plated onto micro cover glass (Matsunami, Tokyo, Japan) in a 12-well plate. The following day, cells were transfected with the indicated plasmids

using Fugene HD (Roche Diagnostics) following the manufacturers' instructions. The total amount of DNA (0.6 μg/well) was kept constant by adding empty vector. Twenty-four hours after transfection, cells were fixed using acetone for 5 min and then permeabilized with PBS containing 0.2% Triton X-100 for 15 min. Fixed cells were blocked in PBS containing 1% BSA, and were labeled with the indicated primary Abs (2–10 μg/ml) for 60 min at room temperature (refer to the legend of Fig. 5). Alexa-conjugated secondary Abs (1:400) were used to visualize staining of the primary Abs. Nuclei were stained with DAPI (2 μg/ml) in PBS for 10 min before mounting the cells onto glass slides using PBS containing 2.3% DABCO and 50% glycerol. Cells were visualized at a magnification of ×63 with an LSM510 META microscope (Zeiss, Jena, Germany).

2.7. Ubiquitination assay

For the ubiquitination assay of TICAM-1, a plasmid encoding two, multiple HA-tagged ubiquitins was used. HEK293FT cells were transfected with pECFP-N1 plasmids containing either CFP-tagged TICAM-1 (or N+TIR) cDNA, pEF-BOS with FLAG-tagged TRAF2 cDNA, or pEF-BOS with 2× HA-tagged ubiquitin. Twenty-four hours after transfection, cells were lysed, and TICAM-1 and other proteins were then immunoprecipitated as described previously (Oshiumi et al., 2009a). The samples were analyzed by SDS-PAGE and stained with anti-HA polyclonal Ab (for detection of ubiquitination), anti-FLAG monoclonal Ab (for detection of TRAF2), or anti-GFP polyclonal Ab. The reproducibility of TICAM-1 ubiquitination was confirmed with additional experiments using purified protein components (McKenna et al., 2001) and K63R- and K48R-ubiquitins (Shieh et al., 2001).

The *in vitro* ubiquitination assay was performed with E1, His-tagged E2 (Mms2/Ubc13), and E3 (TRAF2) and the substrate TICAM-1, which were purified from protein-containing *E. coli* lysates by Ni–NTA column as described previously (McKenna et al., 2001).

2.8. Statistical analysis

Statistical analysis was performed using Student's *t*-test, the practical method of which was described previously (Hirano et al., 2002). Differences were considered significant when the *P* value was less than 0.05.

3. Results

3.1. Identification of proteins which bind the N-terminal region of TICAM-1

Yeast two-hybrid screening using human lung cDNA libraries and partial TICAM-1 fragments as bait allowed the identification of six human molecules which specifically bound the N-terminal fragment (aa 1–359) of TICAM-1: collagen type VIII alpha1, adenovirus E1A-binding protein (BS69), lamin A/C, TRAF1, TRAF2, and TRAF6 (data not shown). Interestingly, three of the six positive molecules were TRAF family proteins. Representative binding profiles of TRAF proteins to TICAM-1 are shown in Fig. 1a and b. Positive clones that bound the C-terminal fragment of TICAM-1 were also obtained, although none were TRAF proteins (data not shown). TRAF3, which acts as a crucial signaling adaptor for TICAM-1-mediated signaling (Häcker et al., 2006; Oganesyan et al., 2006), was not identified in the yeast two-hybrid assay (Supplementary data, Fig. S2). Although

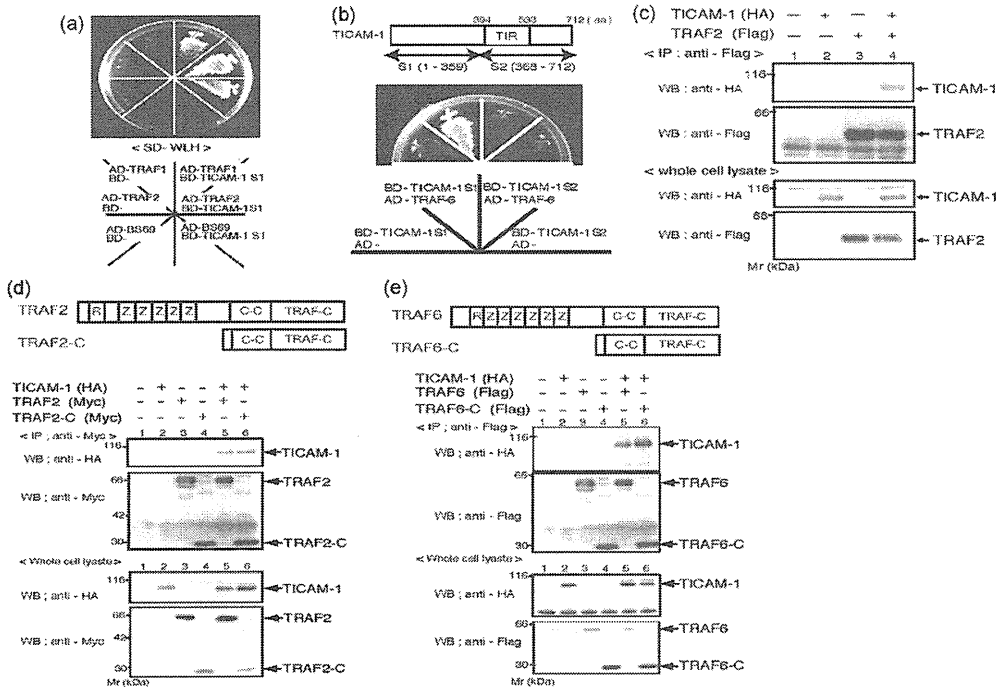

Fig. 1. Molecular interaction of TICAM-1 with TRAF2 and 6. (a) Direct interaction between the N-terminal region of TICAM-1 and either TRAF2 or TRAF6 as identified by yeast two-hybrid screening. Full-length TRAF2 and TRAF6 were cloned into pGADT7, transformed into yeast strain AH109, and then cultured on SD-WL plate for 3 days. Yeast cells transformed with both plasmids were selected on SD-WLH plates and the protein interactions were analyzed by yeast growth. (b) TRAF6 directly interacts with the TICAM-1 N-terminal region. Full-length TRAF6 cDNA was cloned into the pGADT7 vector and co-transformed with the TICAM-1 N-terminal (TICAM-1 S1: 1–359 aa) and C-terminal regions (TICAM-1 S2: 368–712 aa). The analysis method was identical to that indicated in (a). (c–e) Physiological binding of TRAF2 and TRAF6 to TICAM-1 in human cells. HEK293 cells were transfected with vectors for expression of the indicated proteins. Twenty-four hours after transfection, cells lysates were collected, immunoprecipitated, resolved on SDS-PAGE gels, and then subjected to immunoblotting. Control lanes with samples with IgG isotype i.p. had no significant bands (data not shown). Structural information about TRAF2 and TRAF6 is shown atop of (c) and (d). R, RING domain; Z, zinc finger domain, C-C, coiled-coil region; TRAF-C, the C-terminal domain unique to each TRAF.

the possibility of direct binding of TRAF3 to TICAM-1 in human cells cannot be ruled out, a direct interaction could not be confirmed using yeast.

To confirm the associations identified in the yeast two-hybrid assay, immunoprecipitation (I.P.) analyses was performed and supported the interaction of TRAF2 and TICAM-1 (Fig. 1c and d). A similar coprecipitation was observed between TICAM-1 and either TRAF2 or TRAF6 (Fig. 1d and e). Subsequent i.p. analyses revealed that the C-terminal domains, which are highly conserved in TRAFs (Chung et al., 2002), of TRAF2 and TRAF6 bind TICAM-1 (Fig. 1d and e) and indicates that this region of TRAF1, 2, and 6 directly interacts with the N-terminal region of TICAM-1.

We next attempted to determine the precise region of TICAM-1 responsible for TRAF2 binding. The TRAF domain, a conserved region of approximately 180 aa, in the C-terminus of TRAF2 interacts with target molecules through the binding consensus sequence motifs (P/S/A/T)x(Q/E)E, PxQxxD, and PxQx(T/S) (Pullen et al., 1998; Lu et al., 2003). There are two such motifs in the N-terminal region of TICAM-1, represented by AYQE and PLQLS which are located at aa 117–120 and aa 333–337, respectively. To determine if TRAF2 requires these consensus sequences for interacting with TICAM-1, we constructed several truncated mutants of the TICAM-1 N-terminal region and analyzed their interaction with TRAF2 using the yeast two-hybrid system (Fig. 2a). Deletion of the first 200 aa in the N-terminus of the TICAM-1 S1 fragment (dN200) did not affect its binding ability to TRAF2, however, deletion of aa 200–359 (N200) did prevent its association. The dN300 fragment, containing only aa 300–359, was sufficient for binding TRAF2. Hence, while the consensus sequence PLQLS in TICAM-1 is critical for binding TRAF2, the AYQE sequence is dispensable for the association.

It has been reported that there are two pattern mutations in the PxQxS consensus sequence, represented by PxQxA and AxAxS (Lu et al., 2003). We therefore constructed both mutations in TICAM-1 dN300 (TICdN300 PQA and TICdN300 AAS) and examined the ability of these mutated proteins to bind TRAF2 in yeast. It was observed that either mutation of the PxQxS motif in TICAM-1 abolished the binding to TRAF2 (Fig. 2b). These data clearly demonstrate that TRAF2 directly binds the PLQLS sequence of TICAM-1.

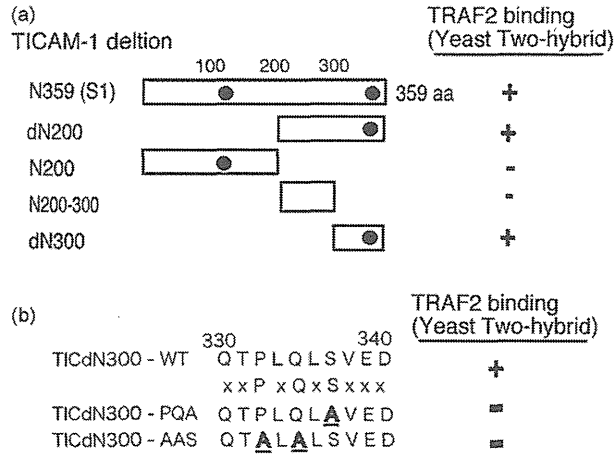

Fig. 2. Identification of the TRAF2-binding site in TICAM-1. (a) Scheme of TICAM-1 truncated mutants and location of the TRAF2 binding motif (black dot). TRAF2 in pGADT7 and each TICAM-1 construct in pGBKT7 were transformed into yeast. TRAF2 binding was assessed on SD-WLH plates as described in Fig. 1. (b) Specific consensus motif of TICAM-1 that directly binds to TRAF2. The predicted TRAF2 binding motif in TICAM-1 (TICdN300) was identified as the PxQxS sequence (300–359 aa). TICAM-1dN300 contained two alanine substitutions (TICdN300-PQA and TICdN300-AAS). These two alanine mutants were examined for their ability to bind TRAF2 by the yeast two-hybrid system. TRAF2 binding was assessed on SD-WLH plates.

We also confirmed a previous report which demonstrated that TRAF1 is a TICAM-1-interacting protein (Su et al., 2006). The TRAF-C domain of TRAF1 and the N + TIR domain of TICAM-1 were responsible for their interaction. In addition, it was shown that TRAF6 failed to couple with the E252A TICAM-1 mutant (data not shown) (Ye et al., 2002; Sato et al., 2003).

3.2. *The function of TRAF2 binding to TICAM-1*

As it has been reported that overexpression of TICAM-1 induces massive IFN-β promoter activation (Oshiumi et al., 2003a), the importance of TRAF binding in TICAM-1 signaling was examined by the ability of TICAM-1 mutant proteins to induce IFN-β promoter activation. As the C-terminal TICAM-1 region (containing the RHIM domain) recruits RIP1 and also activates NF-κB (Meylan et al., 2004), which is involved in IFN-β transcription and apoptosis signaling, we used a C-terminal-deleted TICAM-1 fragment, designated N + TIR (1–533 aa TICAM-1) (Funami et al., 2004) to eliminate the induced effects caused by C-terminal activity. Compared to the N + TIR fragment, which maintained wild-type levels of TICAM-1 IFN-β-inducing activity (Funami et al., 2004), the TICAM-1 PQA (S335A) mutation exhibited slightly reduced IFN-β promoter activation. However, the E252A mutation in TICAM-1, which is located in one of the TRAF6 binding motifs and facilitates TRAF6-TICAM-1 interaction (Jiang et al., 2004), largely impaired IFN-β promoter activation. Interestingly, a double mutation of E252A and PQA further reduced the activation compared to the E252A mutation alone (Fig. 3a). The reduction of IFN-β promoter activation was not caused by protein instability/degradation induced by the mutations, as the amount of N + TIR protein was nearly identical in the wild-type, PQA, E252A, and double-mutant TICAM-1 samples (Fig. 3a, inset). These data indicate that TRAF2 plays a role in TICAM-1-binding and activation of the IFN-β promoter, a conclusion which is supported by the effects of the TRAF6 site-mutation TICAM-1. Previous analysis concerning the role of TRAF6 in TICAM-1 signaling was performed with TRAF6- deficient mouse macrophages (Sato et al., 2003), and in those studies, TRAF2 was found to be intact. It is likely that TRAF6 was dispensable for TICAM-1 signaling due to the compensatory function of TRAF2 and suggests that TRAF2 expression levels would have affected the degree of activation of TICAM-1 signaling in *traf6* mutant cells.

We next examined IFN-β promoter activation and transcription of endogenous IFN-β mediated by full-length TICAM-1. In these experiments, a RHIM-mutated TICAM-1 (Meylan et al., 2004; Kaiser and Offermann, 2005) was used to circumvent apoptotic signaling by TICAM-1 and NF-κB activation through RIP1. A triple mutant of TICAM-1, consisting of E252A, PQA, and RHIM domain mutations, displayed a nearly complete abrogation of reporter activation (Fig. 3b) and induction of IFN-β transcription (Fig. 3c) compared to the wild-type and TICAM-1 RHIM-mutant. Taken together, these results indicate that the interaction of TRAF2 and TRAF6 with TICAM-1 is indispensable for IFN-β induction by overexpressed TICAM-1.

3.3. *Transcription factors activated by TRAF2/6*

IFN-β is transcribed by three transcription factors: NF-κB, IRF-3, and AP-1. To analyze which transcription factors are regulated by TRAF2/6 on TICAM-1 signaling, we performed a reporter gene assay for each of the three transcription factors using the TICAM-1 mutants (Fig. 4 and Supplementary data, Fig. S3). Although TRAF2 and TRAF6 are known to possess the ability to activate NF-κB, TICAM-1 with a mutated TRAF2-binding site (AAS and PQA) had increased activation of NF-κB compared to the control. The PQA/E252A double mutant displayed reduced NF-κB activation compared to the E252A mutant (Fig. 4b). Unexpectedly, the

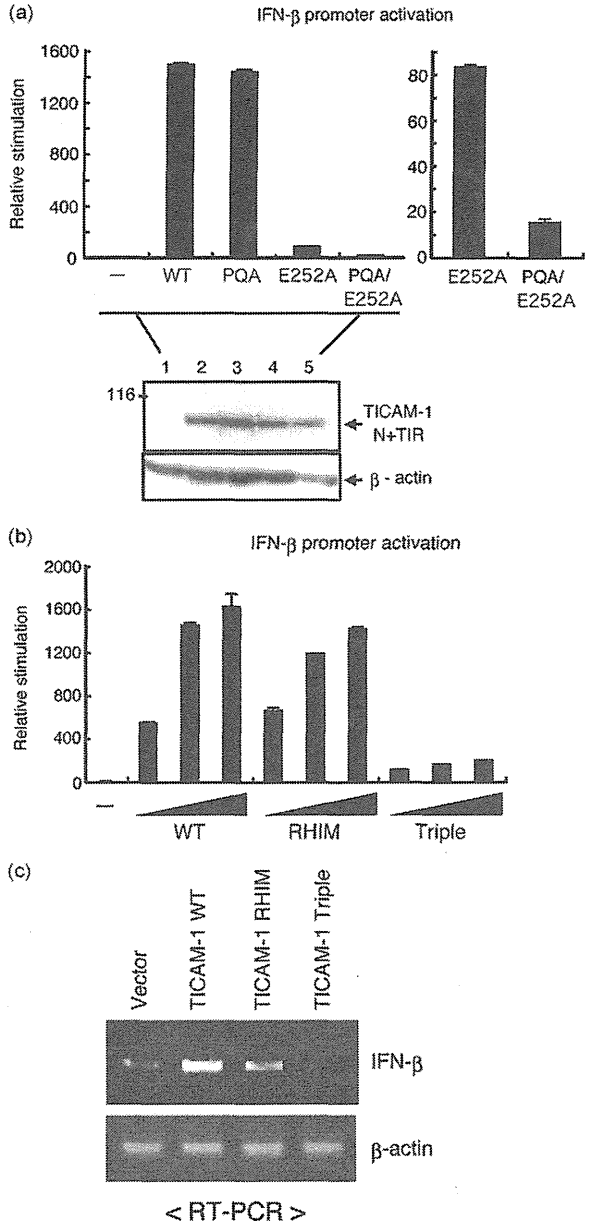

Fig. 3. TRAF2 and TRAF6 binding affect TICAM-1-mediated IFN-β induction. (a and b) HEK293 cells were transiently transfected with 10 ng (a) or 5, 25, 50 ng (b) of the indicated TICAM-1 mutant together with the p125-luc IFN-β promoter reporter plasmid (100 ng). Twenty-four hours after transfection, cells were harvested and the luciferase activities were measured. Data indicate the relative stimulation compared to vector transfection. Assays were performed three times in triplicate. One representative data set of three trials is shown. Bottom panel of (a) shows the expression level of each mutated TICAM-1. After the measurement of luciferase activity, each of the lysate samples were separated using SDS-PAGE, and the expression level of each mutant TICAM-1 was detected by anti-HA rabbit polyclonal antibody, and applied protein levels were detected by anti-β-actin mouse monoclonal antibody. (c) RAW 264.7 cells were transiently transfected with the indicated TICAM-1 constructs. Twenty-four hours after transfection, total RNA from the cells was isolated using RNeasy, and then reverse transcribed into cDNA. PCR was performed using primer sets for mouse IFN-β and β-actin, and PCR products were separated by gel electrophoresis on 1% agarose gels.

E252A/PQA double mutant also had impaired activation of IRF-3 (Fig. 4a) and perhaps to a lesser extent, AP-1 (Fig. 4c). For each transcription factor except for AP-1, a more profound suppression of transcriptional activation was observed in the E252A/PQA double mutant than in the E252A single mutant (Supplementary data, Fig. S3). These results indicate that the IFN-β-inducing signal is due to TICAM-1 oligomerization (Funami et al., 2008) which is regulated by the presence of TRAF2/6 proteins. Although endogenous TICAM-1 is usually present at low levels in non-stimulated cells and may still affect the reporter output, efficient inhibition of reporter activation by the double mutant was reproducible in repetitive experiments (Fig. 4 and Supplementary data, Fig. S3).

3.4. $PQ^{337}S$ and ^{252}E of TICAM-1 N+TIR are critical for TRAF2- and 6-recruitment

To clarify the participation of the single site of TICAM-1 N+TIR for TRAF2- and TRAF6-binding, confocal microscopy imaging analysis using labeled TRAF2, TRAF6, and TICAM-1 N+TIR was used (Fig. 5). While TRAF2 and TRAF6 merged with TICAM-1 N+TIR (upper panels) in overlayed images, under identical test conditions, both largely failed to overlap with the triple mutant (N+TIR/PQA/E252A) (lower panel). Using the TICAM-1 N+TIR mutants, it was confirmed that the PQA and E252A mutations resulted in non-overlapped images with TRAF2 and TRAF6, respectively (data not shown). Thus, there is a single site for TRAF2- and TRAF6-binding in N+TIR of TICAM-1 which is critical for IFN-β promoter activation and TICAM-1 N-terminal oligomerization.

Full-length TICAM-1 containing even single N-terminal mutations was still observed to merge with TRAF2 and TRAF6 in confocal images (data not shown), suggesting that TRAF2 and 6 bind to the C-terminal region of TICAM-1 in addition to the N-terminal sites. Under the same conditions, TRAF3 was recruited to TICAM-1 N+TIR as well as the triple mutant (Fig. 5, right panels). Since TICAM-1 has a TBK1-binding site apart from these TRAF-binding sites (Funami et al., 2007), TRAF3 may bind multiple regions of TICAM-1. Ultimately, the function of TICAM-1 may not be reflecting merely binding to TRAF3 as TICAM-1 overexpression induces TRAF2/6-mediated IFN-β promoter activation.

3.5. TICAM-1 ubiquitination induced by TRAF2

TRAF proteins are E3 ligases involved in the ubiquitination of proteins, an event which is often necessary for the activation of IRF-3. Although ubiquitination is prominent in RIG-I-mediated IRF-3 activation, TICAM-1 is also ubiquitinated during polyI:C stimulation or overexpression (Oshiumi et al., 2003a). We therefore tested whether TRAF2 ubiquitinates TICAM-1 in a similar manner to the activation of RIG-I by TRIM25, which leads to IRF-3 dimerization. When the N+TIR TICAM-1 fragment was co-expressed with TRAF2, the slow migration form of TICAM-1 was observed by SDS-PAGE and immunoblotting (Fig. 6a). To determine whether TICAM-1 was ubiquitinated, CFP-tagged TICAM-1, Flag-tagged TRAF2, and HA-tagged ubiquitin were first co-expressed in HEK293 cells, and lysates were immunoprecipitated with anti-GFP Ab to precipitate TICAM-1-CFP before being subjected to SDS-PAGE and i.p. analyses (Fig. 6b). After staining the blots with anti-HA Ab, high-molecular weight smeared HA-ubiquitin bands appeared when TICAM-1 was immunoprecipitated with anti-GFP Ab, while smeared ubiquitin bands were only slightly visible in the absence of TICAM-1 or TRAF2 (Fig. 6b).

To confirm the TRAF2-mediated polyubiquitination of TICAM-1, an *in vitro* ubiquitination assay was conducted using purified ubiquitin, E1, E2, TRAF2, and TICAM-1 proteins. The protein purity was determined by CBB staining of SDS-PAGE gels to be over 80% (Fig. 6c and data not shown). As revealed by immunoblotting using

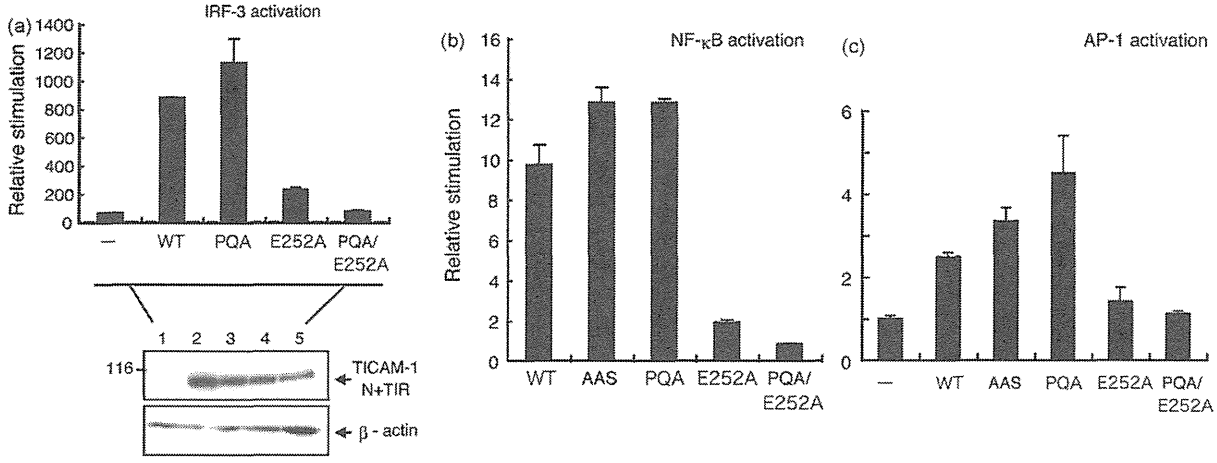

Fig. 4. Complete TRAF2/6 binding to TICAM-1 leads to activation of the transcription factors IRF-3, NF-κ B, and AP-1. (a–c) HEK293 cells were transiently transfected with 10 ng (a) or 200 ng (b and c) of the indicated TICAM-1 mutant plasmid together with p55 UASG-Luc reporter and either GAL4-IRF3 (a), NF-κB reporter (b) or AP-1 reporter (c) plasmid. Twenty-four hours after transfection, cells were harvested and the luciferase activities were measured. Data indicate the relative stimulation compared to empty vector transfection. Assays were performed three times in triplicate. The data are representative of three independent experiments. The bottom panel of (a) shows the expression level of TICAM-1 N + TIR. The method was identical to that described in Fig. 3.

anti-TICAM-1 Ab, *in vitro* TICAM-1 polyubiquitination was clearly observed only in the presence of the ubiquitin ligases and TRAF2 (Fig. 6d). Taken together, these data suggest that TRAF2 polyubiquitnates TICAM-1 to modify its function, although we could not determine whether TRAF2-mediated ubiquitination is specifically induced on TICAM-1.

Next, to determine which lysine residue of ubiquitin is used for polymerization, CFP-labeled TICAM-1 was transfected into HEK293 cells together with HA-labeled wild-type or mutant ubiquitin (K48R or K63R). After comparing the TICAM-1 ubiquitination profiles, K48R ubiquitin only marginally reduced polyubiquitination of TICAM-1 by TRAF2 (6.2 vs. 4.4) while ubiquitination by K63R was rarely observed on TICAM-1 by TRAF2 (6.2 vs. 1.4) in HEK cells (Fig. 6e). These data suggest that K63-linked polyubiquitination is dominant on TICAM-1 through the action of TRAF2 E3 ligase. The predominance of K63-linked polyubiquitination of TICAM-1 is consistent with the observation that TRAF2 and TRAF6 are important for sustaining (rather than impairing) TICAM-1 signaling. To confirm these results, TICAM-1 N + TIR with mutated TRAF2- and/or TRAF6-binding sites were used as substrates of ubiquitination in HEK293 cells. Although both the PQA and E252A mutants displayed reduced polyubiquitination, the modification was still observed at a higher frequency than the control (Fig. 6f). The PQA/E252A double mutation almost completely abrogated ubiquitination in parallel with the failure to recruit TRAF2/6 to TICAM-1 N + TIR (Fig. 6f). These data indicate that TRAF2 and TRAF6 have redundant functions with

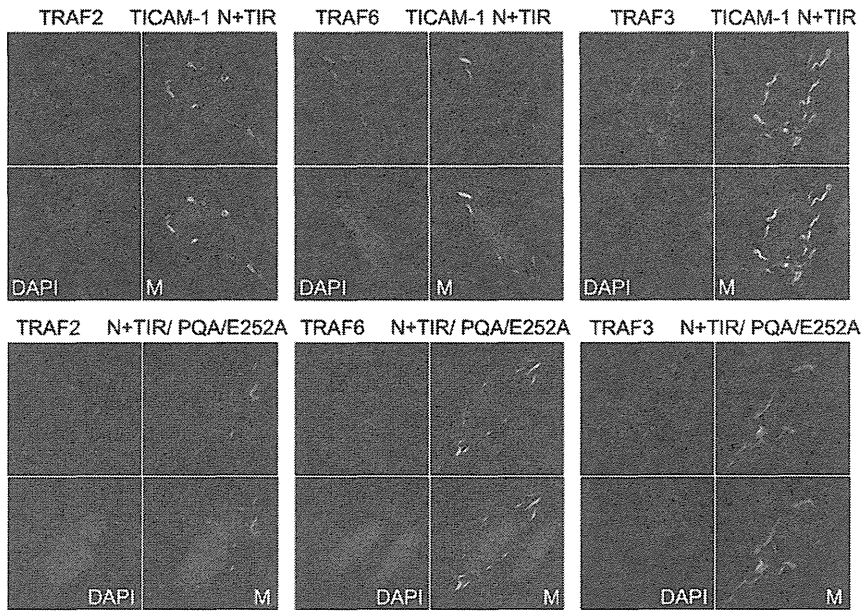

Fig. 5. Co-localization analysis of TICAM-1 N + TIR and TRAF proteins by confocal microscopy. HeLa cells were transfected with 30 ng of pECFP-N1 TICAM-1 and either 500 ng of pEF-BOS Flag-tagged TRAF2, TRAF6, or TRAF3. After 24 h, the cells were fixed, stained with anti-FLAG Ab, and then visualized with Alexa Fluor 568-conjugated secondary Ab. The same slide was also treated with DAPI for the staining of nuclei. M, Merging profile.

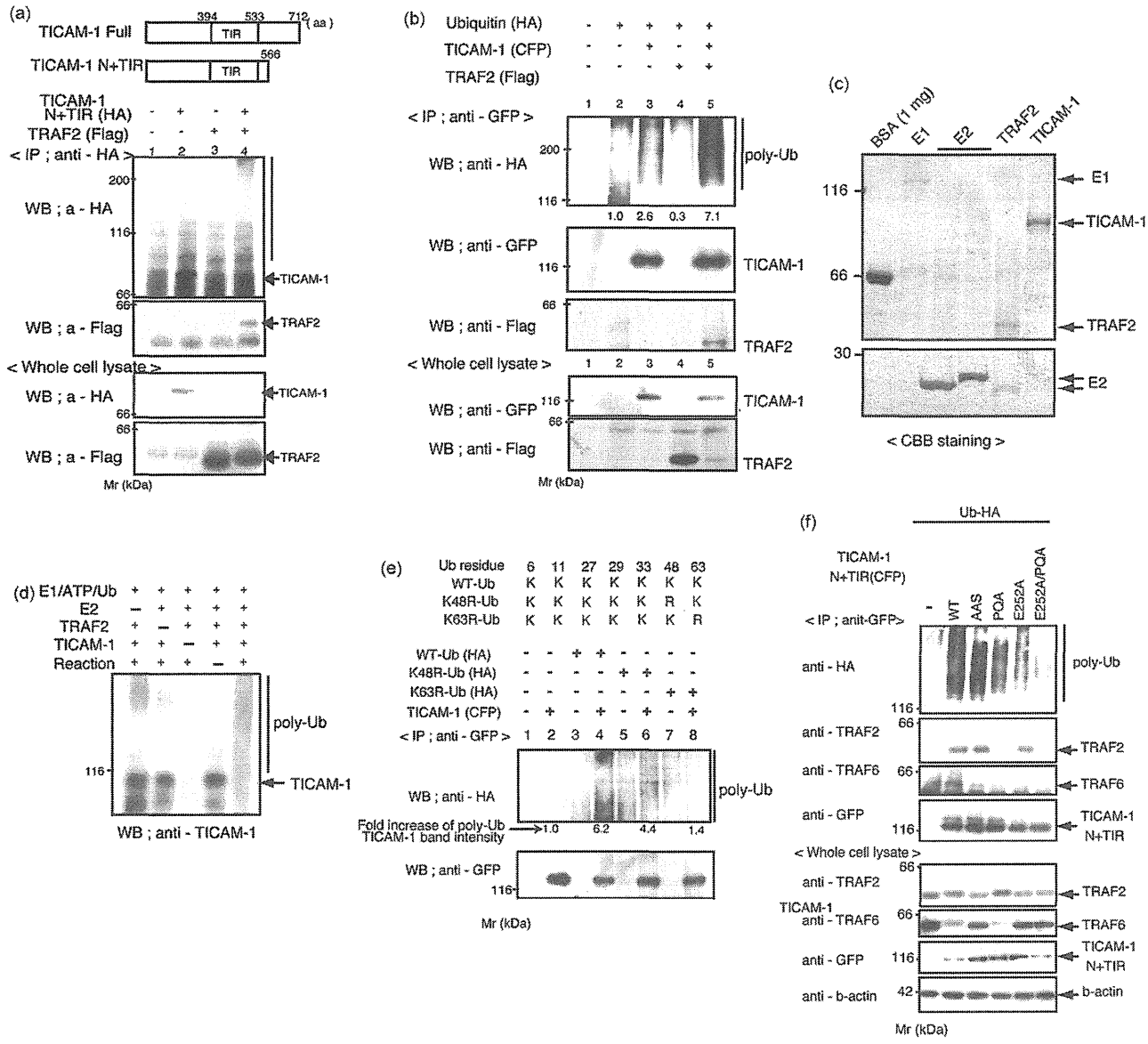

Fig. 6. TRAF2-mediated polyubiquitination of TICAM-1. (a) Interaction between TICAM-1, TICAM-1 N+TIR, and TRAF2. HEK293 cells were transfected with TICAM-1 N+TIR (HA) plasmid (100 ng) and TRAF2 (Flag) plasmid (2 μg) for expression of the indicated proteins. The total amount of DNA (4 μg/well) was kept constant by adding empty vector. Twenty-four hours after transfection, cells lysates were collected, proteins were immunoprecipitated, and the samples were analyzed by SDS-PAGE followed by immunoblotting. The upper two panels show immunoblots using the indicated Abs indicated, while the lower two panels show immunoblots of total cell lysates. (b) TICAM-1 polyubiquitination induced by TRAF2. HEK293 cells were transfected with plasmids for expression of TICAM-1 (CFP) (1 μg), TRAF2 (Flag) (1 μg) and/or ubiquitin (HA) (1 μg) as indicated. After 24 h, cells were lysed and proteins were immunoprecipitated with anti-GFP Ab. Ubiquitin (HA), TICAM-1 (GFP), and TRAF2 (FLAG) were probed with anti-HA, ant-GFP and anti-FLAG Abs, respectively, in the above blots. The protein content in each lysate is indicated at the bottom of the first immunoblot. The relative intensity of ubiquitination was measured using a densitometer. (c) Purity of the proteins used for *in vitro* ubiquitination. Purified proteins were resolved on SDS-PAGE gels (8%) and stained with Commassie brilliant blue to assess the protein concentration in each sample. (d) *In vitro* polyubiquitination of TICAM-1. The combinations of proteins used are indicated above the immuoblot. For the assay, 0.1 μg of E1, 0.5 μg of E2 (MMS2 0.25 μg, Ubc13 0.25 μg), 0.5 μg of TRAF2, 1 μg of ubiquitin and/or 1 μg of TICAM-1 proteins were incubated for 12 h at 30 °C in 20 μl of the reaction buffer (30 mM HEPES (pH7.5), 2 mM ATP, 5 mM $MgCl_2$, 0.2 mM DTT, 1 mM creatine phosphate, 10 U phosphocreatine kinase, and 10 mM phosphocreatine). The proteins were analyzed by SDS-PAGE, and immunoblotting was performed with anti-TICAM-1 Ab. The positions of TICAM-1 and ubiquitination are indicated to the right of the immunoblot. (e) K63-mediated polyubiquitination is dominant in TICAM-1. HEK293 cells were transfected with plasmids encoding TICAM-1 (CFP) (1 μg) and each ubiquitin (HA) plasmid (1 μg). The K48R and K63R ubiquitins were tested for site-specific ubiquitination by immunoprecipitation and blotting with anti-HA. TICAM-1 contents in the lysates are shown to the bottom. The fold increase in polyubiquitination (poly-Ub) intensities of the TICAM-1 band is indicated between the two immunoblots. (f) Effect of mutations of the TRAF2/6 binding site on the degree of TICAM-1 ubiquitination. HEK293 cells were transfected with plasmids encoding TICAM-1 N+TIR (CFP) (100 ng) and ubiquitin (HA) (1 μg). Cell lysates were analyzed by immunoprecipitation and blotting as indicated for each immunoblot. The positions of TRAF2, TRAF6, N+TIR, and β-actin (control) are indicated to the right of each immunoblot.

respect to protein modification by K63 ubiquitination, an observation which correlates with the ability of the N+TIR fragment to activate the IFN-β promoter and the IRF-3 and NF-κB transcription factors.

4. Discussion

Here we demonstrated that the N-terminal region of TICAM-1 recruits TRAF2 and TRAF6 through activation/oligomerization

and that both the TRAF6- and TRAF2-binding sites participate in TICAM-1-mediated IFN-β-induction. Although other TRAF2- and TRAF6-binding sites in the C-terminal region may further modify TICAM-1 function, we focused on the functional modulation of TICAM-1 N+TIR by TRAF2/6, and revealed that the TRAF2 site of TICAM-1 serves as a functional modulator in the absence of the TRAF6 site (Figs. 3 and 4 and Supplementary data, Fig. S3). Tantalized points are that (1) TICAM-1 polyubiquitination occurs essentially in parallel with TRAF2/6 function (Fig. 6), although the role of the ubiquitination has yet to be decisively revealed; (2) TRAF3 binding to TICAM-1 N+TIR unexpectedly appears to remain intact even by the mutation of TRAF2/6 sites in TICAM-1, which suggests that other sites mediate TRAF3-TICAM-1 N+TIR interaction (Fig. 5). Although beyond the scope of this study, experiments are underway to address these unsettled points.

Sato et al. (2003) reported that TRAF6 binds to TICAM-1 and plays an important role in NF-κB activation in TICAM-1-mediated signaling. However, *traf6*−/− knockout analysis showed that deletion of the TRAF6 gene does not impair cytokine production or transcription factor activation by TLR3 (Häcker et al., 2006; Oganesyan et al., 2006). Although this difference was attributed to preferential usage of TRAF subtypes by specific cell types (Gohda et al., 2004), these results suggest that TRAF6 is not the only TRAF that satisfy TLR3 signaling. We therefore constructed TICAM-1 mutants with disrupted TRAF6- or TRAF2-binding sites and demonstrated these sites are critical for mediating the function of TICAM-1, including IFN-β induction. To the best of our knowledge, this is the first study to provide evidence for the redundancy of TRAF2 and TRAF6 in TICAM-1-stimulated NF-κB and IRF-3 activation, and the possibility of K63-linked polyubiqutination for modifying the function of the TLR adaptor protein TICAM-1.

By mutational analysis, we found that disruption of the TRAF2-binding motif of TICAM-1 alone had very little effect on the activation of the IFN-β promoter. However, mutations in both the TRAF2- and TRAF6-binding sites resulted in reduced activation of the TICAM-1 pathway as well as TICAM-1 polyubiquitination. Synergistic activation of TRAF2 and TRAF6 may therefore increase the activation of the IFN-β promoter by the TICAM-1 pathway. Although the importance of TRAF2 has been demonstrated in TRAF2 KO mice, which are embryonic lethal due to apoptosis of hepatocytes, the definitive *in vivo* role of TRAF2 in this pathway is unknown. As previously suggested using *traf6*-disrupted cells, different results were obtained (Kawai and Akira, 2007; Sato et al., 2003; Gohda et al., 2004). Indeed, the expression levels of TRAF2 and TRAF6 differ depending on the cell type (Chung et al., 2002). In addition, our results further demonstrate the involvement of TRAF2 in the TICAM-1 pathway, although TRAF2 activity may be masked in cells with sufficient levels of TRAF6. The unsettled discrepancy in previous reports (Sato et al., 2003; Gohda et al., 2004) regarding the relative importance of TRAF6 in the TICAM-1 pathway may be explained by the joining of TRAF2 and TRAF6 in this pathway. A similar synergistic action of TRAF2 and TRAF6 was reported with CD40, which also recruits TRAF3 and induces the activation of multiple pathways (Davies et al., 2005). It can be concluded that in at least some cell lines and organs, TRAF2 may play a significant role in the TICAM-1 pathway, as in the case of CD40.

TRAF2 and TRAF6 are involved in many cytokine-producing pathways, such as IL-1R and RIG-I signaling. In the cytoplasmic virus recognition system, the adaptor of RIG-I/MDA5, termed IPS-1 (MAVS/Cardif/VISA), requires both TRAF2 and TRAF6 to activate NF-κB, but not for the activation of IRF-3 (Guo and Cheng, 2007). In contrast, TICAM-1 unequivocally employs both TRAF2 and TRAF6 for activation of both these transcription factors. TRAF2 and TRAF6 were also shown to act as E3 ligases for K63 ubiquitination of TICAM-1. It has been reported that TICAM-1 is an essential adaptor for the IFN-inducing pathway and has a unique role in driving predominant NK cell activation in dendritic cells (Akazawa et al., 2007). The functions of TICAM-1 may be dependent on the cell type and require the differential usage of TRAF2 and TRAF6 for signaling, as demonstrated in the IPS-1 pathway.

TRAF1 has been characterized as a TICAM-1-binding protein capable of negatively regulating TICAM-1 function (Su et al., 2006). The TRAF-C domain of TRAF1 and the TIR domain of TICAM-1 are responsible for their interaction, and overexpression of TRAF1 inhibits TLR3/TICAM-1-mediated activation of NF-κB, IFN-stimulated response element, and the IFN-β promoter. TRAF4 is also involved in the underlying mechanisms for silencing TLR-mediated signaling through the interaction with molecules harboring phagosome/endosome membrane (Takeshita et al., 2005). These TRAF family proteins thus bind TLRs to exert their inhibitory functions. As most TRAF family proteins bind TICAM-1, many modes of compensation might be involved in TICAM-1 ubiquitination and IFN-inducing signal modification.

Although TRAF3 is essential for TICAM-1 mediated IRF-3 activation (Häcker et al., 2006; Oganesyan et al., 2006), we were unable to detect the direct binding of TRAF3 to TICAM-1 in yeast cells. Confocal analysis did, however, suggest that TRAF3 is involved in the molecular complex containing TICAM-1, an observation which is consistent with previous reports (Häcker et al., 2006; Oganesyan et al., 2006). Although TRAF2 and TRAF3 compete for the same site in CD40 for their binding (Sanada et al., 2008), this determination was not feasible in the TICAM-1 molecule due to the fact that even in the N+TIR mutant, multiple TRAF3-binding sites are present, making it difficult to analyze TRAF2/6-mediated TRAF3 recruitment. Since TRAF3-binding to TICAM-1 is not completely abrogated by mutation of the TRAF2/6 sites in TICAM-1, it is most likely that TICAM-1 indirectly interacts with TRAF3 besides TRAF2/6. This TRAF3 recruitment may independently occur in addition to the event such that the TRAF2/6 associated with TICAM-1 in turn recruits TRAF3. TRAF3 can also couple with TAK1 and MAP to deliver signals to NEMO (IKKγ) (Leo et al., 2002) which suggests the reported IRF-3-activating kinase complex NAP1-IKKε/TBK1 (Sasai et al., 2005), consisting of a regulatory subunit and kinases, functions downstream of TRAF3 to form the TICAM-1 signalosome (Funami et al., 2007). This would explain why TBK1 and IKKε coprecipitate with TICAM-1 in polyI:C-activated cells (Oshiumi et al., 2010).

From the observed activation-mediated coprecipitation of TICAM-1 and IRF-3-activating kinases in this study, we speculate that TRAF proteins polyubiquitinate TICAM-1, as well as other assembled proteins, leading to the formation of a tight complex. Our protein-expression studies also suggest that TICAM-1 polyubiquitination and IRF-3 activation are correlated in HEK293 cells (Fig. 6). Furthermore, the involvement of A20 K63 deubiquitination enzyme in the regulation of TLR3 signaling have been reported (Wang et al., 2004; Saitoh et al., 2005). Although the specific details of time-dependence between TICAM-1 K63 ubiquitination and the induction of IFN-β remain to be investigated, we speculate that polyubiquitination of TICAM-1 by TRAF2 and TRAF6 is required for TICAM-1 to induce IRF-3 and NF-κB activation. This is supported by the observation that polyubiquitination of TICAM-1 was required for TRAF3-binding to TICAM-1 (Oganesyan et al., 2006). However, which E3 ligase is the best enhancer for TICAM-1 is a topic to be addressed in future experiments.

Acknowledgements

We thank the members of our laboratory for invaluable discussions. This work was supported in part by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT, Sapporo Biocluster "Bio-S", the Knowledge Cluster Initiative

of the MEXT, Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan, Mitsubishi Foundation, Mochida Foundation, NorthTec Foundation and Yakult Foundation. M.S was supported by a Research Fellowship of the Japan Society for the Promotion of Science. Dr. Greg Newton reviewed the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.12.002.

References

Akazawa, T., Ebihara, T., Okuno, M., Okuda, Y., Shingai, M., Tsujimura, K., Takahashi, T., Ikawa, M., Okabe, M., Inoue, N., Okamoto-Tanaka, M., Ishizaki, H., Miyoshi, J., Matsumoto, M., Seya, T., 2007. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc. Natl. Acad. Sci. U.S.A. 104, 252–257.

Chung, J.Y., Park, Y.C., Ye, H., Wu, H., 2002. All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. J. Cell. Sci. 115, 679–688 (Review).

Davies, C.C., Mak, T.W., Young, L.S., Eliopoulos, A.G., 2005. TRAF6 is required for TRAF2-dependent CD40 signal transduction in nonhemopoietic cells. Mol. Cell. Biol. 25, 9806–9819.

Ermolaeva, M.A., Michallet, M.C., Papadopoulou, N., Utermöhlen, O., Kranidioti, K., Kollias, G., Tschopp, J., Pasparakis, M., 2008. Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat. Immunol. 9, 1037–1046.

Funami, K., Matsumoto, M., Oshiumi, H., Akazawa, T., Yamamoto, A., Seya, T., 2004. The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. Int. Immunol. 16, 1143–1154.

Funami, K., Sasai, M., Ohba, Y., Oshiumi, H., Seya, T., Matsumoto, M., 2007. Spatiotemporal mobilization of Toll/1L-1 receptor domain-containing adaptor molecule-1 in response to dsRNA. J. Immunol. 179, 6867–6872.

Funami, K., Sasai, M., Oshiumi, H., Seya, T., Matsumoto, M., 2008. Homo-oligomerization is essential for Toll/interleukin-1 receptor domain-containing adaptor molecule-1-mediated NF-kappaB and interferon regulatory factor-3 activation. J. Biol. Chem. 283, 18283–18291.

Gohda, J., Matsumura, T., Inoue, J., 2004. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not Toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. J. Immunol. 173, 2913–2917.

Guo, B., Cheng, G., 2007. Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK. J. Biol. Chem. 282, 11817–11826.

Häcker, H., Vabulas, R.M., Takeuchi, O., Hoshino, K., Akira, S., Wagner, H., 2000. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. J. Exp. Med. 192, 595–600.

Häcker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.C., Wang, G.G., Kamps, M.P., Raz, E., Wagner, H., Häcker, G., Mann, M., Karin, M., 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 439, 204–207.

Hirano, A., Kurita-Taniguchi, M., Katayama, Y., Matsumoto, M., Wong, T.C., Seya, T., 2002. Ligation of human CD46 with purified complement C3b or F(ab')(2) of monoclonal antibodies enhances isoform-specific interferon gamma-dependent nitric oxide production in macrophages. J. Biochem. 132, 83–91.

Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T., Inoue, J., 1996. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. J. Biol. Chem. 271, 28745–28748.

Jiang, Z., Mak, T.W., Sen, G., Li, X., 2004. Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. Proc. Natl. Acad. Sci. U.S.A. 101, 3533–3538.

Kaiser, W.J., Offermann, M.K., 2005. Apoptosis induced by the Toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. J. Immunol. 174, 4942–4952.

Kawai, T., Akira, S., 2007. Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. 13, 460–469 (Review).

Leo, A., Wienands, J., Baier, G., Horejsi, V., Schraven, B., 2002. Adapters in lymphocyte signaling. J. Clin. Invest. 109, 301–309 (Review).

Lu, L.F., Cook, W.J., Lin, L.L., Noelle, R.J., 2003. CD40 signaling through a newly identified tumor necrosis factor receptor-associated factor 2 (TRAF2) binding site. J. Biol. Chem. 278, 45414–45418.

Mansell, A., Brint, E., Gould, J.A., O'Neill, L.A., Hertzog, P.J., 2004. Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)-6 to mediate NF-kappaB activation by Toll-like receptor (TLR)-2 and TLR4. J. Biol. Chem. 279, 37227–37230.

McKenna, S., Spyracopoulos, L., Moraes, T., Pastushok, L., Ptak, C., Xiao, W., Ellison, M.J., 2001. Noncovalent interaction between ubiquitin and the human DNA repair protein Mms2 is required for Ubc13-mediated polyubiquitination. J. Biol. Chem. 276, 40120–40126.

Meylan, E., Burns, K., Hofmann, K., Blancheteau, V., Martinon, F., Kelliher, M., Tschopp, J., 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat. Immunol. 5, 503–507.

Oganesyan, G., Saha, S.K., Guo, B., He, J.Q., Shahangian, A., Zarnegar, B., Perry, A., Cheng, G., 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature 439, 208–211.

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., Seya, T., 2003a. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat. Immunol. 4, 161–167.

Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M., Seya, T., 2003b. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-beta. J. Biol. Chem. 278, 49751–49762.

Oshiumi, H., Matsumoto, M., Hatakeyama, S., Seya, T., 2009a. Riplet/RNF135, a RING-finger protein, ubiquitinates RIG-I to promote interferon-b induction during the early phase of viral infection. J. Biol. Chem. 284, 807–817.

Oshiumi, H., Sakai, K., Matsumoto, M., Seya, T. 2010. DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-β inducing potential. Eur. J. Immunol (in press).

Pullen, S.S., Miller, H.G., Everdeen, D.S., Dang, T.T., Crute, J.J., Kehry, M.R., 1998. CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. Biochemistry 37, 11836–11845.

Saitoh, T., Yamamoto, M., Miyagishi, M., Taira, K., Nakanishi, M., Fujita, T., Akira, S., Yamamoto, N., Yamaoka, S., 2005. A20 is a negative regulator of IFN regulatory factor 3 signaling. J. Immunol. 174, 1507–1512.

Sanada, T., Takaesu, G., Mashima, R., Yoshida, R., Kobayashi, T., Yoshimura, A., 2008. FLN29 deficiency reveals its negative regulatory role in the Toll-like receptor (TLR) and retinoic acid-inducible gene I (RIG-I)-like helicase signaling pathway. J. Biol. Chem. 283, 33858–33864.

Sasai, M., Oshiumi, H., Matsumoto, M., Inoue, N., Fujita, F., Nakanishi, M., Seya, T., 2005. Cutting edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. J. Immunol. 174, 27–30.

Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K., Akira, S., 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J. Immunol. 171, 4304–4310.

Shieh, H.L., Chen, Y., Brown, C.R., Chiang, H.L., 2001. Biochemical analysis of fructose-1,6-bisphosphatase import into vacuole import and degradation vesicles reveals a role for UBC1 in vesicle biogenesis. J. Biol. Chem. 276, 10398–10406.

Su, X., Li, S., Meng, M., Qian, W., Xie, W., Chen, D., Zhai, Z., Shu, H.B., 2006. TNF receptor-associated factor-1 (TRAF1) negatively regulates Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF)-mediated signaling. Eur. J. Immunol. 36, 199–206.

Takeshita, F., Ishii, K.J., Kobiyama, K., Kojima, Y., Coban, C., Sasaki, S., Ishii, N., Klinman, D.M., Okuda, K., Akira, S., Suzuki, K., 2005. TRAF4 acts as a silencer in TLR-mediated signaling through the association with TRAF6 and TRIF. Eur. J. Immunol. 35, 2477–2485.

Wang, Y.Y., Li, L., Han, K.J., Zhai, Z., Shu, H.B., 2004. A20 is a potent inhibitor of TLR3- and Sendai virus-induced activation of NF-kappaB and ISRE and IFN-beta promoter. FEBS Lett. 576, 86–90.

Ye, H., Arron, J.R., Lamothe, B., Cirilli, M., Kobayashi, T., Shevde, N.K., Segal, D., Dzivenu, O.K., Vologodskaia, M., Yim, M., Du, K., Singh, S., Pike, J.W., Darnay, B.G., Choi, Y., Wu, H., 2002. Distinct molecular mechanism for initiating TRAF6 signalling. Nature 418, 443–447.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., Fujita, T., 1998. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. EMBO J. 17, 1087–1095.

Review Article

Pattern recognition receptors of innate immunity and their application to tumor immunotherapy

Tsukasa Seya,[1] Hiroaki Shime, Takashi Ebihara, Hiroyuki Oshiumi and Misako Matsumoto

Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

(Received October 7, 2009/Accepted November 10, 2009/Online publication January 4, 2010)

Dendritic cells (DC) begin maturation in response to complex stimuli consisting of antigens and pattern molecules (PAMP) for the activation of the immune system. Immune adjuvant usually contains PAMP. Infection represents one event that is capable of inducing such a complex set of stimuli. Recently, DC were subdivided into a number of subsets with distinct cell-surface markers, with each subset displaying unique differential maturation in response to pattern molecules to induce various types of effector cells. In the present study, we review how pattern recognition molecules and adaptors in each DC subset drive immune effector cells and their effect in the stimulated DC. Although tumor cells harbor tumor-associated antigens, they usually lack PAMP. Hence, we outline the properties of exogenously-added PAMP in the modulation of raising tumor immunity. In addition, we describe the mechanism by which DC-dependent natural killer activation is triggered for the induction of antitumor immunity. (*Cancer Sci* 2010; 101: 313–320)

Adjuvants are typically administered with target antigens in order to enhance the host immune response. Freund complete adjuvant (FCA), Freund incomplete adjuvant (FIA), and hydrated alumina (alum) are representative adjuvants that are used as antigen conjugates to potentiate immune responses and antibody production in animals. Although the mechanism by which these reagents enhance immunity was not completely understood, it appeared that the addition of adjuvants to antigens potentially induced immunity by "making it dirty".[1] However, more recently the agonistic features of adjuvants for pattern-recognition receptors (PRR) have been highlighted based on elucidation of the ligand properties of Toll-like receptors (TLR) and TLR-mediated dendritic cell (DC) maturation. The accumulated evidence on TLR-dependent DC maturation has solidified the current understanding that DC TLR confer the direction of the effector driving on the DC that present antigens. We hold that antigens determine the object toward which immune cells are proliferated, whereas adjuvants determine what effectors will be selected for immunological output.[2] The fundamental concepts of the immune system should be re-evaluated through the understanding of TLR-mediated DC immune responses, which will also revolutionize the concepts related to antitumor immunity.

The two major arms of the innate immune signaling pathway, the MyD88 and toll-interleukin 1 receptor domain (TIR)-containing adoptor molecule (TICAM-1) pathways (Fig. 1), have been identified through the investigation of TLR signaling.[3] Although MyD88 is dominant in mammals living on land, most aquatic vertebrates preferentially use TICAM-1 for TLR signaling.[4] TLR employing MyD88 adaptors usually recognize bacterial patterns, whereas TLR taking TICAM-1 recognize virus products, including nucleic acids. In addition to these PRR, the retinoic acid-inducible protein I (RIG-I)-like receptor and nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor (NLR) systems are located in the cytoplasm[5,6] and are inherent in most animals.[7] PRR systems are also distributed across the cell membrane and cytoplasm. The mineral oil component of FIA, crystallized uric acid, and alum are able to activate the NLR-inflammasome pathway,[5] which yields interleukin (IL)-1β and IL-18. These cytokines in turn stimulate their respective receptors to activate the MyD88 pathway in myeloid DC (mDC).[8] The activation of the MyD88 pathway in mDC is a common feature in bacterial stimulation.

The MyD88 pathway of plasmacytoid DC (pDC) is unique, as TLR7 and TLR9 predominantly activate interferon-regulatory factor (IRF)-7 and induce interferon (IFN)-α.[9] Human mDC lack TLR7 and TLR9 and the IFN-inducing MyD88 pathway, although mouse mDC harbor the TLR7 and TLR9 MyD88 pathway, which are inducible by RNA and CpG DNA respectively.[10] In contrast, TICAM-1 links the type I IFN-inducing pathways in the mDC of both humans and mice,[11] while TLR3 represents the sensor of dsRNA of viral origin.[12] In addition, viral products, double-stranded (ds) RNA, and 5'-triphosphate RNA stimulate the intracytoplasmic helicases melanoma-differentiation-associated gene 5 (MDA5) and RIG-I, which in turn activate the IRF-3- and IRF-7-activating kinases (TANK-binding kinase (TBK1)/I kappa B kinase (IKK)ε.[5,11] The adaptor of this pathway is IPS-1,[5] and it is therefore known as the interferon-beta promoter stimulator 1 (IPS-1) pathway. The IPS-1 pathway shares the downstream signaling components, including the kinases, with the TICAM-1 pathway to activate the IFN-inducing pathway.[13] Thus, the representative inflammatory responses in pattern recognition are rooted in the properties of the adaptors in the case of TLR, MyD88, and TICAM-1. In DC, these pathways play a significant role in differential maturation.

Bacterial and viral pattern molecules revisited

It is known that FCA contains heat-killed mycobacteria (the causative agent of tuberculosis), which functions as a ligand of TLR.[14] These are MyD88-dependent properties and the features of the DC maturation profiles with these TLR ligands have been examined (Table 1). Although the toxicity of the TLR agonists is not removed, their role in triggering antitumor immunity, including cytokine- and effector-inducing abilities, are being examined with respect to their practical use for patients with cancer. Alum (aluminum hydroxide) acts as an NLR agonist involving the secondary activation of MyD88[15] and is currently used as a standard adjuvant in humans. However, a sufficient immune potential may not be accomplished with a

[1]To whom correspondence should be addressed.
E-mail: seya-tu@pop.med.hokudai.ac.jp

doi: 10.1111/j.1349-7006.2009.01442.x

single stimulation of the NLR system. The adjuvant BCG– cell wall skeleton (CWS), which contains mycolic acid, arabinogalactan, and peptidoglycan (PGN), has been used for patients with cancer, and a good prognosis was reported after BCG–CWS treatment.[16] This adjuvant contains muramyl dipeptide (MDP) as a center for the activation of TLR2 and TLR4 and also involves MyD88 activation.[17] The DC maturation profile induced by BCG-CWS is comparable to that induced by Pam2 peptides that activates TLR2 (4) BCG–CWS does not contain DNA, which excludes the possibility of activating TLR9. Only rare examples of fatal shock and interstitial pneumonia have been reported with BCG–CWS that stimulates TLR2 and TLR4.[18]

In contrast, viral products, including dsRNA (and its analog polyI:C), and the lipopolysaccharide (LPS) of Gram-negative bacteria were identified as TLR ligands with TICAM-1 agonistic function.[3] dsRNA and LPS stimulate TLR3 and TLR4, respectively, both of which link the adaptor TICAM-1.[3,11] As they activate nuclear factor (NF)-κB and IRF-3, cytokine storm (hypercytokinemia) or endotoxin-like shock tends to occur *in vivo*.[19] It is therefore mandatory to reduce their toxic properties before they are applied to human patients. Importantly, polyI:CLC (TLR3-complexed poly inosinic: polycytidylic (IC) with carboxymethylcellulose and poly-L-lysine to improve resistance to ribonucleases (i.e. TLR3),[20] and monophosphoryl lipid A (i.e. TLR4)[21] have been considered promising candidates for immunotherapy. These TLR agonists mainly stimulate the TICAM-1 pathway without the robust activation of the MyD88 pathway[20,21] and rarely induce side-effects, such as cytokine storms, skin festering, and the symptoms of inflammation during preclinical trials. It is important that the differential view of the MyD88 and TICAM-1 adjuvants in terms of their DC maturation and effector-driving properties be examined. The development of TLR agonists with properties superior to those of alum can be expected to be revealed through these studies. In this review, the molecular mechanisms of effector activation by DC TLR are outlined and discussed.

Adjuvants stimulate tumor-associated myeloid cells and DC

We have speculated from *in vitro* studies that immature mDC are matured to antigen-presenting mDC by BCG–CWS, a TLR2 agonist,[22] which also induces a variety of immune effector cells, including CD8+ T cells (CTL)[23] and NK cells.[24] These effector cells can damage tumor cells under high effector target (E/T) ratios *in vitro*.[23,24] Indeed, tumor B16 mutanoma growth is retarded in tumor-bearing mice (C57BL/6) when BCG–CWS-matured mDC or secondary-induced CTL are injected in the area surrounding the tumor. It is the CTL, but not NK, cells that are the main effector responsible for tumor regression *in vivo*.[23] Unexpectedly, however, the immune cells which infiltrate into the tumor largely consist of macrophages and not lymphocytes or mDC in mouse models (Shime H and Seya T, unpublished observation, 2009). The properties of these macrophages remain experimentally undetermined. As the tumor-infiltrating macrophages contain many subsets, and some of them often possess immune suppressing properties,[25] these macrophages could be related to myeloid-derived suppressor cells (MDSC) and act as inflammation inducers to sustain tumor growth. Thus, BCG-CWS-mediated functional modification of these macrophages and their effect on tumor growth in mice remains to be determined. Specific questions also remain concerning adjuvant administration to patients. How myeloid cells mature to DC after they are phagocytozing tumor-associated antigens, how mature mDC are located by effector cells, and how tumors regress in such situations still remain unanswered.

Treg, a regulatory population of CD4 T cells, has an inhibitory activity against antitumor immunity[26] and has been shown to inhibit CD8 CTL tumoricidal activity *in vitro*.[26] Several reports indicate that Treg cells infiltrate into tumors and support tumor progression.[27,28] However, mDC are present at very low levels in the tumor masses where Treg cells invade. Again, the functional modulation of Treg cells in the local tumor environment by adjuvants or mDC is unclearly illustrated.

Recently, several myeloid cell populations have been discovered that are associated with tumor cell progression, including interferon-producing killer DC (IKDC),[29] MDSC,[25,30] and tumor-associated macrophages (TAM).[31] Although the maturation or activation of these myeloid cells is likely crucial for tumor progression, only a few reports have investigated their maturation mechanism and effect on tumors by adjuvant treatment. Early-acting pattern molecules can act on tumor cells to release late-acting substances. In fact, damage-associated molecular patterns (DAMP), such as high-modify group box protein (HMGB1), uric acids, heat-shock protein (HSP), and DNA complexes,[32] are secondary liberated from tumors, and stimulate the TAM. Whether these stimuli alter the tumor-progressing ability of the macrophages should be a point of consideration for adjuvant therapy. The types of TLR present in these myeloid cells and the effect of administered adjuvants are topics that need to be investigated.

Many studies on TLR knockout mice allowed us to describe the properties of mouse bone marrow-derived DC (BMDC) treated with a variety of adjuvants[33] and to show the points for induction of immune effector cells through the adjuvant immunotherapy of cancer. Ambivalent functions between mDC and MDSC in a tumor environment can affect the conformation of antitumor immunity.

MyD88- and TICAM-1-mediated DC maturation

Soon after the discovery of the TLR,[34] it was shown that TLR agonists have a DC maturation activity.[35] DC maturation is characterized by TLR adaptors, which have common features, including the upregulation of major histocompatibility complex (MHC), costimulators and NK-activating ligands, and the following features which are unique to each adaptor in mDC.[36] MyD88-dependent DC maturation has two modes, with NK activation and CTL induction occurring concomitantly with the activation of NF-κB, followed by the induction of inflammatory cytokines.[37] Using BCG–CWS as an adjuvant for the TLR2 agonist, we examined how the TLR2 agonist acts on mDC and tumor cells.[23] While NK activation by MyD88 is feasible *in vitro*, TLR2 agonists exhibit minimal NK-mediated tumor-suppression activity in tumor-implant mice.[24] The TLR2-dependent antitumor NK activity is abrogated in MyD88–/– mice, suggesting the presence of a NK-activation pathway via MyD88.[24] However, following an *in vitro* analysis, it was revealed that TLR2–MyD88 in NK cells, but not in mDC, is rather dominant in this mode of NK activation, and that activated NK cells barely enter the tumor mass. For this reason, the subcutaneous administration of BCG–CWS marginally retards tumor growth in mice via the activation of NK cells.

In contrast, mDC maturation is accompanied with potent antigen presentation secondary to cross-priming in TLR2-primed mDC.[23] Tumor antigen-specific CTL induction is facilitated in mice with an implant tumor burden, concomitant with the retardation of tumor growth. This CTL induction is MyD88 dependent, since TLR2-mediated cross-priming does not occur in MyD88–/– mDC. Neither CTL induction nor the retardation of tumor growth significantly occurs in MyD88-deficient mice. Thus, MyD88 in mDC preferentially participates in cross-priming and driving CTL *in vivo*. The downstream molecules of MyD88 associated with mDC CTL driving are unknown.

doi: 10.1111/j.1349-7006.2009.01442.x

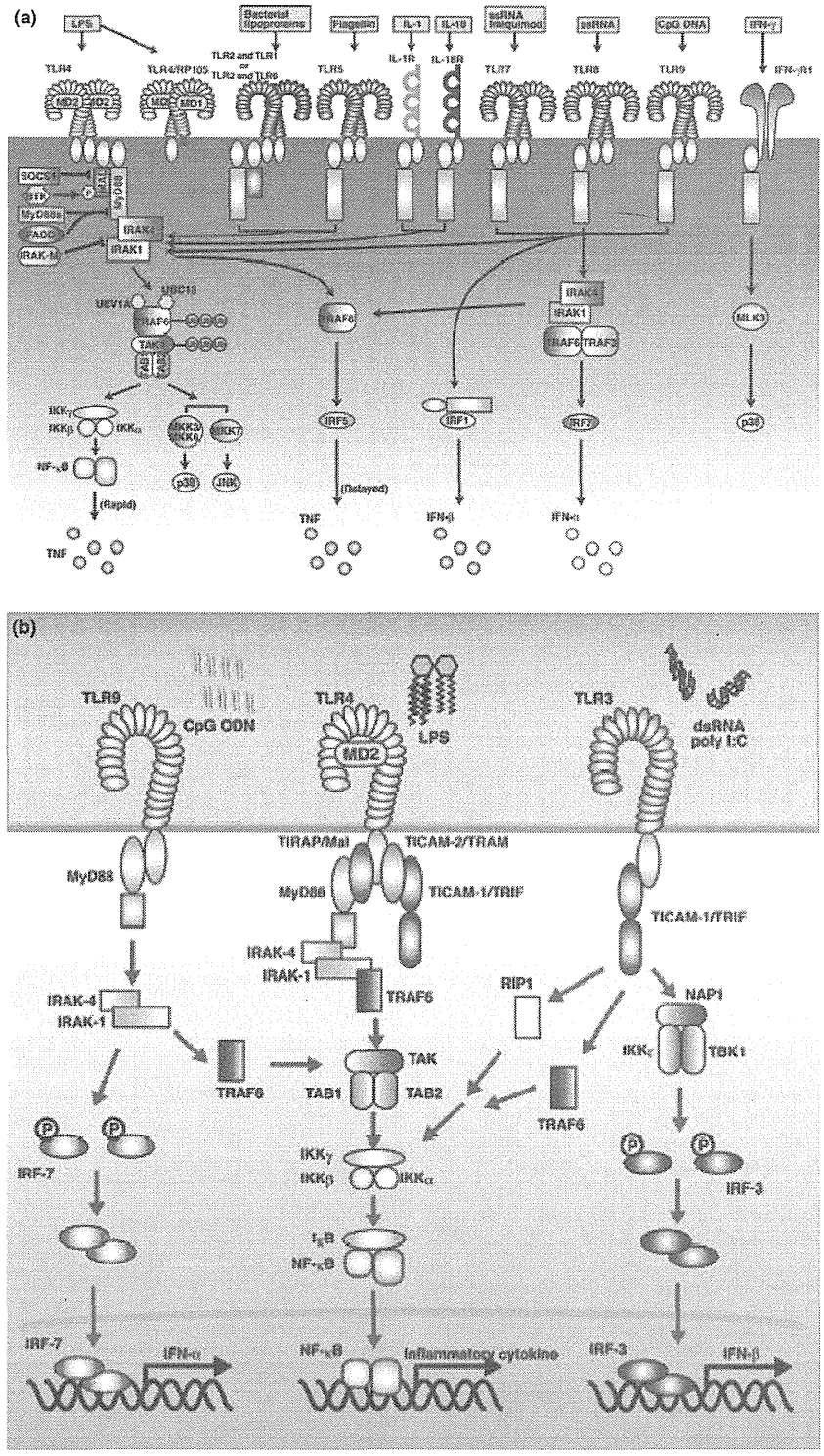

Fig. 1. MyD88 and Toll-interleukin I receptor-domain (TIR)-containing adoptor molecule (TICAM-1) pathways. MyD88 is an adaptor for all Toll-like receptors (TLR), except TLR3 (a). TLR2 and TLR4 recruit MyD88 via the bridging adaptor Toll-interleukin I receptor-domain (TIR)-containing adoptor protein (TIRAP) (MAL) (b). Other TLR directly recruit MyD88. MyD88 activates nuclear factor-kappa β (NF-κB) in most cell types, except plasmacytoid dendritic cells (pDC), which activate the interferan-regulatory factor (IRF-7) transcription factor. MyD88 pathway is involved in the production of pro-inflammatory cytokines in most cells. In contrast, the MyD88 pathway in pDC and the TICAM-1 pathway in myeloid dendritic cells (DC) activate the type interferon (IFN) promoter via IRF-3 or IRF-7 (b). TLR4 can recruit both MyD88 and TICAM-1, whereas other TLR recruit either of them. Each TLR responds to different agonistic stimuli, as shown in Table 1. DC, dendritic cells; IFN, interferon; IRF, interferon-regulatory factor; pDC, plasmacytoid DC; TICAM-1, Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule; TIRAP, Toll-interleukin 1 receptor (TIR) domain-containing adapter protein; TLR, toll like receptor.

Table 1. Human TLR and pattern molecules with MyD88- or Toll-interleukin I receptor domain (TIR)-containing adoptor molecule (TICAM-1)-activating properties

Human TLR	Ligands
TLR1	Pam3
TLR2	Pam2, Pam3, PGN
TLR3	dsRNA
TLR4	LPS, virus fusion units
TLR5	Flagellin
TLR6	Pam2
TLR7	ssRNA
TLR8	ssRNA
TLR9	CpG DNA
TLR10	–

MyD88 activators (Lipoproteins, PGN)	Reference
M161Ag (MALP-2)	(62)
TAN33	(63)
OM-174	(64)
BCG-CWS (Azuma lot)	(22)
SMP105	(65)

TICAM-1 activators (RNA, lipid A)	
DI RNA (stem loop)	(66)
Poly(A:U)	(67)
Poly(I:C_{12}U)	(68)
Polyl:C(LC)	(20)
MPLA	(21)

Anti-human TLR monoclonal antibodies		
TLR1	TLR1.136	(58)
TLR2	TLR2.45	(59)
TLR3	TLR3.7	(60)
TLR4	HTA125	(61)
TLR6	TLR6.127	(58)

dsRNA, double-stranded RNA; TLR, Toll-like receptor. SMP105 is a lot of BCG-CWS that activates only TLR2.

In the present study, we used polyI:C for evaluating the TICAM-1 potential in mDC maturation and antitumor immunity.[38] The TICAM-1 pathway allows mDC to activate IRF-1 and IRF-3, which in turn activate the IFN-β promoter, as well as unidentified antitumor factors (Fig. 1). The data imply that cross-priming and the NK-driving signal are also dependent upon TICAM-1, but the transcription factors utilized by TICAM-1 are wholly distinct from those of MyD88. The search for the molecules that participate in the TICAM-1 CTL driving is underway, and a molecule downstream of IRF-1, but not IRF-3, has been shown to be crucial for *in vivo* CTL induction. In contrast, TICAM-1-mediated antitumor NK activation largely relies on the IRF-3-derived NK-activating molecule (INAM), in addition to the reported cytokines IL-15, IFN-α, and IL-12p70.[39]

MyD88 and TICAM-1 activate different signaling platforms for the recruitment of second adaptors.[3] In mDC, TLR2 and TLR4 recruit the combined adaptor Mal/Toll-interleukin I receptor domain-containing adoptor protein (TIRAP)–MyD88 to signal the transcription factor NF-κB.[4] In contrast, TLR3 and TLR4 can utilize TICAM-1 as the adaptor.[3] TLR4 recruits the combined adaptor Toll-IL-IR domain-containing adoptor inducing IFN-beta-related adaptor molecule (TRAM) (TICAM-2)–TICAM-1 while TLR3 directly recruits TICAM-1 for signaling.[3] TLR4 is unique in that it uses both MyD88 and TICAM-1 adaptors (Fig. 1). The classic example in which both routes are activated is during LPS-induced endotoxic shock.[40] Like BCG-CWS and PolyI:C, activation of either one route would be required for a condition of less toxic adjurants. Studies of the TICAM-1 signalosome suggest that upon TLR3 activation, TICAM-1 recruits a variety of molecules as secondary adaptors, including NAK-associated protein I (NAP1),[41] receptor-interacting protein I (RIP1),[42] similar to NAP I TBK adoptor (SINTBAD),[43] adenovirus 5 E1A-binding protein (BS69),[44] and TNF receptor-associated factor (TRAF) family proteins.[45] Whether or not these molecules are associated with antitumor CTL or NK induction remains to be determined.

The mode by which mDC are matured differs in the MyD88 and the TICAM-1 pathways. The TICAM-1 pathway preferentially induces IL-12 and type I IFN in mDC and drives NK activation.[38] Type I IFN induction by MyD88 has been observed only in pDC.[9,10] In contrast, mDC MyD88 strongly induces pro-inflammatory cytokines, such as tumor necrosis factor-α, IL-1β, and IL-6.[9,46] The molecular mechanism that facilitates the cross-presentation ability in mDC is currently unknown.

DC subsets and TLR expression

BMDC (representative of mDC) and pDC can be prepared from mouse bone marrow cells by using granulocyte-macrophage colony-stimulating factor (GM–CSF) and the Flt3 ligand[46] while Langerhans cells can be generated by the addition of transforming growth factor-β to GM–CSF and IL-4.[47] The DC subsets in the spleen and the intestinal tract can be separated using a flow cytometry (FACS) sorter. The characteristics of these mouse DC subsets have been described previously.[36] In humans, monocyte-derived DC can be used as mDC, but their characteristics are somewhat different from mDC prepared in the peripheral blood using the mDC marker plasmocytoid DC antigen (PDCA1). Human peripheral blood pDC can be isolated from whole blood using PDCA4.

The distribution of TLR of the DC subset were examined by using human TLR-specific monoclonal antibodies generated in our laboratory, and the TLR repertoires of monocyte-derived DC and pDC were determined (Table 2). The TLR distribution roughly resembles mouse DC, although a clear result could not be obtained with mouse BMDC and pDC because of a lack of appropriate specific antibodies against mouse TLR.[36] The discrepancy of appropriate TLR7 levels in mouse BMDC and human mDC could be a result of differences in the inducible nature of mouse, but not human, TLR7. It was also shown that human mDC express TLR8, while mouse mDC do not.[36] The

Table 2. TLR expression profiles in human DC subsets

	Freshly isolated			*In vitro*-differentiated	
	Monocytes	mDC*	pDC**	DCs	Macrophages
TLR1	++	+	–	+	++
TLR2	++	++	–	++	++
TLR3	–	++	–	++	+
TLR4	++	+	–	+	+
TLR6	++	+	–	+	+
TLR7	–	–	+	–	–
TLR8	+	+	–	+	+
TLR9	–	–	+	–	–

Positive and negative symbols denote the results of the flow cytometry (FACS) analyses using monoclonal antibodies, except TLR7, TLF8, and TLR9. Results were determined by reverse transcription–polymerase chain reaction. TLR3, TLR7, TLR8, and TLR9 reside in the endosome to recognize nucleotide derivatives. (*) PDCA1+ cells; (**) PDCA4+ cells. PDCA, plasmacytoid dendritic cell antigen; DC, dendritic cell; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; TLR, Toll-like receptor.

doi: 10.1111/j.1349-7006.2009.01442.x

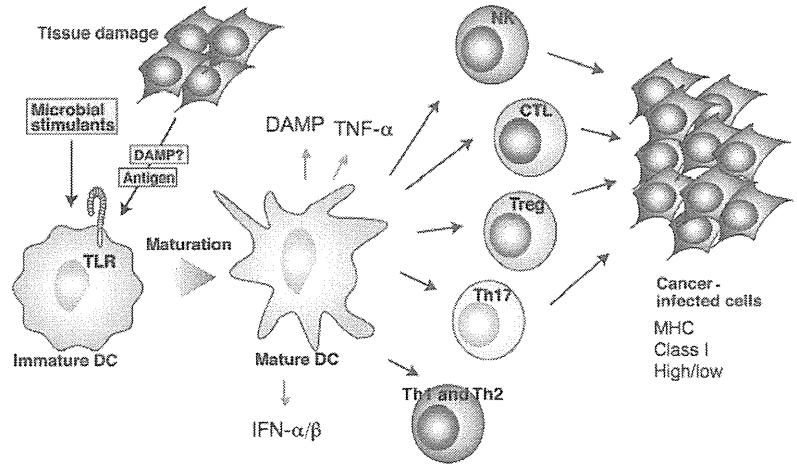

Fig. 2. Selective induction of immune effector lymphocytes by different agonistic stimuli. Each pattern molecule (PAMP) has its own uniqueness in myeloid dendritic cell (mDC) maturation. Differential maturation of mDC results in different effector driving as shown. CD8 T, various CD4 T, and B cells are proliferated by myeloid DC (mDC) with different properties. Tumor regression is a marker for evaluating which lymphocytes are activated in response to pathogen-associated molecular patterns (PAMP).

failure of CpG DNA to raise effective antitumor immunity can be attributable to the low or absent induction of TLR9 in human mDC, unlike the situation in mouse mDC.

DC subsets and effector induction

CTL and NK cells can be induced by mature mDC, with or without the presentation of MHC class I antigens, while CD4 T-cell subsets are induced by the presentation of class II antigens. In addition to CTL and NK cells, the tumor-modulating functions of Th1, Th2, Th17, and Treg were evaluated (Fig. 2). NK activation is a result of the balance between NK-activating and inhibitory ligands on mDC. NK cells can also be activated with cytokines, such as IL-2, IL-15, IFN-α/β, and IL-12.[48] CTL is a result of the activation of the CD8+ T cell by the presentation of class I antigens on mDC. Other effectors are the result of the activation of CD4+ T cells by MHC class II antigen presentation on mDC. A master transcription factor in addition to T-bet, GATA-3, RORgT, and Foxp3 are known to exist for Th1, Th2, Th17, and each Treg on the CD4 lymphocyte side.[49] However, there is little information concerning the mDC properties driving these effector cells.

Each DC subset seems to correspond to a specific effector, although the selection mechanism by which DC induce various effectors is not clear in most instances. However, it is known that CD8+ DC induce Treg[50] and NK cells[51] in the mouse spleen, and lamina propria pDC in the mouse enteric canal promotes immunoglobulin A production.[52] In addition, CD70+/CD11c+ DC induce Th17 cells by the adenosine tri phosphate (ATP) of enterobacteria,[53] and BMDC activate NK cells via the TICAM-1 pathway.[54] Further examples of DC subsets that preferentially function with specific effectors will likely be demonstrated through practical experiments.

Mechanism of DC-mediated antitumor NK activation

It has been reported that BMDC drive antitumor NK activation in a TICAM-1-dependent manner.[38,54] This NK activation does not rely upon a soluble factor, such as a cytokine, but instead was generated by BMDC–NK cell–cell contact.[39] Therefore, there must be an NK-activating molecule that is induced on the BMDC surface in response to TICAM-1 signaling (Fig. 3a). We focused our attention on this key molecule, which is crucial for antitumor NK immunity and found that DC-mediated NK activation occurred normally in IRF-7 −/− BMDC stimulated with polyI:C, but this response was absent in IRF-3 −/− BMDC.[39] Therefore, the putative NK-driving signal in mDC involves transcription factor IRF-3 downstream of the activated TICAM-1. Ultimately, the NK activation molecule was identified using a screening method in which candidate molecules were expressed in IRF-3 −/− BMDC using a lentiviral vector.[39] We named this molecule

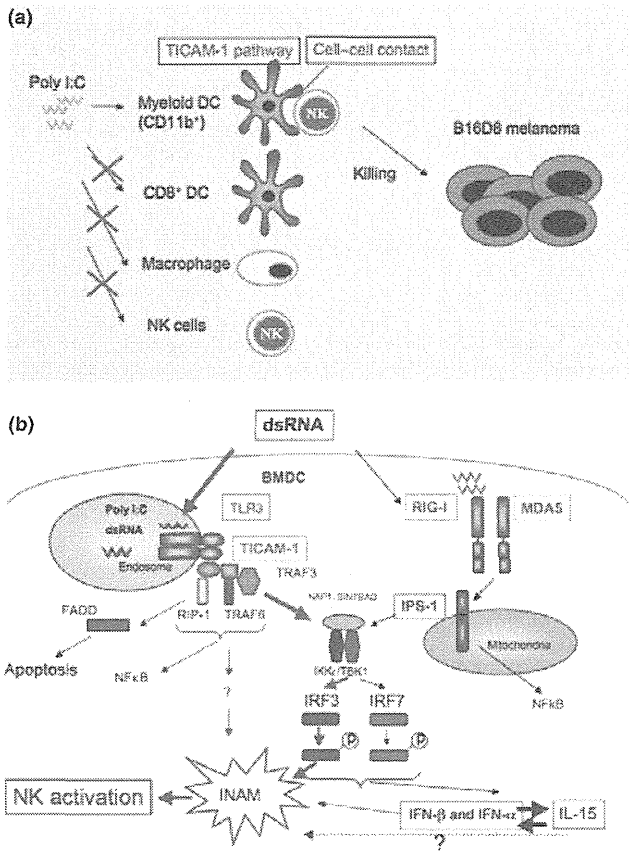

Fig. 3. A molecular mechanism of myeloid dendritic cell (mDC)-mediated natural killer (NK) activation. (a) CD11b+ bone marrow-derived dendritic cells (BMDC) act for natural killer (NK) activation by double-stranded (ds) RNA. NK cells express tumoricidal activity against major histocompatibility complex (MHC) low implant tumors if they are primed by polyI:C plus bone marrow-derived dendritic cells (BMDC), but not other myeloid cells. Dendritic cell–NK cell–cell contact is essential for the induction of polyI:C-mediated antitumor NK cells. (b) Route for mDC maturation for the induction of NK activation.[39]

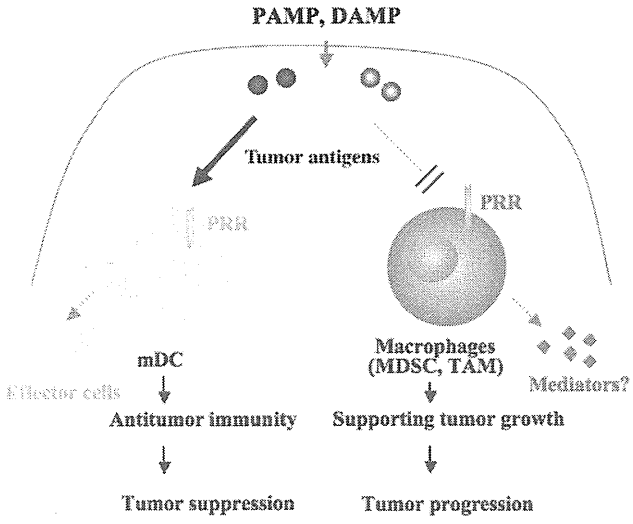

Fig. 4. Diverged functions of myeloid cells in tumor mass. A variety of myeloid subsets reside in tumor masses. Some of the subsets exhibit an immune suppressive feature that facilitates escape of tumor cells from immune effectors. Since pattern molecules (PAMP) act on both myeloid dendritic cells (mDC) and myeloid-derived immune suppressing cells, complicated immune responses occur in tumors. Selective maturation of mDC circumventing the exacerbation of tumor progression by myeloid suppressor cells should be considered as adjuvant therapy of cancer. DAMP, damage-associated molecular patterns; MDSC, myeloid-derived suppressor cells; PRR, pattern-recognition receptors; TAM, tumor-associated macrophages.

INAM. When INAM was expressed in mDC, it promoted NK activation in the mixture of mDC (expressing INAM) and NK cells; however, INAM did not exhibit an NK-activating function on BaF3 cells. INAM is an NK-activating molecule peculiar to BMDC whose TICAM-1 has been activated, and there have been no reports suggesting the presence of this kind of molecule until recently (Fig. 3b). In BMDC, INAM receives a sugar chain modification by a similar membrane protein to tetraspanin with a molecular weight of 45 kDa. INAM is distributed in the spleen and lymph nodes, and is actually expressed by a variety of lymphocyte subsets present in the lymph nodes. It has been predicted to make a loop card structure on the surface of the cell in two portions based on the amino acid sequence.[39]

It is predicted that INAM is related to the composition of immune synapses in the BMDC–NK contact. When BMDC, which forcibly express INAM, are prepared and adoptively transferred around the tumors of tumor-bearing mice, the tumor is efficiently regressed. These results suggest that INAM is the factor directly responsible for driving antitumor NK activation. Humans have an ortholog of INAM, although its distribution profile appears to be somewhat different than to that of mice.

Points to trigger antitumor immune potential

Effector tumor cell–cell contact is essential for tumor damage by immune effector cells. The material liberated from cancer cells on one side generates the modulators of the PRR of mDC and influences the trigger of effector induction. The host molecules that modulate PRR are the previously-mentioned DAMP.[32] For effective tumor damage, the effector must reach the tumor mass. A suitable strategy is needed for determining the basic factor(s) of the immune response involved in cancer, and can be achieved by using immunomodulatory reagents and gene-disrupted mice with abrogated TLR pathways.

We have analyzed how BMDC acquire effector-driving functions by focusing on the innate immune response. The results suggest that PRR stimuli become a trigger that leads to the alteration of precancerous cells to the malignant form. However, PRR are indispensable to the activation of antitumor immunity. In both cases, myeloid cells are intimately involved in the process of tumor–immune cell interaction. Indeed, BCG has high therapeutic potential for patients with bladder transitional epithelial cancer,[55] but it has less of an effect on a variety of other solid cancers. This discrepancy can be rooted in the fact that myeloid cells interact with tumor cells with ambivalent reaction profiles (Fig. 4). An effective strategy for tackling the issue of immune abnormality has yet to be proposed, and even the fundamental immune aberrance present in the microenvironment of tumors is not generally recognized by researchers. It has been speculated that tumor cells produce cytokines that modulate the inflammatory environment as tumor develops. When tumor is surgically excised, many constitutional accidents are often diminished,[56] which can reflect the fact that tumors develop concomitantly with immune modulation. It has become clear that some modulating factors of the innate immune system, such as DAMP, cause cancer-mediated idiosyncrasies (Fig. 2).

Up until now, the effectiveness of cancer immunotherapy has been primarily evaluated based on tumor regression and the survival prognosis of patients. A representative study involved the evaluation of peptide vaccine therapy for cancer treatment. According to the report by Rosenberg,[57] the peptide vaccine administered to melanoma patients had an effective rating of approximately 2.6%. For future studies, it is necessary to determine the potential of peptide-conjugating materials, including adjuvants and inflammation-inducing reagents.[20] A number of reports have suggested that adjuvants can greatly increase the efficiency rate of treatment, although the criteria is prerequistie to fairly evaluate the function of adjuvants in cancer patients.

The method for stimulating DC needs to be carefully selected, as the systemic administration of inflammation-inducing material can also lead to the acceleration or invasion of developing malignancies at the same time (Fig. 4). The adoptive transfer of adjuvant-treated mDC to patients is a promising choice; however, it might be difficult for this treatment to be adapted by the Japanese health insurance system. The molecular manipulation of a specific PRR in DC that is involved in effector driving can lead to effective treatment with minimal side-effects. In this case, the route and molecule that selectively raises the degree of DC maturation without enhancing MDSC should be clarified. If the inflammatory signals that promote carcinogenesis are properly controlled using adjuvants, the design of DC maturation can be manipulated without helping tumor progression. The search for the functional molecule of antitumor effector induction in mDC will help establish an effective treatment of cancer and facilitate the evaluation of the efficacy of peptide vaccines. In the future, we hope that through continued research, cancer patients will have access to convenient and highly effective immunotherapy.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan by the Akiyama Foundation and Yakulto Foundation. Financial support from the Sapporo Biocluster "Bio-S" Knowledge Cluster Initiative of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) is gratefully acknowledged. We are grateful to our laboratory members for their critical discussions, and Ms R. Hatsugai and Ms H. Sato in our laboratory for secretarial assistance.

doi: 10.1111/j.1349-7006.2009.01442.x

References

1 Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology *Cold Spring Harb Symp Quant Biol* 1989; **54**: 1–13.
2 Seya T, Matsumoto M. The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. *Cancer Immunol Immunother* 2009; **58**: 1175–84.
3 Akira S. Toll-like receptor signaling. *J Biol Chem* 2003; **278**: 38105–8.
4 Seya T, Akazawa T, Tsujita T, Matsumoto M. Role of Toll-like receptors in adjuvant-augmented immune therapies. *Evid Based Complement Alternat Med.* 2006; **3**: 31–8.
5 Yoneyama M, Onomoto K, Fujita T. Cytoplasmic recognition of RNA. *Adv Drug Deliv Rev* 2008; **60**: 841–6.
6 Martinon F, Gaide O, Pétrilli V, Mayor A, Tschopp J. NALP inflammasomes: a central role in innate immunity. *Semin Immunopathol* 2007; **29**: 213–29.
7 Ishii A, Kawasaki M, Matsumoto M, Tochinai S, Seya T. Phylogenetic and expression analysis of amphibian Xenopus Toll-like receptors. *Immunogenetics* 2007; **59**: 281–93.
8 De Gregorio E, D'Oro U, Wack A. Immunology of TLR-independent vaccine adjuvants. *Curr Opin Immunol* 2009; **21**: 339–45.
9 Honda K, Taniguchi T. Toll-like receptor signaling and IRF transcription factors. *IUBMB Life* 2006; **58**: 290–5.
10 Hoshino K, Kaisho T. Nucleic acid sensing Toll-like receptors in dendritic cells. *Curr Opin Immunol* 2008; **20**: 408–13.
11 Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv Drug Deliv Rev* 2008; **60**: 805–12.
12 Matsnmoto M, Funami K, Oshiumi H, Seya T. Toll-like receptor 3: a link between toll- like receptor, interferon and viruses. *Microbiol Immunol.* 2004; **48**: 147–54.
13 Sasai M, Shingai M, Funami K *et al.* NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. *J Immunol* 2006; **177**: 8676–83.
14 Freund J, Stone SH. The effectiveness of tuberculo-glycolipid as an adjuvant in eliciting allergic encephalomyelitis and aspermatogenesis. *J Immunol* 1959; **82**: 560–7.
15 Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 2008; **453**: 1122–6.
16 Yasumoto K, Manabe H, Yanagawa E *et al.* Nonspecific adjuvant immunotherapy of lung cancer with cell wall skeleton of Mycobacterium bovis Bacillus Calmette-Guérin. *Cancer Res* 1979; **39**: 3262–7.
17 Uehori J, Fukase K, Akazawa T *et al.* Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation. *J Immunol* 2005; **174**: 7096–103.
18 Diner EK, Verghese M. Interstitial pneumonitis secondary to intravesical bacillus calmette-guerin for carcinoma in-situ of the bladder. *Int Braz J Urol* 2004; **30**: 400–2.
19 Absher M, Stinebring WR. Toxic properties of a synthetic doublestranded RNA. Endotoxin-like properties of poly I. C. an interferon stimulator. *Nature* 1969; **223**: 715–7.
20 Longhi MP, Trumpfheller C, Idoyaga J *et al.* Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J Exp Med* 2009; **206**: 1589–602.
21 Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* 2007; **316**: 1628–32.
22 Tsuji S, Matsumoto M, Takeuchi O *et al.* Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guérin: involvement of toll-like receptors. *Infect Immun* 2000; **68**: 6883–90.
23 Akazawa T, Masuda H, Saeki Y *et al.* Adjuvant-mediated tumor regression and tumor-specific cytotoxic response are impaired in MyD88-deficient mice. *Cancer Res* 2004; **64**: 757–64.
24 Fujimoto Y, Hashimoto M, Furuyashiki M, Katsumoto M, Seya T, Suda Y, Fukase K. Lipopeptides from Staphylococcus aureus as Tlr2 Ligands: prediction with mrna expression, chemical synthesis and immunostimulatory activities. *Chembiochem* 2009; **10**: 2311–5.
25 Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol* 2008; **181**: 5791–802.
26 Cao X. Regulatory T cells and immune tolerance to tumors. *Immunol Res.* 2009; Sep 10. [Epub ahead of print].
27 Chaput N, Conforti R, Viaud S, Spatz A, Zitvogel L. The Janus face of dendritic cells in cancer. *Oncogene* 2008; **27**: 5920–31.
28 Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* 2000; **74**: 181–273.
29 Ullrich E, Bonmort M, Mignot G *et al.* Therapy-induced tumor immunosurveillance involves IFN-producing killer dendritic cells. *Cancer Res* 2007; **67**: 851–3.
30 Nagaraj S, Gabrilovich DI. Tumor Escape Mechanism Governed by Myeloid-Derived Suppressor Cells. *Cancer Res* 2008; **68**: 2561–3.
31 Gallina G, Dolcetti L, Serafini P *et al.* Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* 2006; **116**: 2777–90.
32 Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol* 2008; **8**: 279–89.
33 Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. *C R Biol* 2004; **327**: 581–9.
34 Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; **388**: 394–7.
35 Reise Sousa C. Toll-like receptors and dendritic cells: for whom the bug tolls. *Semin Immunol* 2004; **16**: 27–34.
36 Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; **5**: 987–95.
37 Steinman RM, Hemmi H. Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol* 2006; **311**: 17–58.
38 Akazawa T, Ebihara T, Okuno M *et al.* Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proc Natl Acad Sci U S A* 2007; **104**: 252–7.
39 Ebihara T, Azuma M, Oshiumi H, Taniguchi T, Matsumoto M, Seya T Identification of INAM, a polyI:C-inducible membrane protein, that participates in dendritic cell-mediated natural killer cell activation. *J Exp Med.* 2010; (in press).
40 Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999; **11**: 115–22.
41 Sasai M, Oshiumi H, Matsumoto M *et al.* Cutting Edge: NF-kB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN Regulatory Factor 3 activation. *J Immunol* 2005; **174**: 27–30.
42 Meylan E, Burns K, Hofmann K *et al.* RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nat Immunol* 2004; **5**: 503–7.
43 Ryzhakov G, Randow F. SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. *EMBO J* 2007; **26**: 3180–90.
44 Takaki H, Oshiumi H, Sasai M, Kawanishi T, Matsumoto M, Seya T. Oligomerized TICAM-1(TRIF) in the cytoplasm recruits nuclear Bs69 to enhance NF-kappaB activation and type I IFN induction. *Eur J Immunol.* 2009; **39**: 3469–76.
45 Sasai M, Oshiumi H, Funami K, Matsumoto M, Seya T Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor of the Toll-like receptor 3/4 pathway. *Molec. Immunol* 2010; (in press).
46 Honda K, Ohba Y, Yanai H *et al.* Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 2005; **434**: 1035–40.
47 Geissmann F, Prost C, Monnet JP, Dy M, Brousse N, Hermine O. Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med* 1998; **187**: 961–6.
48 Wu J, Lanier LL. Natural killer cells and cancer. *Adv Cancer Res* 2003; **90**: 127–56.
49 Chen Z, O'Shea JJ. Th17 cells: a new fate for differentiating helper T cells. *Immunol Res* 2008; **41**(2): 87–102.
50 Yamazaki S, Dudziak D, Heidkamp GF *et al.* CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol* 2008; **181**: 6923–33.
51 Miyake T, Kumagai Y, Kato H *et al.* Poly I:C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways. *J Immunol* 2009; **183**: 2522–8.
52 Tezuka H, Abe Y, Iwata M *et al.* Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* 2007; **448**: 929–33.
53 Atarashi K, Nishimura J, Shima T *et al.* ATP drives lamina propria T(H)17 cell differentiation. *Nature* 2008; **455**: 808–12.
54 Ebihara T, Azuma M, Oshiumi H *et al.* Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. *Hepatology* 2008; **48**: 48–58.
55 Alexandroff AB, Jackson AM, O'Donnell MA, James K. BCG immunotherapy of bladder cancer: 20 years on. *Lancet* 1999; **353**: 1689–94.
56 Kodama K, Higashiyama M, Takami K *et al.* Innate immune therapy with a Bacillus Calmette-Guérin cell wall skeleton after radical surgery for non-small cell lung cancer: a case-control study. *Surg Today* 2009; **39**: 194–200.
57 Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 2004; **10**: 909–15.
58 Nakao Y, Funami K, Kikkawa S *et al.* Surface-expressed TLR 6 Participates in the Recognition of Diacylated Lipopeptide and Peptidoglycan in Human Cells. *J Immunol* 2005; **174**: 1566–73.
59 Uehori J, Matsumoto M, Tsuji S *et al.* Simultaneous blocking of human Toll-like receptor 2 and 4 suppresses myeloid dendritic cell activation induced by

Mycobacterium bovis bacillus Calmette-Guérin (BCG)-peptidoglycan (PGN). *Infect Immun* 2003; **71**: 4238–49.
60 Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun* 2002; **293**: 1364–9.
61 Bosisio D, Polentarutti N, Sironi M *et al.* Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. *Blood* 2002; **99**: 3427–31.
62 Nishiguchi M, Matsumoto M, Takao T *et al.* Mycoplasma fermentans lipoprotein M161Ag-induced cell activation is mediated by Toll-like receptor 2: role of N-terminal hydrophobic portion in its multiple functions. *J Immunol* 2001; **166**: 2610–6.
63 Matsuo-Tanabe M, Kawamoto T, Tanida S, Matsumoto M, Seya T Toll-like receptor 2 agonists with unique properties synthesized with reference to a product of *Streptosporangium*. *Immunology* 2004: 235–41.
64 Brandenburg K, Lindner B, Schromm A *et al.* Physicochemical characteristics of triacyl lipid A partial structure OM-174 in relation to biological activity. *Eur J Biochem* 2000; **267**: 3370–7.
65 Murata M. Activation of Toll-like receptor 2 by a novel preparation of cell wall skeleton from Mycobacterium bovis BCG Tokyo (SMP-105) sufficiently enhances immune responses against tumors. *Cancer Sci* 2008; **99**: 1435–40.
66 Shingai M, Ebihara T, Begum NA *et al.* Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J Immunol* 2007; **179**: 6123–33.
67 Sugiyama T, Hoshino K, Saito M *et al.* Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7. *Int Immunol* 2008; **20**: 1–9.
68 Navabi H, Jasani B, Reece A *et al.* A clinical grade poly I:C-analogue (Ampligen) promotes optimal DC maturation and Th1-type T cell responses of healthy donors and cancer patients in vitro. *Vaccine* 2009; **27**: 107–15.

doi: 10.1111/j.1349-7006.2009.01442.x

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: xx November 2010
doi: 10.3389/fmicb.2010.00128

Characterization of natural killer cells in tamarins: a technical basis for studies of innate immunity

Tomoyuki Yoshida[1,2†], Akatsuki Saito[2,3†], Yuki Iwasaki[1,4], Sayuki Iijima[1], Terue Kurosawa[1], Yuko Katakai[5], Yasuhiro Yasutomi[6], Keith A. Reimann[7], Toshiyuki Hayakawa[2] and Hirofumi Akari[1,2]*

[1] *Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan*
[2] *Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan*
[3] *International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan*
[4] *Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*
[5] *Corporation for Production and Research of Laboratory Primates, Tsukuba, Ibaraki, Japan*
[6] *Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan*
[7] *Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*

Edited by:
Yasuko Yokota, National Institute of Infectious Diseases, Japan

Reviewed by:
Koji Ishii, National Institute of Infectious Diseases, Japan
Ikuo Shoji, Kobe University Graduate School of Medicine, Japan

***Correspondence:**
Hirofumi Akari, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan.
e-mail: akari@pri.kyoto-u.ac.jp

†Tomoyuki Yoshida and Akatsuki Saito have contributed equally to this work.

Natural killer (NK) cells are capable of regulating viral infection without major histocompatibility complex restriction. Hepatitis C is caused by chronic infection with hepatitis C virus (HCV), and impaired activity of NK cells may contribute to the control of the disease progression, although the involvement of NK cells *in vivo* remains to be proven. GB virus B (GBV-B), which is genetically most closely related to HCV, induces acute and chronic hepatitis upon experimental infection of tamarins. This non-human primate model seems likely to be useful for unveiling the roles of NK cells *in vivo*. Here we characterized the biological phenotypes of NK cells in tamarins and found that depletion of the $CD16^+$ subset *in vivo* by administration of a monoclonal antibody significantly reduced the number and activity of NK cells.

Keywords: CD16, cynomolgus monkey, tamarin, NK cell

INTRODUCTION

Natural killer (NK) cells are a component of the innate immune system that play a central role in host defense against viral infection and tumor cells. Much of the evidence for a role for NK cells in controlling viral infections has come from experiments with mice that were genetically modified (Lian and Kumar, 2002) or were treated with NK cell-depleting antibodies (Kasai et al., 1980) or from the study of humans with inherited NK cell deficiencies (Biron et al., 1989; Orange, 2002).

NK cells can be rapidly recruited into infected organs and tissue by chemoattractant factors produced by virus-infected cells and activated resident macrophages, which are also a major source of interferon (IFN), which induces NK cell proliferation, NK cell-mediated cytolysis of virus-infected cells, and the secretion of chemokines (Robertson, 2002). NK cells can kill virus-infected cells by using cytotoxic granules or by recognizing and inducing lysis of antibody-coated target cells (antibody-dependent cell cytotoxicity) via antibody binding receptor CD16. For instance, human blood NK cells are cytotoxic against dengue virus-infected cells in target organs via direct cytolysis and antibody-dependent cell-mediated cytotoxicity (reviewed by Navarro-Sánches et al., 2005). Early activity of NK cells may be important for clearing acute infections such as that of dengue virus. However, the effect that NK cells may exert on chronic infections with viruses such as hepatitis C virus (HCV) is less clear.

HCV is the causative agent of chronic hepatitis C, cirrhosis, and finally liver cancer. In general, acquired and innate immunity induced by acute HCV infection is not sufficient for the viral clearance, and persistent HCV infection frequently leads to progression to chronic hepatitis (reviewed by Cheent and Khakoo, 2010). It was reported that dendritic cells (DCs) in HCV infection were not responsive to IFN-α, and thus failed to promote subsequent activation of NK cells as a primary innate immune response (reviewed by Kanto, 2008). This is in agreement with the finding that the killing activity of NK cells in patients with chronic hepatitis C is inactivated in *in vitro* studies (Deignan et al., 2002; Golden-Mason et al., 2008). These data suggest that the dysfunction of NK cells contributes to the persistent infection of HCV and chronic hepatitis. On the other hand, it was suggested that inappropriately activated NK cells caused liver injury after the viral infection (Liu et al., 2000). The population of NK cells is relatively minor in peripheral lymphoid organs but is abundant in liver, raising a question as to their function in the innate immune response to acute and chronic HCV infection in the liver. It is possible that NK cells partially regulate the replication of HCV in this organ during early infection whereas they promote the liver dysfunction in chronic HCV infection. To examine these possibilities, it is necessary to clarify the involvement of NK cells *in vivo* in HCV infection. However, it is questionable whether the results of *ex vivo* analyses of NK cells would reflect their actual roles *in vivo*. Therefore, it might be more informative to study the function of NK cells directly by means of *in vivo* depletion technique in animal models.

A chimpanzee model of HCV infection has frequently been employed to evaluate the role of acquired antiviral immune responses, although the involvement of NK cells has not been fully evaluated because of the limitations on the use of chimpanzees