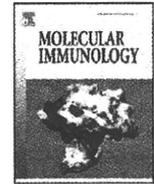


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Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the Toll-like receptor 3/4 pathway

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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- κ B 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- κ B, 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.

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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) super-families (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 I κ B kinase complex and c-jun kinases (Häcker et al.,

2000). TRAF6 also interacts with MyD88 to mediate NF- κ B 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- κ B and MAPKs or produce inflammatory cytokines. TLR4 ligand-induced cytokine production is also markedly reduced in TRAF6^{-/-} cells, although the activation of NF- κ B 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

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rogates 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 AF6 (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; Oganessian 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; Oganessian 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 extracellular 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 IRF-3 induction, which may be independent of the TRAF3 recruitment to TICAM-1.

Materials and methods

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 DMEM 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 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 T. Taniguchi (The University of Tokyo, Tokyo, Japan). Gal4-IRF3-Gal4-DBD, and p53 UASG-Luc were used for IRF-3 activation (Yoneyama et al., 1998). NF- κ B and AP-1 activation were determined as previously described (Oshiumi et al., 2003a).

Yeast two-hybrid screening

The yeast two-hybrid assay was performed as described previously (Oshiumi et al., 2003a). Briefly, the yeast strain AH109 (Stratagene, 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.

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 Na₃VO₄, 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.

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, pRL-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 p53 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 \pm S.D.

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).

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 $\mu\text{g}/\text{well}$) 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 $\mu\text{g}/\text{ml}$) 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 $\mu\text{g}/\text{ml}$) 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 $\times 63$ 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 \times 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; Oganessian et al., 2006), was not identified in the yeast two-hybrid assay (Supplementary data, Fig. S2). Although

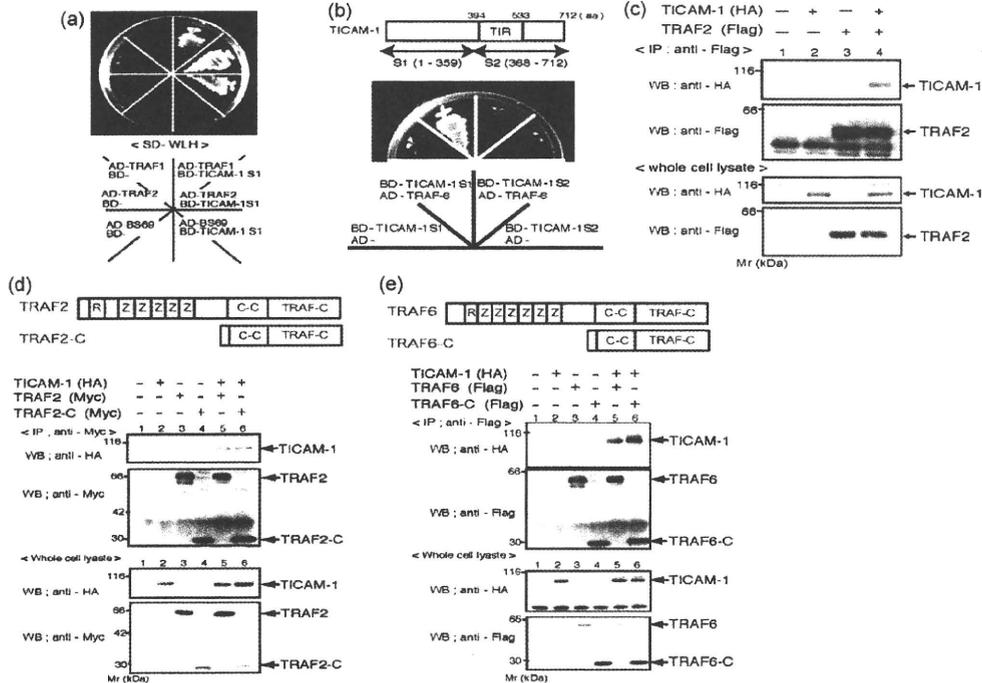


Fig. 1. Molecular interaction of TICAM-1 with TRAF2 and 6. (a) Direct interaction between the N-terminal region of TICAM-1 and either TRAF2 or TRAF6 as identified by yeast two-hybrid screening. Full-length TRAF2 and TRAF6 were cloned into pGADT7, transformed into yeast strain AH109, and then cultured 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 in 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 (Jung 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; Ye 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 a yeast two-hybrid system (Fig. 2a). Deletion of the first 200 aa of the N-terminus of the TICAM-1 S1 fragment (dN200) did not affect its binding ability to TRAF2, however, deletion of aa 200–359 (dN300) 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 QxS consensus sequence, represented by PxQxA and AxAxS (Lu et al., 2003). We therefore constructed both mutations in TICAM-1 (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.

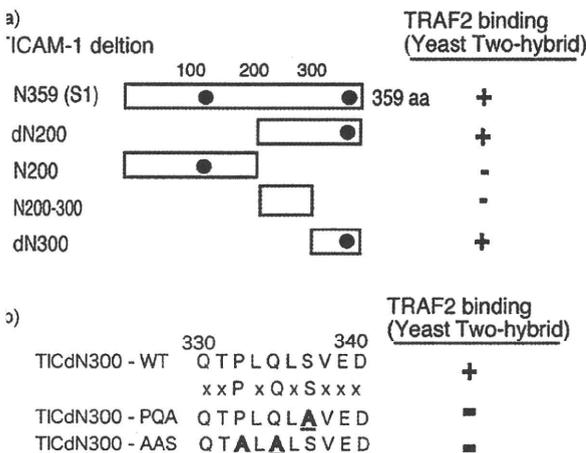


Fig. 2. Identification of the TRAF2-binding site in TICAM-1. (a) Scheme of TICAM-1 truncated mutants and location of the TRAF2 binding motif (black dot). TRAF2 binding was assessed on SD-WLH plates as described in Fig. 1. (b) Specific consensus motif of TICAM-1 that directly binds to TRAF2. The predicted TRAF2 binding motif in TICAM-1 (TICdN300) was identified as the PxQxS sequence (300–359 aa). TICdN300 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

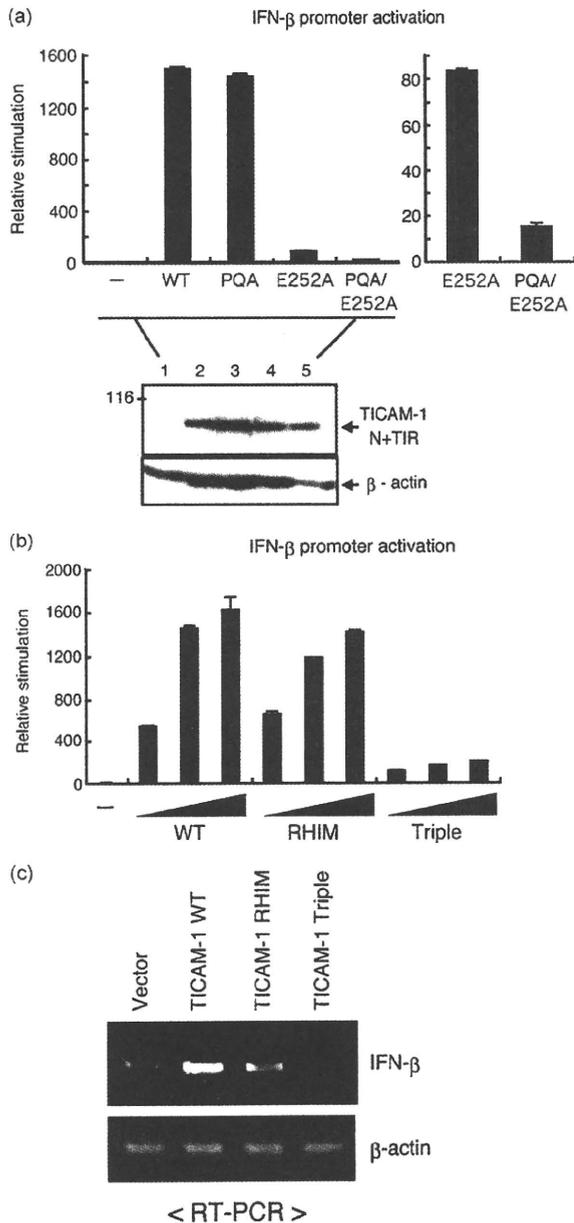


Fig. 3. TRAF2 and TRAF6 binding affect TICAM-1-mediated IFN- β induction. (a and b) HEK293 cells were transiently transfected with 10 ng (a) or 5, 25, 50 ng (b) of the indicated TICAM-1 mutant together with the p125-luc IFN- β promoter reporter plasmid (100 ng). Twenty-four hours after transfection, 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. PQA^{337S} and ^{252E} of TICAM-1 N + TIR are critical for TRAF2- and 6-recruitment

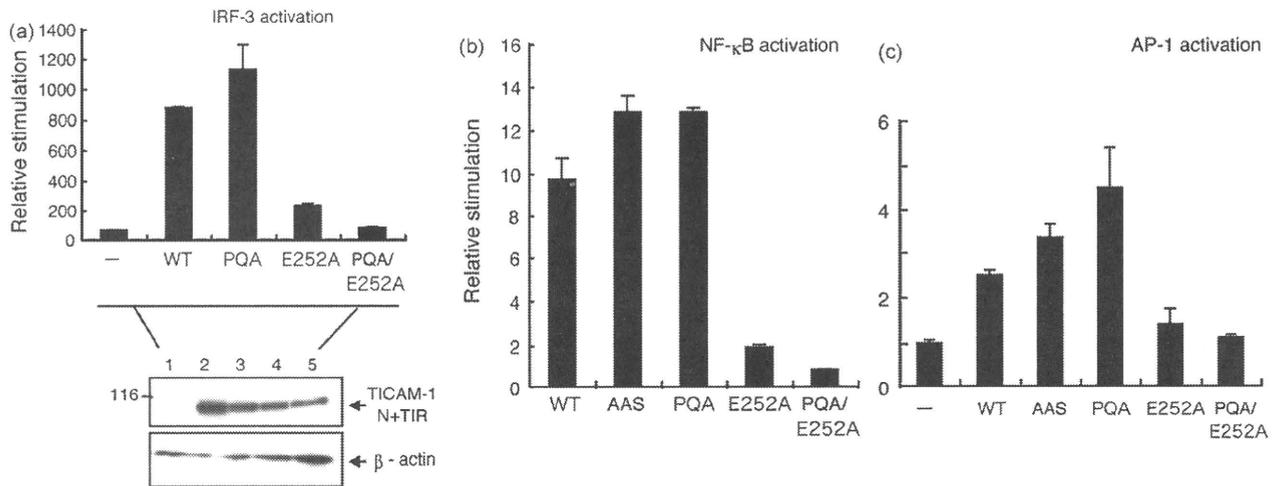
To clarify the participation of the single site of TICAM-1 N + TIR for TRAF2- and TRAF6-binding, confocal microscopy imaging analysis using labeled TRAF2, TRAF6, and TICAM-1 N + TIR was used (Fig. 5). While TRAF2 and TRAF6 merged with TICAM-1 N + TIR (upper panels) in overlaid images, under identical 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

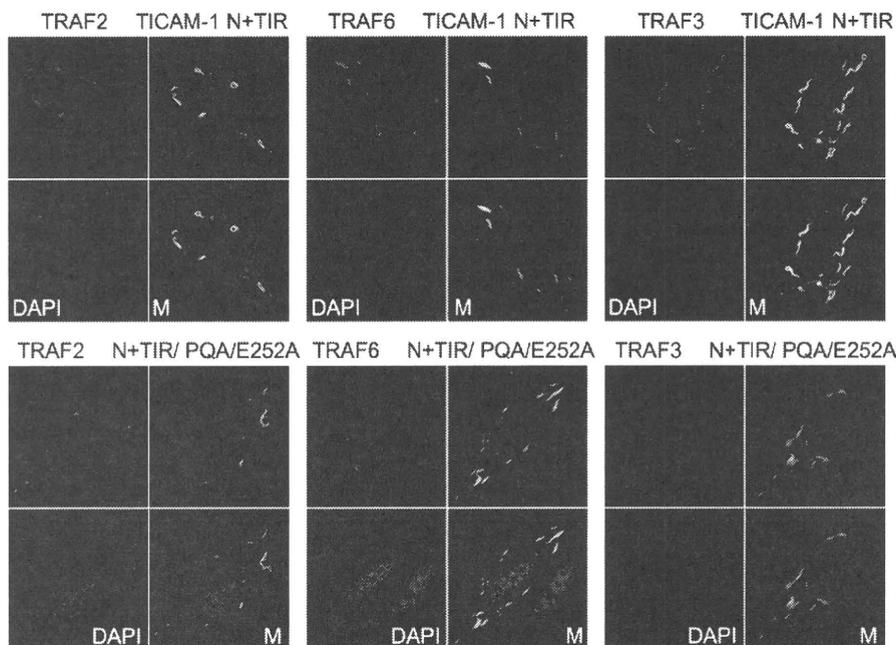


4. Complete TRAF2/6 binding to TICAM-1 leads to activation of the transcription factors IRF-3, NF-κ B, and AP-1. (a–c) HEK293 cells were transiently transfected with 100 ng (a) or 200 ng (b and c) of the indicated TICAM-1 mutant plasmid together with p55 UASG-Luc reporter and either GAL4-IRF3 (a), NF-κB reporter (b) or AP-1 reporter (c) smid. 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 polyubiquitinates 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 is 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



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 antibody. The same slide was also treated with DAPI for the staining of nuclei. M, Merging profile.

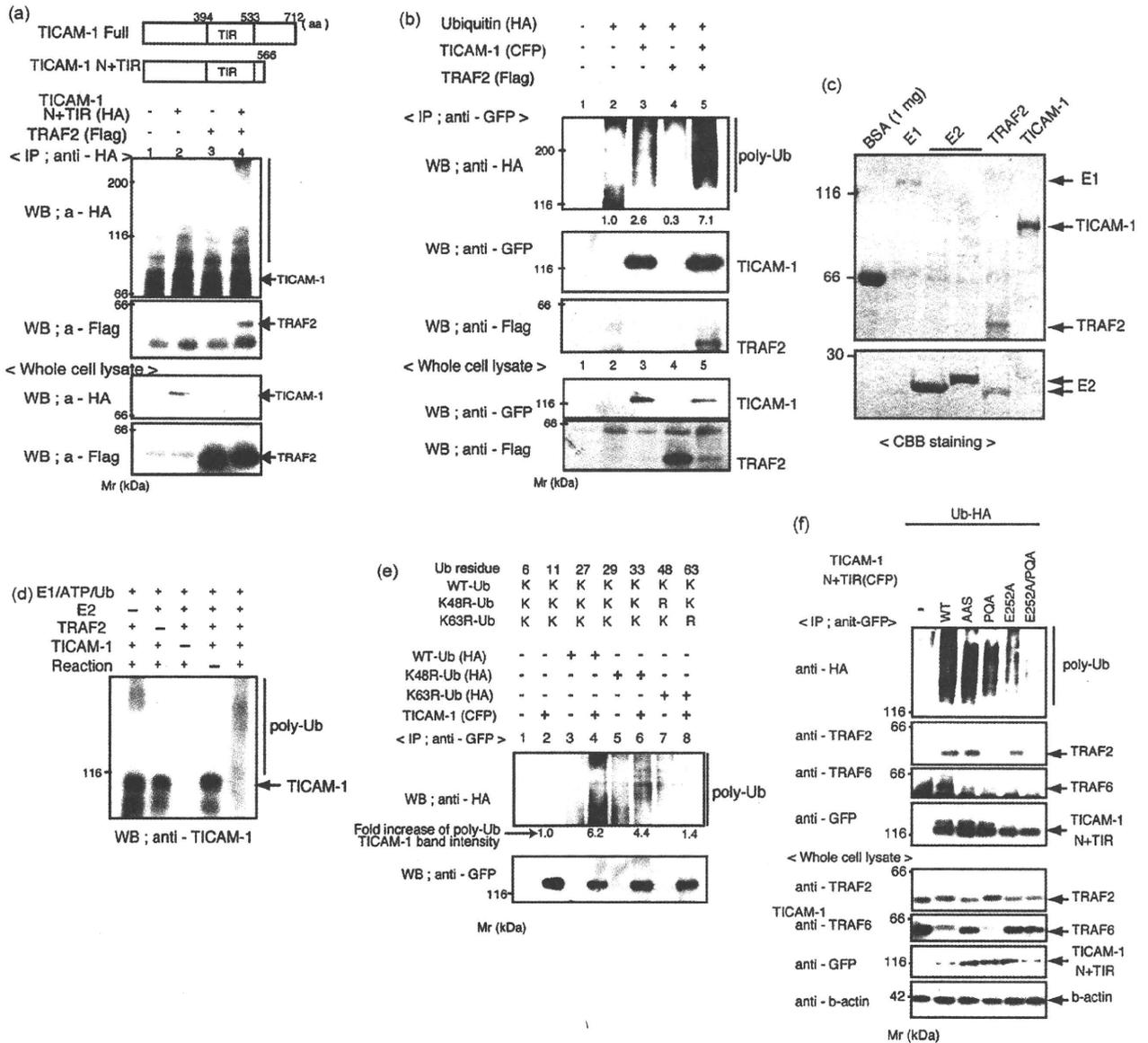


Fig. 6. TRAF2-mediated polyubiquitination of TICAM-1. (a) Interaction between TICAM-1, TICAM-1 N+TIR, and TRAF2. HEK293 cells were transfected with TICAM-1 N+TIR (HA) plasmid (100 ng) and TRAF2 (Flag) plasmid (2 µg) for expression of the indicated proteins. The total amount of DNA (4 µg/well) was kept 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, anti-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 immunoblot. 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 MgCl₂, 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

d 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 modulate TICAM-1 function, we focused on the functional modulation of TICAM-1 N+TIR by TRAF2/6, and revealed that the TRAF2 site on TICAM-1 serves as a functional modulator in the absence of the TRAF6 site (Figs. 3 and 4 and Supplementary data, Fig. S3). Our results indicate that (1) TICAM-1 polyubiquitination occurs essentially in parallel with TRAF2/6 function (Fig. 6), although the mechanism 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- κ B 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; Oganessian et al., 2006). Although this difference was attributed to differential usage of TRAF subtypes by specific cell types (Gohda et al., 2004), these results suggest that TRAF6 is not the only TRAF that is involved in TLR3 signaling. We therefore constructed TICAM-1 mutants that disrupted TRAF6- or TRAF2-binding sites and demonstrated that 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- κ B and IRF-3 activation, and the possibility of K63-linked polyubiquitination 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-deficient mice, which are embryonic lethal due to apoptosis of hepatocytes, the definitive *in vivo* role of TRAF2 in this pathway is known. 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 between 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- κ B, 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 are 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- κ B, 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 to 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; Oganessian 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; Oganessian 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 (IKK γ) (Leo et al., 2002) which suggests the reported IRF-3-activating kinase complex NAP1-IKK ϵ /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 IKK ϵ coprecipitate with TICAM-1 in poly(I: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- κ B activation. This is supported by the observation that polyubiquitination of TICAM-1 was required for TRAF3-binding to TICAM-1 (Oganessian et al., 2006). However, which E3 ligase is the best enhancer for TICAM-1 is a topic to be addressed in future experiments.

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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.

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

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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 adaptor 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 nucleo-

tide-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)-1 β 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

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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)- κ B 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 melanoma 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 phagocytosing 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-mobility 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- κ B, 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.

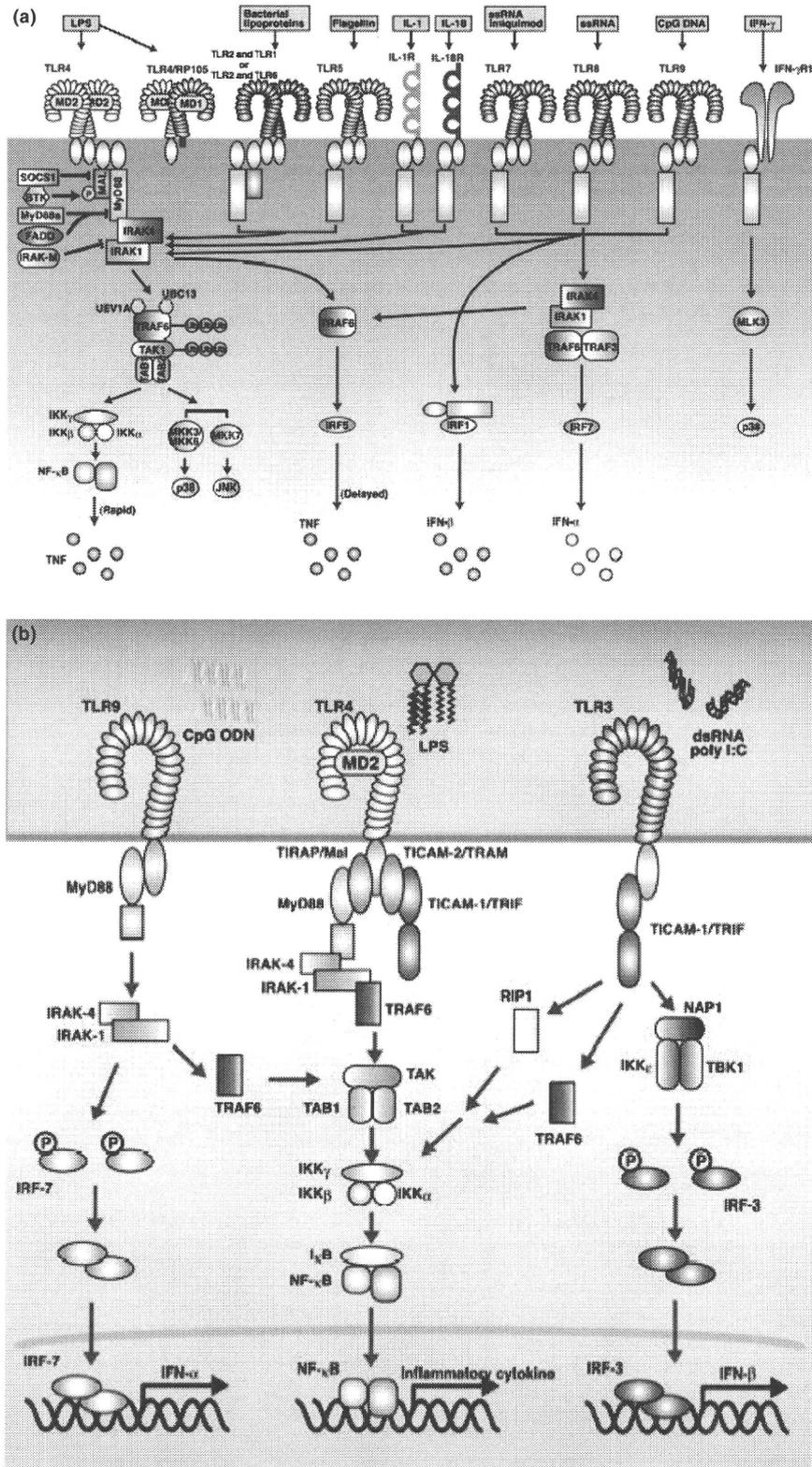


Fig. 1. MyD88 and Toll-interleukin 1 receptor-domain (TIR)-containing adaptor molecule (TICAM-1) pathways. MyD88 is an adaptor for all Toll-like receptors (TLR), except TLR3 (a). TLR2 and TLR4 recruit MyD88 via the bridging adaptor Toll-interleukin 1 receptor-domain (TIR)-containing adaptor protein (TIRAP) (MAL) (b). Other TLR directly recruit MyD88. MyD88 activates nuclear factor-kappa β (NF- κ B) in most cell types, except plasmacytoid dendritic cells (pDC), which activate the interferon-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 I 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 1 receptor domain (TIR)-containing adaptor 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)
Poly(I: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 1 receptor domain-containing adaptor protein (TIRAP)-MyD88 to signal the transcription factor NF- κ B.⁽⁴⁾ In contrast, TLR3 and TLR4 can utilize TICAM-1 as the adaptor.⁽³⁾ TLR4 recruits the combined adaptor Toll-IL-1R domain-containing adaptor 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 adjuvants. 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 1 (NAP1),⁽⁴¹⁾ receptor-interacting protein 1 (RIP1),⁽⁴²⁾ similar to NAP1 TBK adaptor (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-1 β , 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 plasmacytoid 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			<i>In vitro</i> -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, TLR8, 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.

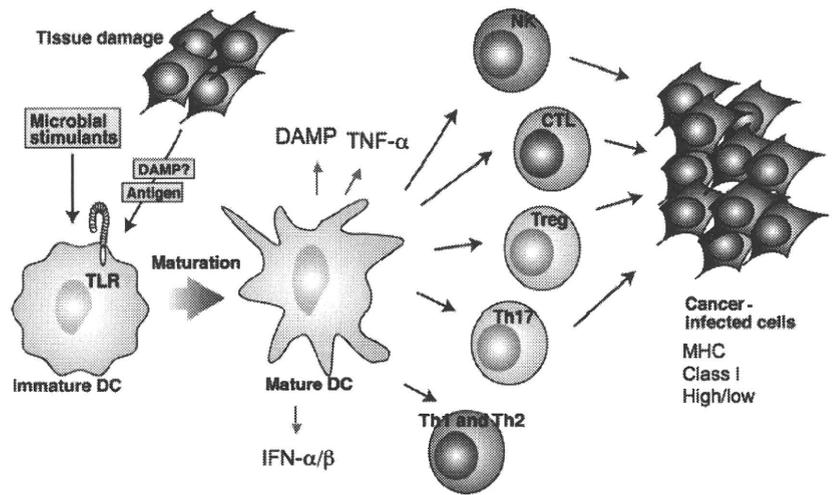


Fig. 2. Selective induction of immune effector lymphocytes by different agonistic stimuli. Each pattern molecule (PAMP) has its own uniqueness in myeloid dendritic cell (mDC) maturation. Differential maturation of mDC results in different effector driving as shown. CD8 T, various CD4 T, and B cells are 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, ROR γ T, 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 triphosphate (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

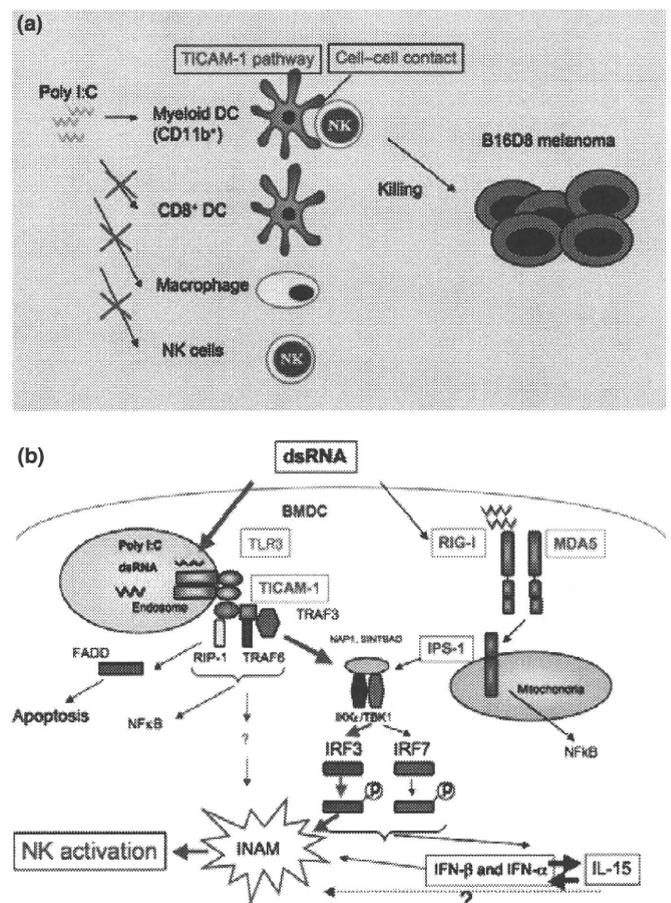


Fig. 3. A molecular mechanism of myeloid dendritic cell (mDC)-mediated natural killer (NK) activation. (a) CD11b+ bone marrow-derived dendritic cells (BMDC) act for natural killer (NK) activation by double-stranded (ds) RNA. NK cells express tumoricidal activity against major histocompatibility complex (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⁾

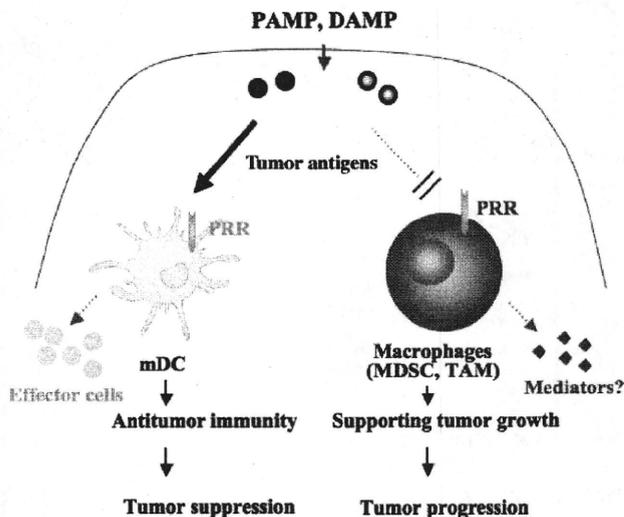


Fig. 4. Diverged functions of myeloid cells in tumor mass. A variety of myeloid subsets reside in tumor masses. Some of the subsets exhibit an immune suppressive feature that facilitates escape of tumor cells from immune effectors. Since pattern molecules (PAMP) act on both myeloid dendritic cells (mDC) 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 prerequisite 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.

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RESEARCH

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An embryo-specific expressing TGF- β family protein, growth-differentiation factor 3 (GDF3), augments progression of B16 melanoma

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Abstract

Malignant tumor cells often express embryonic antigens which share the expression with embryonic stem (ES) cells. The embryonic antigens are usually encoded by ES cell-specific genes, a number of which are associated with tumorigenesis and/or tumor progression. We examined the expression of ES cell-specific genes in the mouse B16 melanoma cell line to identify the factors promoting tumorigenesis. We found that endogenous growth-differentiation factor 3 (GDF3) expression was induced in implant B16 tumor during tumor progression in syngenic C57BL/6 mice. B16 F10, a subline with a high metastatic potential, continuously expressed GDF3 while low metastatic B16 F1 expressed comparatively decreased levels of GDF3. Overexpression of GDF3 promoted growth of implanted melanoma B16 F1 and F10 in syngenic mice. Ectopic expression of GDF3 was accompanied by an increased level of production of CD24/CD44. Such a profile was reported to be characteristic of melanoma stem cell-like cells. GDF3 expression was observed in embryonal carcinomas, primary testicular germ cell tumors, seminomas and breast carcinomas. However, the role of GDF3 in these cancers remains undetermined. Overexpression of GDF3 did not affect the growth of mouse hepatoma high or low metastatic sublines G5 or G1, both of which do not express GDF3. Since GDF3-driven CD24 acts as a receptor for endogenous innate immune ligands that modulate cell proliferation, CD24 is an effective determinant of tumorigenesis in malignant cell transformation. Finally, our results support the view that GDF3 has the ability to induce progression of CD24-inducible melanoma in mice.

Introduction

Growth-differentiation factor 3 (GDF3) belongs to the transforming growth factor (TGF)- β superfamily, and is also called Vgr-2 [1,2]. Human GDF3 was first identified during a study of cDNAs expressed in human embryonal carcinoma cells [3]. GDF3 expression is also found in primary testicular germ cell tumors, seminomas, and breast carcinomas. Despite its ubiquitous expression the role of GDF3 in cancer remains undetermined [4-6]. In normal tissues, GDF3 is expressed in embryonic stem (ES) cells and the early embryo [7-10]. Chen et al. have demonstrated that mice with null mutation on GDF3 exhibit developmental abnormalities [11].

Cancers are composed of heterogeneous cell populations. The cancer stem cell (CSC) hypothesis was

advocated for acute myeloid leukemia (AML) system [12] and recent studies have provided evidence that solid cancers can also originate from CSCs [13]. A previous report has shown that human melanomas also contain CSCs, and these tumor derived CSCs express ABCB5 [14]. This investigation also reported that the CSC population despite being very low could generate a tumor in human melanomas [14]. A recent work has shown that approximately 27% of human melanoma cells could initiate a tumor [15].

Mouse melanoma B16-F10 cells also contain CSC-like cells, which express CD133, CD44, and CD24 [16]. The mouse melanoma CSC-like cells, when injected subcutaneously into syngenic mice display tumorigenic ability [16]. Initial reports showed that the mouse CSC-like cells are a very small population, while most cells within the B16-F10 cell line retain the ability to induce malignancy [17].

The expression of ES-specific genes is observed in several human cancers. For example, the ES-specific gene,

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Sall4, is expressed in AML and precursor B-cell lymphoblastic leukemia [18,19]. Sall4 transgenic mice develop AML [19], but the molecular mechanism by which this occurs has not been shown yet. Another ES-specific gene, Klf4, functions as either a tumor suppressor or an oncogene in a tissue type or cell context dependent manner. Klf4 expression is frequently lost in colorectal [20], gastric [21], and bladder cancers [22]. Overexpression of Klf4 can reduce the tumorigenicity of colonic and gastric cancer cells *in vivo* [21,23]. On the other hand, high Klf4 expression levels have been detected in primary ductal carcinomas of the breast and oral squamous cell carcinomas [24,25], and ectopic expression of Klf4 induced squamous epithelial dysplasia in mice [26].

Because several ES-specific genes induce tumor progression, we tried to identify other ES-specific genes that promote tumorigenesis. Using mouse melanoma B16-F1 and B16-F10 cell lines as a model system, we found that GDF3 expression is different in these B16 sublines during tumor progression. We also observed that the ectopic expression of GDF3 promotes B16-F1 and B16-F10 tumorigenesis. Interestingly, B16-F1 and B16-F10 cells induced expression of CD133, ABCB5, CD44 and CD24, which are expressed in mouse melanoma CSC-like cells during tumorigenesis, and ectopic generation of GDF3 increased the CD24 expression. Since CD24 is a pattern-recognition receptor to participate in poor prognosis in cancer patients, we discussed the possible role of the GDF3-CD24 pathway in tumor progression.

Results

The expression of ES cell-specific genes in mouse melanoma B16 cells

We examined the expression of ES cell-specific genes in mouse melanoma B16 cell lines. The mouse melanoma B16-F10 cells were cultured in a 10-cm dish and their total RNA was extracted. Total RNA derived from excised C57BL/6 mouse skin was used as a control. RT-PCR analysis revealed that Sall4, Dppa5, Ecat1, and c-Myc were expressed in B16-F10 cells in culture dish but not in mouse skin (Figure 1A). In addition, Grb2, β -catenin, and Stat3 were expressed more in B16-F10 than in mouse skin (Figure 1A). Klf4 gene expression in B16-F10 cells was almost similar to that seen in mouse skin (Figure 1A). The expression of other genes was not detected under these experimental conditions (Figure 1A).

Expression of ES-specific genes during tumorigenesis

Next, we examined the expression of ES-specific genes in B16 sublines during tumorigenesis. B16-F1 or B16-F10 cells were injected subcutaneously into C57BL/6 mice. Seven days after injection the tumor was excised and total RNA was extracted. RT-PCR analysis revealed that Ecat1, Dppa5, Ecat8, GDF3, Sall4, Klf4, c-Myc,

β -catenin, Stat3, and Grb2 were expressed after tumorigenesis of B16-F1 and/or B16-F10 (Figure 1B,C).

Sall4, Grb2, β -catenin, and Stat3 are known to be expressed in tumor cells and their roles in cancer has been already studied [19,27,28]. Ecat1, Dppa5, and GDF3 genes are expressed in ES cells, but their expression in tumor has not yet been reported. We initially focused on Ecat1 and Dppa5 during tumorigenesis. To investigate the expression kinetics we excised the B16-F1 or B16-F10 tumor 7, 10, or 14 days after implantation, and extracted total RNA. RT-PCR analysis revealed that Ecat1 and Dppa5 expression did not increase during tumorigenesis in both sublines (Figure 2A and 2B).

Next, we focused on GDF3. GDF3 mRNA expression was not detectable in B16-F1 cells cultured in dish (day 0 in Figure 2C) and only a weak expression was detected in B16-F10 cells cultured in dish (day 0 in Figure 2D). Interestingly, GDF3 mRNA expression increased approximately 10-fold 7 days after s.c. inoculation in both B16-F1 and B16-F10 cells (Figure 2C and 2D). Following the increase for 7 days after injection, GDF3 expression gradually decreased in B16F1 cells, but maintained a high level in B16-F10 cells (Figure 2E and 2F).

GDF3 promotes the tumorigenesis of B16 melanoma

GDF3 is a member of TGF- β super family which is expressed in ES cells and in several human tumor cells. However, the role of GDF3 during tumorigenesis remains undetermined. To assess the functional importance of GDF3 expression during tumorigenesis, we examined whether the exogenous expression of GDF3 is sufficient to promote the tumorigenesis. Murine GDF3 cDNA was synthesized from the total RNA of B16-F1 cells and cloned into the pEF-BOS expression vector. The transfection efficiencies of this vector in B16-F1 and B16-F10 cells were ~25% with no difference between the two sublines. F1 or F10 cells were transfected with empty or GDF3-expressing vector. The following day, 1×10^6 of the transfected B16-F1 or B16-F10 cells were challenged subcutaneously into C57BL/6 mice and the tumor diameters were measured. The tumor diameters of the control B16-F1 tumors were larger than the control B16-F10 tumors at days 7, 10, and 14 (Figure 3A). Interestingly, the overexpression of GDF3 increased the tumor diameters in both B16-F1 and B16-F10 cells (Figure 3A). The promotion of tumorigenesis by GDF3 overexpression was also observed in mice injected with 1×10^5 of B16-F1 or B16-F10 cells (Figure 3B).

GDF3 does not promote tumorigenesis of hepatoma G1 or G5 cells

The expression profiles of ES-specific genes from mouse hepatoma G5 cells were different from those from B16-F1 and B16-F10 cells (Figure 4A). We then examined