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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-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

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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

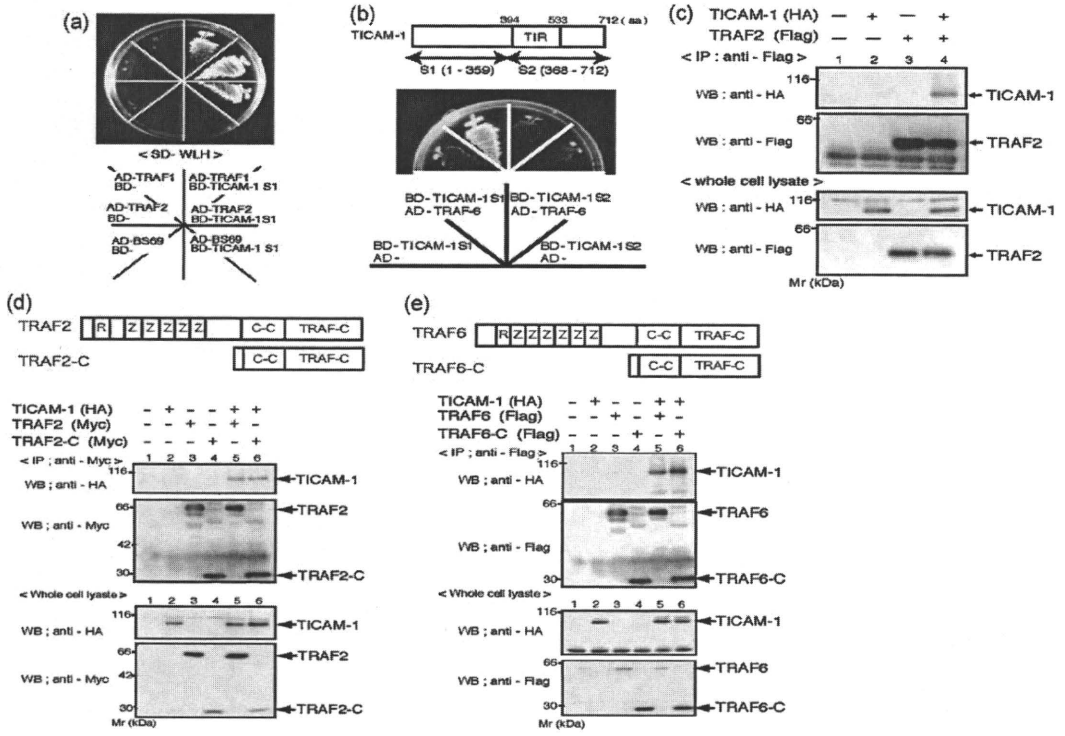

Fig. 1. Molecular interaction of TICAM-1 with TRAF2 and 6. (a) Direct interaction between the N-terminal region of TICAM-1 and either TRAF2 or TRAF6 as identified by yeast two-hybrid screening. Full-length TRAF2 and TRAF6 were cloned into pGADT7, transformed into yeast strain AH109, and then cultured 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.

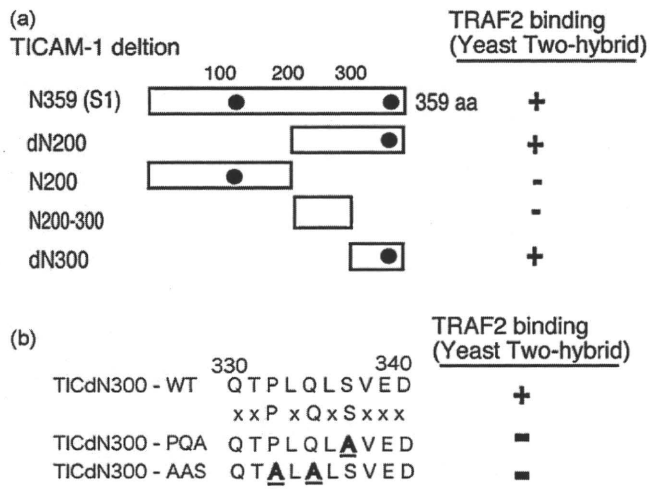

Fig. 2. Identification of the TRAF2-binding site in TICAM-1. (a) Scheme of TICAM-1 truncated mutants and location of the TRAF2 binding motif (black dot). TRAF2 in pGADT7 and each TICAM-1 construct in pGBKT7 were transformed into yeast. TRAF2 binding was assessed on SD-WLH plates as described in Fig. 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

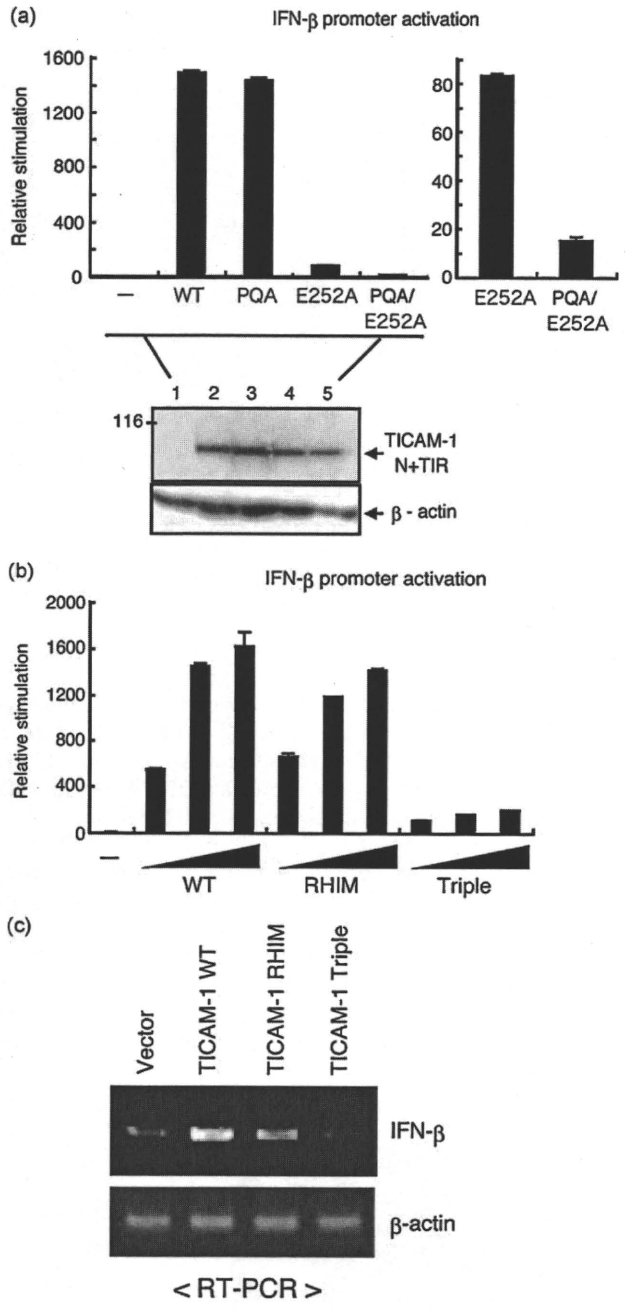

Fig. 3. TRAF2 and TRAF6 binding affect TICAM-1-mediated IFN-β induction. (a and b) HEK293 cells were transiently transfected with 10 ng (a) or 5, 25, 50 ng (b) of the indicated TICAM-1 mutant together with the p125-luc IFN-β promoter reporter plasmid (100 ng). Twenty-four hours after transfection, cells 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

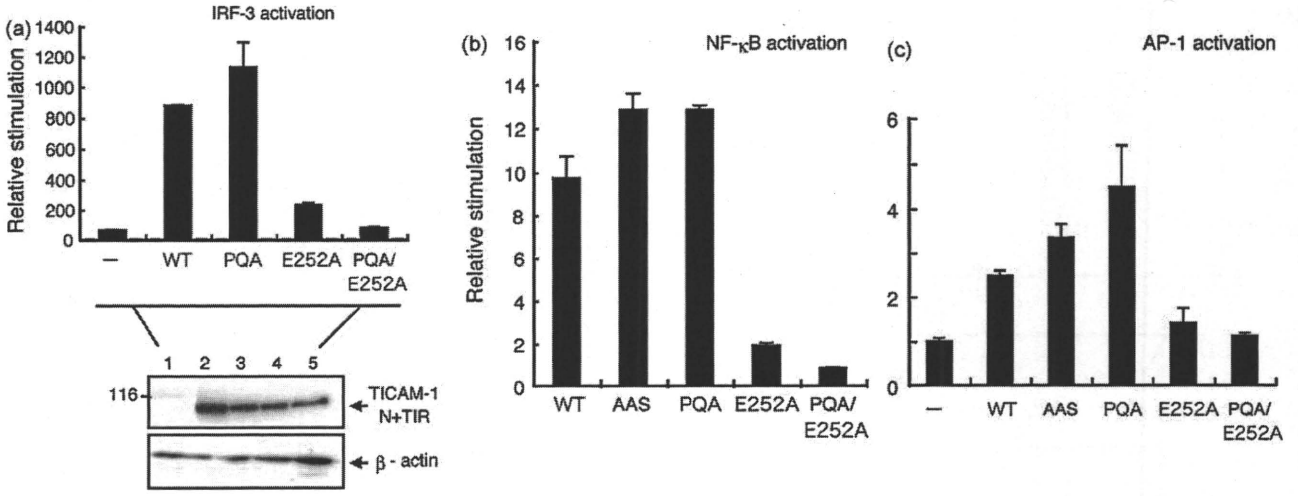

Fig. 4. Complete TRAF2/6 binding to TICAM-1 leads to activation of the transcription factors IRF-3, NF-κ B, and AP-1. (a–c) HEK293 cells were transiently transfected with 10 ng (a) or 200 ng (b and c) of the indicated TICAM-1 mutant plasmid together with p55 UASG-Luc reporter and either GAL4-IRF3 (a), 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

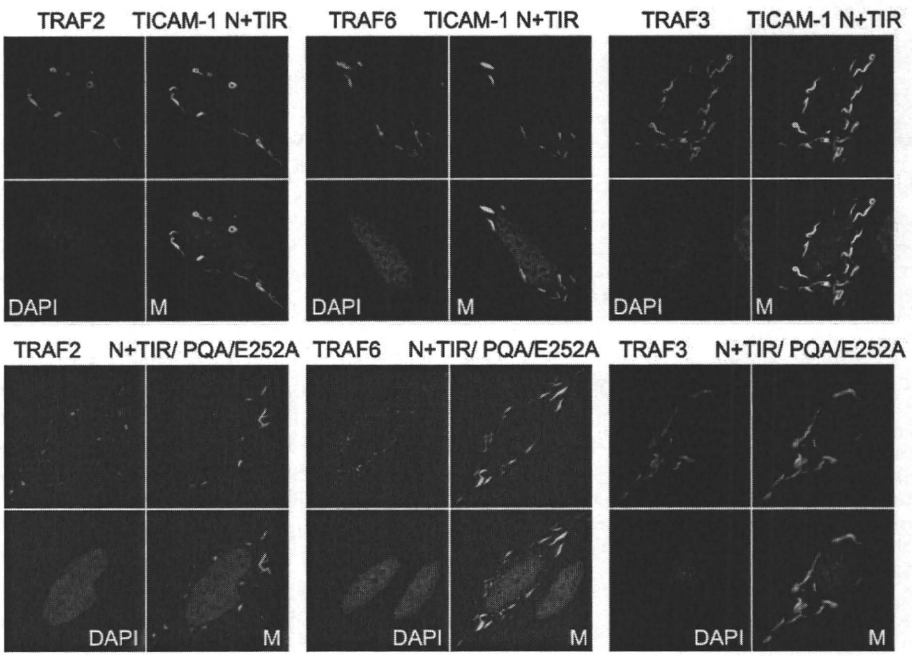

Fig. 5. Co-localization analysis of TICAM-1 N+TIR and TRAF proteins by confocal microscopy. HeLa cells were transfected with 30 ng of pECFP-N1 TICAM-1 and either 500 ng of pEF-BOS Flag-tagged TRAF2, TRAF6, or TRAF3. After 24 h, the cells were fixed, stained with anti-FLAG Ab, and then visualized with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

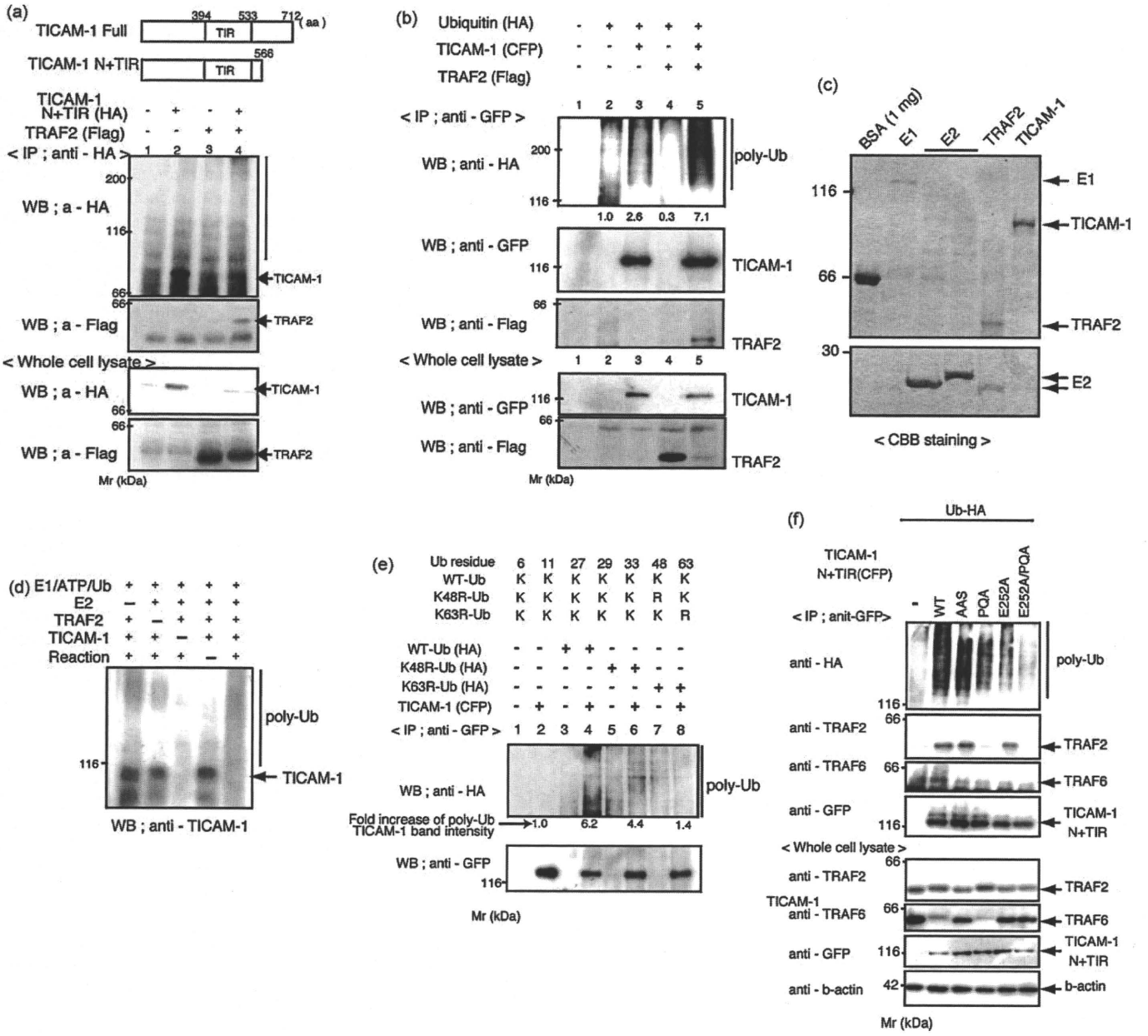

Fig. 6. TRAF2-mediated polyubiquitination of TICAM-1. (a) Interaction between TICAM-1, TICAM-1 N+TIR, and TRAF2. HEK293 cells were transfected with TICAM-1 N+TIR (HA) plasmid (100 ng) and TRAF2 (Flag) plasmid (2 μg) for expression of the indicated proteins. The total amount of DNA (4 μg/well) was kept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

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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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IL-6 and IFN-α from dsRNA-stimulated dendritic cells control expansion of regulatory T cells

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ABSTRACT

Foxp3$^+$CD4$^+$ regulatory T cells (Treg) control not only autoimmunity but also the effective immune response against RNA virus infections, which produces virus-derived double-stranded RNA (dsRNA). To induce effective anti-viral immunity, it is a key issue to learn how Treg respond to dsRNA *in vitro* and *in vivo*. We here showed that synthetic dsRNA, polyI:C, caused peripheral expansion of functional Treg in a TICAM-1- and IL-6-dependent manner *in vivo*. PolyI:C did not expand Treg directly, but promoted the expansion of naturally occurring Treg indirectly through IL-6 produced from dendritic cells (DCs). In addition, the expansion of Treg by IL-6 was inhibited by IFN-α from polyI:C-stimulated DCs. These data suggest that the balance of IL-6 and IFN-α in the region of RNA virus infection may determine the number of peripheral Treg, which affects the effective immune responses against viruses.

Introduction

CD4$^+$CD25$^+$ regulatory T cells (Treg) are crucial to control autoimmunity and maintain immunological self-tolerance [1,2]. The development and function of Treg is controlled by the forkhead/winged helix transcription factor Foxp3 [1,2]. Naturally occurring Treg cells (nTreg) are arising from thymus, while induced Treg (iTreg) are converted from peripheral CD4$^+$CD25$^-$ T cells [3,4]. Both Treg constitute 5–15% of peripheral CD4$^+$ cells and control not only immunological self-tolerance but also immune response to pathogens [4,5]. In RNA virus infections, during which virus-specific RNA patterns are generated in infected cells, many researchers suggest that peripheral Treg are increased to cause persistent infection of viruses [6].

Innate and adaptive immune responses against RNA virus infections are controlled by dendritic cells (DCs) [7]. For sensing virus-derived RNAs, murine DCs are armed with Toll-like receptor (TLR)3, TLR7 and TLR8, and RIG-I-like receptors (RLRs), which include RIG-I, MDA5 and LGP2 [8,9]. Myeloid DCs express TLR3 and TLR8, whereas plasmacytoid DCs (pDCs) exclusively express TLR7 [10]. TLR7 and TLR8 recognize single-stranded RNAs (ssRNAs), whereas TLR3 detects virus-derived dsRNAs. These three TLRs reside in the endosome to encounter exogenous RNAs [11]. While TLR7 and TLR8 require MyD88 as an adaptor molecule for its signaling, TLR3 recruits TIR-containing adaptor molecule (TICAM)-1 (also called TRIF) which induces type I IFN through IRF-3 activation and inflammatory cytokines (IL-6, TNF-α, etc.) by NF-κB activation [11].

In contrast, RLRs are distributed in a variety of cells including DCs. RIG-I and MDA5 are cytosolic sensors of RNAs and interact with a downstream mitochondrial protein, IFN-β promoter stimulator 1 (IPS-1, also called MAVS/VISA/CARDIF), which activates IRF-3 (interferon-regulatory factor 3), NF-κB (nuclear factor-kappaB), and AP-1 (activator protein 1) and induces IFN-β and inflammatory cytokines [9].

TLRs are also known to be expressed on CD4$^+$CD25$^+$Foxp3$^+$ Treg and directly modulate the proliferation and suppressive functions [12,13]. CD4$^+$CD25$^+$ Treg selectively expresses TLR4, TLR5, TLR7 and TLR8 [12]. In contrast, TLR1, TLR2, TLR3 and TLR6 are more widely expressed on CD4$^+$ T cells. TLR8 ligand is known to work on Treg directly and reverse the Treg suppressive activity [14]. However, the response of Treg against dsRNA is poorly understood neither *in vivo* nor *in vitro*.

Here, we examined the effect of synthetic dsRNA, polyI:C, on Treg expansion. PolyI:C increased peripheral Treg in a bone marrow-derived DC (BMDC)-dependent manner *in vivo* and *in vitro*.

Abbreviations: Treg, regulatory T cells; DC, dendritic cell; BMDC, bone marrow-derived dendritic cell; TICAM-1, Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule; Foxp3, forkhead box P3; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; IPS-1, IFN-β promoter stimulator 1; RLRs, RIG-I-like receptors.

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The polyI:C plus BMDCs expanded Treg in a TICAM-1- and IL-6-dependent manner. We also found that IFN-α from BMDCs suppressed the proliferation of nTreg. These indicate that myeloid DCs play a regulatory role in nTreg proliferation by producing IL-6 and IFN-α upon polyI:C stimulation.

Materials and methods

Mice and reagents. C57BL/6J mice and IL-6$^{-/-}$ mice were purchased from Charles River (Yokohama, Japan). TICAM-1$^{-/-}$ mice were generated in our laboratory [15]. IFNAR$^{-/-}$ mice were kindly provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). All mice were bred and housed pathogen-free in our facility with the approval of the Hokkaido University Animal Experiments Committee. PolyI:C was purchased from GE Healthcare (Chalfont St. Giles, UK). Recombinant murine IL-2 was purchased from Pepro Tech (Rocky Hill, NJ, USA). Recombinant murine IL-6 (097-04431) and IFN-α (130-093-131) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Miltenyi Biotec (Bergisch Gladbach, Germany), respectively. FITC anti-Foxp3 mAb (11-5773), PE anti-CD4 mAb (12-0042), PE-Cy5 anti-CD4 mAb (15-0042), FITC Rat IgG2a isotype control (11-4321), PE Rat IgG2a isotype control (12-4321), PE-Cy5 Rat IgG2a isotype control (15-4031) and functional grade anti-CD3 mAb (14-0033) were from eBioscience (San Diego, CA, USA).

Cells. CD4$^+$CD25$^+$ (Treg) cells and CD4$^+$CD25$^-$ cells were purified from mouse splenocytes using a MACS CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). BMDCs were generated from bone marrow cells by culture for 6 days in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (JRH Biosciences, Lenexa, KS, USA) in the presence of 500 IU/ml recombinant murine granulocyte macrophage colony-stimulating factor (Pepro Tech). Sometimes, BMDCs (1×10^6/ml) were incubated with or without 50 μg/ml polyI:C for 24 h and the supernatants were collected for ELISA. The concentrations of cytokines (IL-6 and IFN-α) were measured by commercial ELISA kits (Invitrogen, Carlsbad, CA, USA; PBL Biomedical Laboratories, Piscataway, NJ, USA). PolyI:C (1.25 mg/ml:200 μl) was injected intraperitoneally and inguinal lymph nodes were excised for FACS analysis. The ratio of Treg cells (CD4$^+$Foxp3$^+$/CD4$^+$) was determined by analysis from FlowJo (Tree Star Inc., OR, USA).

In vivo polyI:C administration. PolyI:C (250 mg/200 ml) or control phosphate-buffered saline (PBS) was intraperitoneally administered into mice twice at three days interval. Twenty-four hours after the last injection, the spleen and lymph nodes were extracted and total cell numbers were counted. Then, the numbers of the CD4$^+$ and CD4$^+$Foxp3 populations were assessed by FACS as described [16] and the scales of the CD4$^+$ and CD4$^+$Foxp3 fractions were evaluated.

Treg proliferation assay. Treg cells (5×10^4) were cultured in 96 wells round bottom-shaped plate in the presence of 1 μg/ml anti-CD3 antibody and 100 U/ml recombinant IL-2 with or without 50 μg/ml polyI:C for 2 days. For the Treg/BMDCs coculture, 1×10^6 BMDCs were added to the well. Occasionally, IL-6 (10 ng/ml) and/or IFN-α (10–10^4 IU/ml) were added to the culture. During the last 6 h of culturing, [^{3}H]thymidine (1 μCi/well) was mixed in the culture medium. The cells and medium were harvested separately by cell-harvester, and the radioactivity was measured by a liquid scintillation counter (Aloca, Tokyo, Japan).

Treg suppression assay. Treg cells were incubated with BMDCs for 2 days as described above, and subsequently only the Treg cells were resorted by MACS system. Splenocytes (1×10^5) were treated with mytomycin C (20 μg/ml, 45 min) and cultured with freshly isolated CD4$^+$CD25$^-$ T cells (responder, 2.5×10^4) for 2 days. The ratio of CD4$^+$CD25$^-$/CD4$^+$CD25$^+$ was indicated in the figure. The proliferation of responder cells was measured by [^{3}H]thymidine uptake assay.

Results

PolyI:C induces the proliferation of Treg in vivo and in vitro

To examine the effect of dsRNA on Treg function *in vivo*, we administered polyI:C intraperitoneally into mice and evaluated the absolute numbers and increase of Treg cells (CD4$^+$Foxp3$^+$) compared to CD4$^+$ T cells in the inguinal lymph nodes (LN) and spleen. Treg numbers were increased after polyI:C administration in LN (Fig. 1A and B), and spleen (data not shown). The results were confirmed with additional experiments (Fig. S1) where the numbers of the Treg cells in spleens and indicated lymph nodes were counted with mice treated with or without polyI:C as in Fig. 1A.

To investigate the mechanisms of Treg expansion by polyI:C, we first examined whether polyI:C acts on nTreg cells (CD4$^+$CD25$^+$ T cell) directly as a proliferation stimulator or whether polyI:C converts CD4$^+$CD25$^-$ T cells into CD4$^+$CD25$^+$ T cells (iTreg) *in vitro*. We observed that polyI:C stimulated Treg to activate the transcription factors downstream the TLR3/TICAM-1 pathway (data not shown), although polyI:C neither elicited proliferation of nTreg cells (Fig. 1C) nor induced CD4$^+$CD25$^+$ T cells from CD4$^+$CD25$^-$ T cells *in vitro* (Fig. S2). These results suggest that polyI:C may act on cells other than Treg to initiate Treg expansion.

To see if polyI:C expands Treg through myeloid DCs, we cultured nTreg and BMDCs in the presence of polyI:C *in vitro*. BMDC is the most likely candidate because it has been reported that LPS-matured BMDCs expand nTreg [16–18], and polyI:C induces maturation of BMDCs through TLR3 [7,19]. As a result, polyI:C plus BMDCs triggered Treg expansion (Fig. 1D). We next injected polyI:C-stimulated BMDCs intraperitoneally and examined the ratio of Treg/CD4$^+$ cells in LN. PolyI:C-stimulated BMDCs actually mediated peripheral Treg expansion *in vivo* (Fig. 1E). These results suggest that polyI:C-stimulated BMDCs help Treg expand *in vivo* and *in vitro*.

The Treg proliferation by polyI:C-stimulated DCs requires TICAM-1 signal and IL-6

Next we examined whether IL-6 induced by the TLR3/TICAM-1 pathway influences the Treg maintenance using IL-6$^{-/-}$ and TICAM-1$^{-/-}$ mice. When we injected polyI:C into IL-6$^{-/-}$ mice or TICAM-1$^{-/-}$ mice, there was no significant increase of Treg in LN (Fig. 2A). Consistent with our previous report [15], we found that TICAM-1$^{-/-}$ mice impaired full production of IL-6 in response to polyI:C *in vitro* and *in vivo* (Fig. 2B). These results suggest that the Treg expansion by polyI:C injection may require IL-6, which is produced through TICAM-1 signaling.

To see if IL-6- or TICAM-1-signaling is essential for polyI:C-stimulated BMDCs to expand Treg, Treg cells were cultured with BMDCs from TICAM-1$^{-/-}$, IL-6$^{-/-}$ or wild-type mice with or without polyI:C. The Treg expansion by polyI:C was largely suppressed with TICAM-1$^{-/-}$ BMDCs and more severely abrogated in IL-6$^{-/-}$ BMDCs (Fig. 2C). When we checked the IL-6 production from each culture, the Treg proliferation appeared to be associated with the IL-6 production from BMDCs (Fig. 2D). To see if the reconstitution of IL-6 can recover the reduced Treg proliferation by TICAM-1$^{-/-}$ or IL-6$^{-/-}$ BMDCs plus polyI:C, IL-6 was added into the BMDC/Treg coculture. The exogenous IL-6 could recover the Treg proliferation by BMDCs from TICAM-1$^{-/-}$ and IL-6$^{-/-}$ mice in the presence of polyI:C (Fig. 2E).

These data suggest that the Treg proliferation by BMDC plus polyI:C is dependent on IL-6 produced by BMDCs through the TLR3/TICAM-1 pathway.

DC produced IFN-α to inhibit the Treg expansion induced by IL-6

Next we cultured Treg with BMDCs with or without polyI:C in the presence or absence of exogenous IL-6. Treg was expanded

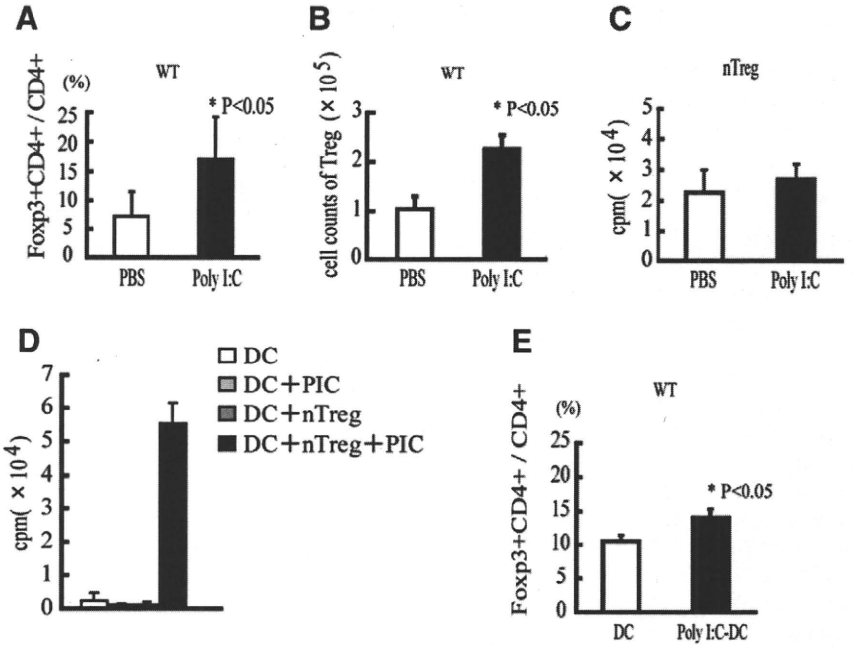

Fig. 1. PolyI:C induces the proliferation of Treg *in vivo* and *in vitro*. (A,B) C57BL/6J wild-type (WT) mice were intraperitoneally injected with polyI:C (1.25 mg/ml:200 μl) or PBS twice every 3 days throughout the experiments. Inguinal lymph nodes were excised, and the ratio of $CD4^+Foxp3^+$ /$CD4^+$ T cells (A) and the absolute number of $CD4^+Foxp3^+$ (B) cells were determined by FACS at 1 day after the final administration. (C) Freshly isolated $CD4^+CD25^+$ Treg (5×10^4) from WT mice were cultured in the presence of 1 μg/ml anti-CD3 antibody and 100 U/ml recombinant IL-2 with or without 50 μg/ml polyI:C. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. There was no statistical difference between them. (D) As in (C), but 1×10^6 WT BMDCs were added to each well. (E) The ratio of $CD4^+Foxp3^+/CD4^+$ T cells in LN was analyzed at 24 h after injection of non-treated BMDCs (DC) or BMDCs incubated with 50 μg/ml polyI:C (polyI:C DC) for 24 h. Data represented the mean ± SD of three independent experiments.

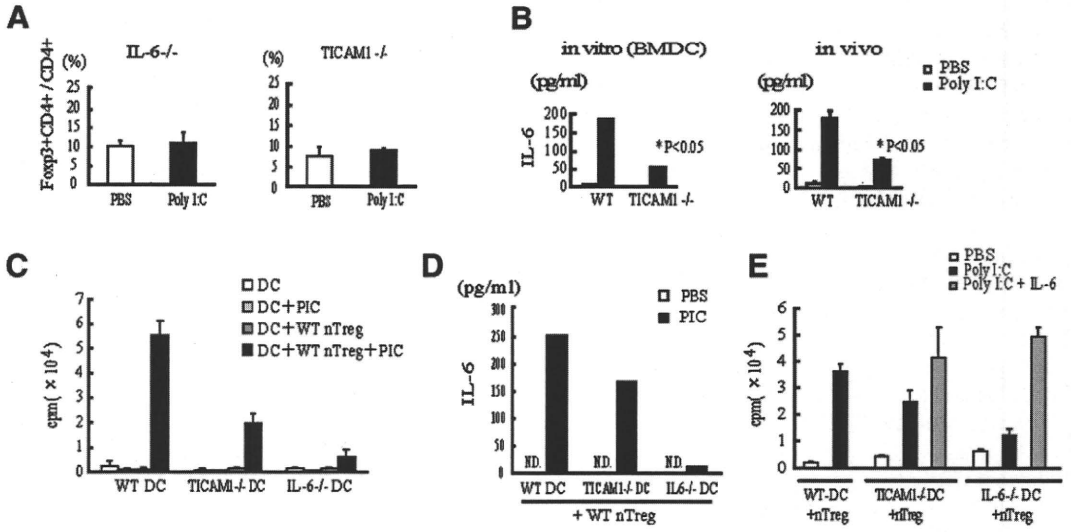

Fig. 2. The Treg proliferation by polyI:C plus BMDCs requires TICAM-1 signaling and IL-6. (A) TICAM-$1^{-/-}$ mice and IL-$6^{-/-}$ mice were intraperitoneally injected with polyI:C or PBS as in Fig. 1A and the ratio of $Foxp3^+CD4^+$ Treg/$CD4^+$ T cells was determined. There was no statistical difference between PBS-group and polyI:C-group. (B) The supernatants and sera were assayed for the production of IL-6. BMDCs were incubated with or without 50 μg/ml polyI:C for 24 h, and the supernatants were collected. The sera were collected at 24 h after injection of polyI:C. (C) BMDCs from WT, TICAM-$1^{-/-}$ or IL-$6^{-/-}$ mice (1×10^6) were cultured in the presence of 1 μg/ml anti-CD3 antibody and 100 U/ml recombinant IL-2 with or without Treg (5×10^4) from WT mice in the presence or absence of 50 μg/ml polyI:C. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (D) As in (C), but Treg from WT mice were cultured with BMDCs from WT, TICAM-$1^{-/-}$ or IL-$6^{-/-}$ mice. After 24 h culture, supernatants were collected and measured for IL-6 production. (E) As in (C), but Treg from WT mice were cultured with BMDCs from WT, TICAM-$1^{-/-}$ or IL-$6^{-/-}$ mice with or without 50 μg/ml polyI:C or polyI:C plus 10 ng/ml IL-6. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. Data represented the mean ± SD of three independent experiments.

by polyI:C plus BMDCs as described above, and Treg proliferated better in the presence of both polyI:C and IL-6 (Fig. 3A). However, interestingly, we found that Treg was expanded much better by IL-6 alone (Fig. 3A). This indicates that Treg-proliferation induced by IL-6 seems to be suppressed by polyI:C.

Since type I IFN is a critical factor for Th1-dominant CD4 response against dsRNA [20], we hypothesized that IFN-α produced by polyI:C-stimulated BMDCs may induce proliferation of Th1 cells and suppress the Treg-proliferation induced by IL-6 from polyI:C-stimulated BMDCs. To test this possibility, we first measured

IFN-α production in serum from polyI:C-injected wild-type and TICAM-1$^{-/-}$ mice. As shown in Fig. 3B left, IFN-α production was intact in TICAM-1$^{-/-}$ mice after the polyI:C injection. IFN-α production in culture supernatants was also similar between BMDCs from wild-type mice stimulated with polyI:C and those from TICAM-1$^{-/-}$ mice (Fig. 3B right). The results infer that cytoplasmic MDA5 rather than TLR3 preferentially induces IFN-α in response to polyI:C in our setting *in vivo* and *in vitro*.

Next, we checked if exogenous IFN-α could inhibit the Treg proliferation. When Treg were cultured with BMDCs in the presence of polyI:C and graded doses of IFN-α, IFN-α actually inhibited the Treg proliferation in a dose-dependent manner (Fig. 3C). IFN-α also abolished the proliferation of Treg induced by BMDCs plus IL-6 in a dose-dependent manner (Fig. 3D). To see if IFN-α derived from BMDCs is responsible for the suppression of the Treg-proliferation induced by IL-6 from polyI:C-stimulated BMDCs, we used IFNAR$^{-/-}$ BMDCs which barely amplify type I IFN production but can activate the MDA5/IPS-1 pathway [15]. We found that IFNAR$^{-/-}$ BMDCs did not suppress IL-6-mediated Treg expansion induced by polyI:C-stimulated BMDCs (Fig. 3E). These indicate that IFN-α has negative effect on Treg-proliferation induced by IL-6 derived from polyI:C-stimulated BMDCs.

We next examined which cells were required to be stimulated by these two cytokines for Treg expansion. BMDCs were treated with mitomycin C after stimulation with IL-6 and/or IFN-α and co-cultured with Treg cells in the presence of IL-6 and/or IFN-α. In this series of experiments, we could not observe any effects of IL-6 and IFN-α on direct Treg expansion (Fig. 3F), suggesting that IL-6 and IFN-α modulate the BMDC function to adjust the Treg number in the periphery.

Treg cells expanded by polyI:C-stimulated DCs are functional in vitro

Finally, we tested whether polyI:C-stimulated BMDC-driven Treg cells sustain the suppressive activity against responder cells. Treg suppressive activity was not altered after co-culturing with BMDC in the presence of polyI:C, IL-6 and IFN-α (Fig. 4A and B). Hence, IL-6 and type I IFN from BMDCs control the number of Treg cells but not the ability to suppress naïve T cells.

Discussion

We demonstrated in this study that BMDCs control proliferation of Treg by secreting IL-6 and IFN-α after sensing dsRNA. Although IFN-α negatively acts on Treg expansion, IL-6 overwhelmed the inhibitory effects of IFN-α on Treg. As a result, dsRNA caused proliferation of Treg with competent suppressive activity. Although the cytoplasmic polyI:C response governs the level of type I IFN in BMDCs and *in vivo*, the TICAM-1 pathway in BMDCs participates in proliferation of Treg in the periphery.

IFN-α is a main anti-viral cytokine that induces many IFN-inducible gene products, such as OAS, Mx1, and ISG15, leading to the limitation of RNA virus replication [8,20]. Here we describe a new anti-viral function of IFN-α. IFN-α suppressed Treg-proliferation induced by IL-6 derived from polyI:C-treated myeloid DCs. Treg cells suppress DC function and T-cell activation as well as NK activation [4]. Therefore, type I IFN including IFN-α may work to enforce the anti-viral cellular immunity by inhibiting Treg proliferation. In RNA virus infections, not only myeloid DCs but also pDCs and other virus-infected cells systematically produce type I IFN [8], which can contribute to the inhibition of Treg proliferation *in vivo*. Our data suggest that the tissue-specific cytokine balance between IL-6 and IFN-α is a determinant factor of Treg expansion.

IFN-α and IL-6 are known to up-regulate co-stimulatory molecules such as CD80 and CD86 on DCs. We have shown that CD8$^+$CD205$^+$ splenic DCs in the steady state induce antigen-specific Foxp3$^+$ Treg from Foxp3$^-$CD25$^-$CD4$^+$ T cells using endogenous TGF-β [21]. Thus, specific resident DC subsets govern iTreg induction. Our present data speculate that bone marrow-supplied DC subsets in the inflammatory states also regulate the peripheral Treg balance. The Treg control by polyI:C-stimulated BMDCs is IL-6- and IFN-α-dependent and may modally distinct from that of the splenic DCs. Although what pathogenic states preferentially enhance nTreg expansion remain to be elucidated, it is interesting

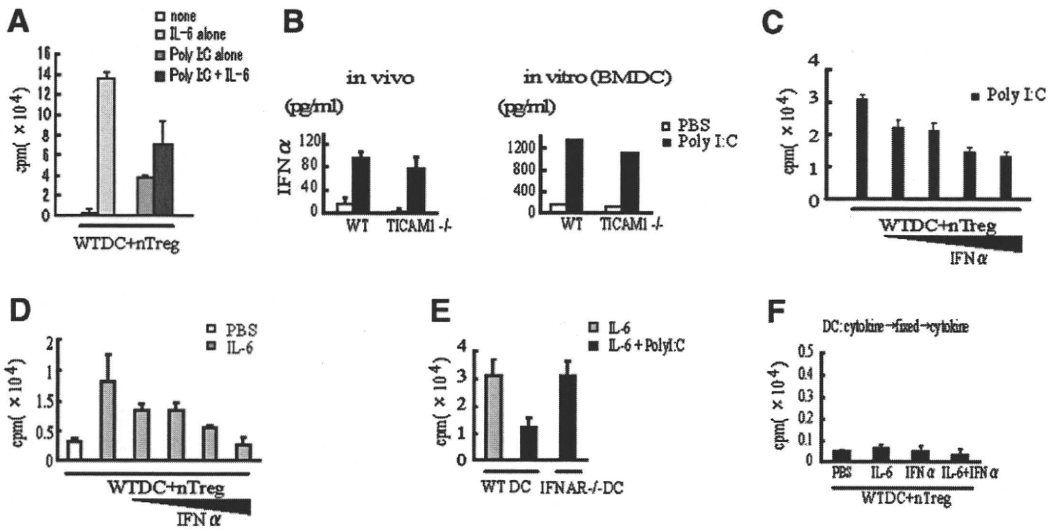

Fig. 3. Effect of IFN-α and IL-6 on Treg expansion. (A) As in Fig. 2C, but Treg from WT mice were cultured with WT BMDCs with or without 50 μg/ml polyI:C or 10 ng/ml IL-6. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (B) As in Fig. 2B, but the supernatants and sera were assayed for production of IFN-α. (C) As in (A), but graded doses of IFN-α (10–10^4 IU/ml) was added to the culture with 50 μg/ml polyI:C. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (D) As in (C), but graded doses of IFN-α (10–10^4 IU/ml) was added to the culture with or without IL-6 (10 ng/ml). The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (E) As in Fig. 2C, but Treg from WT mice were cultured with BMDCs were from IFNAR$^{-/-}$ or WT mice in the presence of 10 ng/ml IL-6 with or without 50 μg/ml polyI:C. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (F) WT BMDCs were incubated with IFN-α (10^3 IU/ml) and/or IL-6 (10 ng/ml) for 24 h and fixed by mitomycin C subsequently. Then, nTreg were cultured with these fixed BMDCs for 2 days in the presence of the same cytokines used with stimulating BMDCs. Data represented the mean ± SD of three independent experiments.

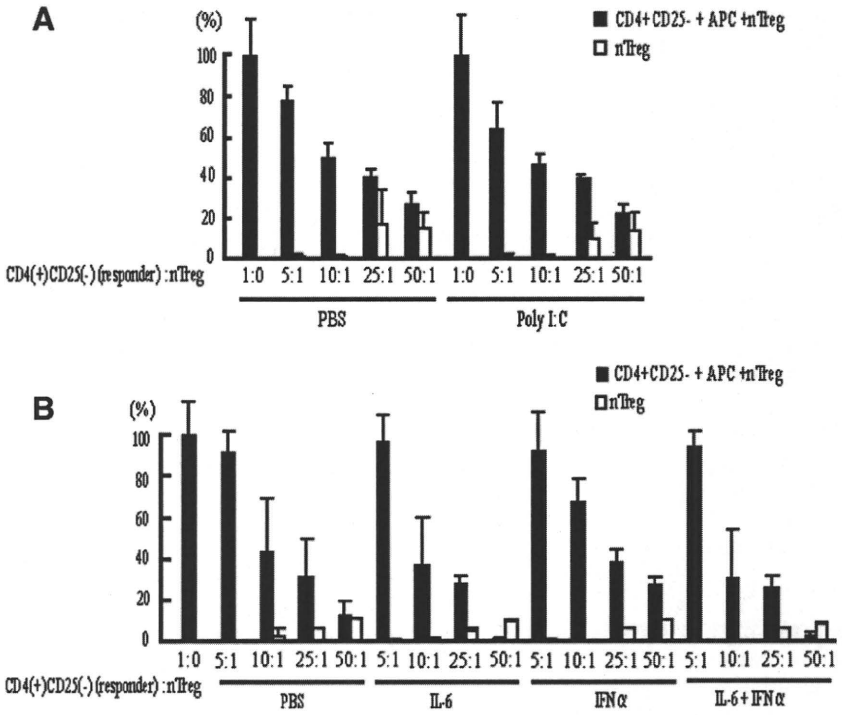

Fig. 4. Treg expanded by polyI:C plus BMDCs are suppressive *in vitro*. (A) Treg were isolated after 2-day culture with BMDCs in the absence (PBS) or presence of 50 μg/ml polyI:C (polyI:C). Then, these nTreg (suppressor) were cultured with freshly isolated $CD4^+CD25^-$ T cells (responder, 2.5×10^4), mitomycin C-treated splenocytes (1×10^5) and anti-CD3 Ab for 2 days. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (B) As in (A), but Treg were cultured with BMDCs with or without IL-6 (10 ng/ml) or IFN-α (10^3 IU/ml), and used for the suppression assay.

that IL-6 and IFN-α differentially regulate myeloid DC function to stimulate nTreg.

Our data showed that peripheral expansion of Treg is dependent on IL-6 induced by polyI:C, though an *in vivo* Treg increase is less efficient than *in vitro*. IL-6 has been shown to play a multifarious role to expand and maintain Treg. IL-6 has contrasting effects against nTreg and iTreg [15,17,22,23]. IL-1 and IL-6 production by myeloid DC is required to enhance nTreg proliferation after LPS stimulation [17]. Treg can be induced from $CD4^+CD25^-$ T cells, and peripheral Treg number is controlled in the balance between iTreg and pro-inflammatory IL-17-secreting cells (Th17) [5]. IL-6 and TGF-β together induce the differentiation of Th17 cells from naive T cells [24,25]. Moreover, IL-6 can convert nTreg to Th17 cells [26]. Therefore, in this line, pro-inflammatory effects of IL-6 promote differentiation of Th17, but not that of Treg.

In our experiments, $CD4^+CD25^+Foxp3^+$ cells were not induced from $CD4^+CD25^-$ T cells by function of polyI:C-stimulated BMDCs (Fig. S2). However, in contrast, the polyI:C-stimulated BMDCs could expand Treg (Fig. 1D). Moreover, although TGF-β is a key cytokine for differentiation of iTreg and Th17, serum level of TGF-β did not increase after i.p. polyI:C administration, and BMDC did not produce TGF-β (data not shown). Therefore, we prefer the interpretation that the peripheral increase of Treg numbers by polyI:C is due to the proliferation of nTreg *in vivo*. However, since there is no marker to distinguish nTreg from iTreg, we have no way to examine the actual proportion of these two subsets *in vivo*.

TLR ligands including TLR2, TLR4, TLR5, and TLR8 directly modulate the Treg suppressive function and number of nTreg [12–14]. TLR-signaling through TLR2 or TLR4 in nTreg enhances proliferation and suppressive activity of nTreg [12,13]. In our investigation, nTreg did not proliferate in direct response to polyI:C, a TLR3 ligand alone; however, polyI:C enhances nTreg expansion in the presence of BMDCs by the DC TICAM-1-mediated pathway. Previous reports showed that TLRs in BMDCs control Treg expansion and function, using a TLR4 ligand, LPS [16–18]. Since TLR4 signaling induces type I IFN and IL-6 mainly through the TICAM-1 pathway, it is possible that these two cytokines produced by TLR4 signaling may also exert its suppressive or enhancing effects on Treg proliferation as in the case of polyI:C stimulation.

It is an intriguing idea to control Treg for the induction of effective anti-viral immunity against persistent RNA virus infections. We found that IFN-α-treated mDCs actually suppress Treg growth, whereas signaling of IL-6 on mDCs overcomes the IFN-α-mediated suppression of Treg expansion. Investigating how Treg are controlled by these two cytokines may shed light on developing a new way to induce powerful anti-virus immunity on RNA virus infection.

Acknowledgments

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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.bbrc.2009.12.081.

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Dendritic cell-derived TNF-α is responsible for development of IL-10-producing CD4$^+$ T cells

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ABSTRACT

Immature dendritic cells (DCs) appear to be involved in peripheral immune tolerance via induction of IL-10-producing CD4$^+$ T cells. We examined the role of TNF-α in generation of the IL-10-producing CD4$^+$ T cells by immature DCs. Immature bone marrow-derived DCs from wild type (WT) or TNF-α$^{-/-}$ mice were cocultured with CD4$^+$ T cells from OVA specific TCR transgenic mice (OT-II) in the presence of $OVA_{323-339}$ peptide. The WT DCs efficiently induced the antigen-specific IL-10-producing CD4$^+$ T cells, while the ability of the TNF-α$^{-/-}$ DCs to induce these CD4$^+$ T cells was considerably depressed. Addition of exogenous TNF-α recovered the impaired ability of the TNF-α$^{-/-}$ DCs to induce IL-10-producing T cells. However, no difference in this ability was observed between TNF-α$^{-/-}$ and WT DCs after their maturation by LPS. Thus, TNF-α appears to be critical for the generation of IL-10-producing CD4$^+$ T cells during the antigen presentation by immature DCs.

1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells which are primarily responsible for the initiation and regulation of immune responses against various antigens [1–3]. DCs exhibit a unique ability to activate naive T cells. The DC ability for the antigen presentation to T cells depends on their maturation stage. Immature DCs are present in almost all tissues as sentinels of the immune system. When encountering pathogens, immature DCs recognize the pathogen-derived components via pattern recognition receptors such as Toll-like receptors (TLRs) and differentiate to mature DCs [4]. The mature DCs highly express major histocompatibility complex (MHC) and co-stimulatory molecules on their surface and potently activate the antigen-specific CD4$^+$ T cells to eliminate the pathogens.

DCs also play a role in the maintenance of peripheral tolerance to self-antigens in the steady state. Actually, immature DCs induce T cell anergy or IL-10-producing regulatory T cells in vitro and in vivo [5–8]. Thus, it has been considered that interaction of T cells with immature DCs cause immune tolerance, while the interaction with mature DCs generates T cell immunity. However, the precise mechanism underlying the immature DC-mediated induction of IL-10 producing T cells remains unclear.

Tumor necrosis factor (TNF)-α is a major inflammatory cytokine and promotes various inflammatory responses. However, it has been reported that TNF-α-pretreated DCs ameliorate experimental autoimmune encephalomyelitis [9]. It seems that TNF-α exhibits not only proinflammatory functions but also displays immunoregulatory properties.

In this study, we examined the role of DC-produced TNF-α in the development of IL-10-producing CD4$^+$ T cells in vitro using bone marrow-derived DCs (BMDCs) and ovalbumin (OVA)-specific T-cell receptor (TCR) transgenic T cells (OT-II transgenic T cells). We demonstrate herein that the DC-derived TNF-α is responsible for the development of the IL-10-producing CD4$^+$ T cells by immature DCs.

2. Materials and methods

2.1. Mice

Wild type (WT) C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). TNF-α knock out (TNF-α$^{-/-}$) mice and

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OT-II TCR ($OVA_{323-339}$ peptide specific) transgenic mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a specific pathogen-free condition of our animal facility at Hokkaido University. All experiments were approved by regulations of Hokkaido University Animal Care and Use Committee.

2.2. Flow cytometry

FITC-conjugated anti-mouse CD86 mAb (GL1), FITC-conjugated anti-mouse IFN-γ mAb (XF G1.2), PE-conjugated anti-mouse CD40 mAb (3/23), biotin-conjugated anti-mouse I-A^b, PerCP™-conjugated anti-mouse CD4 (RM4–5) and streptavidin-PerCP™ were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-mouse IL-10 mAb (JES5-16E3) and allophycocyanin-conjugated anti-mouse IL-4 mAb (11B11) were obtained from BioLegend, Inc. (San Diego, CA). Cells were stained using FITC-, PE-, PerCP™-, allophycocyanin-, or biotin-conjugated mAbs and streptavidin-PerCP™. The fluorescence intensity of the cells was analyzed by flow cytometry on EPICS XL (Beckman Coulter, Inc., Miami, FL) or FACSCanto II (BD Biosciences, San Jose, CA).

2.3. DC culture

Murine BMDCs were generated by a well-established method as previously described [10–12]. Bone marrow cells were prepared from femur and tibial bone marrow of WT or TNF-$\alpha^{-/-}$ mice. After lysis of erythrocytes, MHC class II-, CD45R (B220)-, CD4- and CD8-positive cells were removed by killing with mAbs (1E4, RA3-6B2, GK1.5 and 53-6.7) and rabbit complement. The cells were extensively washed to remove mAbs, complement, and cell debris. The cells were cultured in 5% FCS RPMI-1640 containing 20 ng/ml GM-CSF (BMDC medium) at a density of 1×10^6 cells/ml/well (24-well plate). On day 2, the medium was gently exchanged to fresh medium. On day 4, non-adherent granulocytes were removed without dislodging clusters of developing DCs, and fresh medium was added. On day 6, free-floating and loosely adherent cells were collected and were used as BMDCs (>95% $CD11c^+$ $B220^-$). Unstimulated BMDCs were used as immature DCs. BMDCs cultured with 1 μg/ml LPS for 24 h were used as mature DCs.

2.4. T cell differentiation in the presence of WT or TNF-$\alpha^{-/-}$ DCs

$CD4^+$ T cells were isolated from spleens and LNs of OT-II TCR transgenic mice. After lysis of erythrocytes, $CD4^+$ T cells were selected using anti-CD4 (L3T4) MicroBeads and a MACS column (Miltenyi Biotec, Auburn, CA). Purity of the $CD4^+$ T cells ($CD4^+$ $TCR\beta^+$) was >95%. The purified OT-II $CD4^+$ T cells (5×10^4 cells) were cocultured with WT or TNF-$\alpha^{-/-}$ DCs (1×10^4 cells) in the presence of 0.1 μM $OVA_{323-339}$ peptide in 200 μl RPMI-1640 containing 10% FCS and 50 μM 2-ME for 5 days using a 96-well round-bottom plate. In some experiment, 100 ng/ml TNF-α was added to the culture. The culture supernatants were subjected to quantification of cytokines by ELISA using OptEIA Set (BD Biosciences). For intracellular cytokine staining, the cells were harvested and restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug (BD Biosciences) for 5 h. The intracellular staining of IL-4 and IL-10 was performed using the Cytofix/Cytoperm Kit (BD Biosciences). Proportion of IL-4 and IL-10 positive cells in the $CD4^+$ T cells were determined by flow cytometry.

3. Results

3.1. Cell surface expressions of maturation markers on TNF-$\alpha^{-/-}$ DCs

In this study, we used BMDCs (>95% $CD11c^+$) from WT and TNF-$\alpha^{-/-}$ mice to stimulate antigen-specific $CD4^+$ T cells (OT-II). These BMDCs were positive for CD11b and negative for CD8 and B220 (data not shown), a pattern typical of conventional DCs. We first examined the cell surface expression of maturation markers, CD86, CD40 and I-A^b, on these DCs (Fig. 1). Both types of DCs from WT and TNF-$\alpha^{-/-}$ mice showed immature phenotype, low expression of CD86 and CD40 and moderate expression of I-A^b. No significant difference was detected in CD86 and I-A^b expressions between WT and TNF-$\alpha^{-/-}$ DCs. CD40 expression on TNF-$\alpha^{-/-}$ DCs was slightly but significantly lower than that of WT DCs.

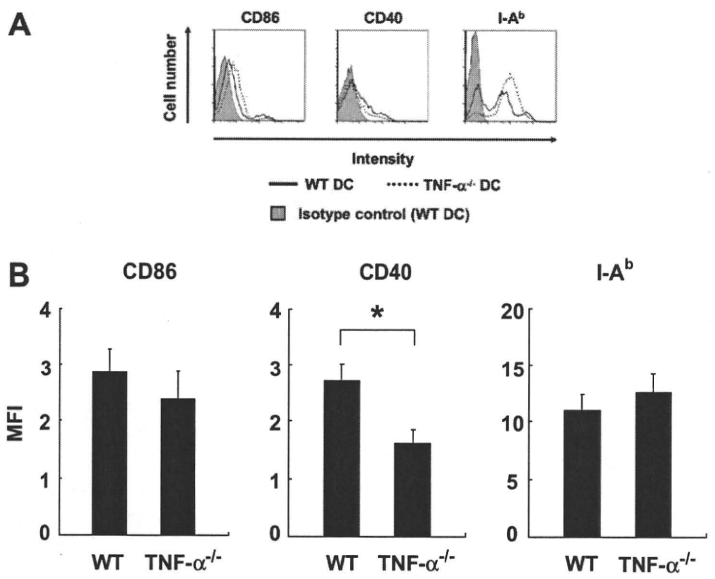

Fig. 1. Expressions of surface molecules on WT and TNF-$\alpha^{-/-}$ DCs. BMDCs were generated from WT or TNF-$\alpha^{-/-}$ mice. Expressions of CD86, CD40 and I-A^b were analyzed by flow cytometry. (A) Representative histogram of each molecule on DCs. (B) Each column represents the means ± SE of three independent experiments. MFI, mean fluorescence intensity. Statistical significance was calculated by Student's *t*-test (*: <0.05).

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However, this change was negligible compared to that induced by treatment with LPS (data not shown) [13].

3.2. IL-10 production in the culture of antigen-specific CD4$^+$ T cells with WT or TNF-$\alpha^{-/-}$ DCs

It has been reported that immature DCs induced IL-10-producing CD4$^+$ T cells [4–6]. To explore the role of DC-derived TNF-α to induce the antigen-specific IL-10-producing CD4$^+$ T cells, WT or TNF-$\alpha^{-/-}$ immature DCs were cocultured with OT-II CD4$^+$ T cells in the presence of $OVA_{323-339}$ peptide (0.1 μM) for 5 days, and cytokine levels in the culture supernatant were quantitated using ELISA (Fig. 2). Significant IL-10 and IL-4 production was observed in the T cell culture with WT DCs in the presence of $OVA_{323-339}$ peptide (Fig. 2A and B). Interestingly, TNF-$\alpha^{-/-}$ DCs with $OVA_{323-339}$ peptide induced considerably lower level of IL-10 production in the culture with CD4$^+$ T cells than WT DCs (Fig. 2A). In contrast, no significant differences in IL-4 production were detected between WT and TNF-$\alpha^{-/-}$ DCs (Fig. 2B). Negligible production of IL-10 or IL-4 was detected in the absence of $OVA_{323-339}$ peptide (Fig. 2A and B). Either type of DCs with $OVA_{323-339}$ peptide failed to induce substantial level of IFN-γ in the culture with CD4$^+$ T cells (data not shown).

We also analyzed TNF-α level in the culture of OT-II CD4$^+$ T cells with WT or TNF-$\alpha^{-/-}$ immature DCs (Fig. 2C). TNF-α was detected in the culture of CD4$^+$ T cells and WT DCs in the absence of $OVA_{323-339}$ peptide. The TNF-α level was markedly increased by addition of the antigen. No TNF-α production was detected in the culture with TNF-$\alpha^{-/-}$ DCs in the absence of the antigen. However, addition of the antigen resulted in significant TNF-α production in the culture of CD4$^+$ T cells and TNF-$\alpha^{-/-}$ DCs.

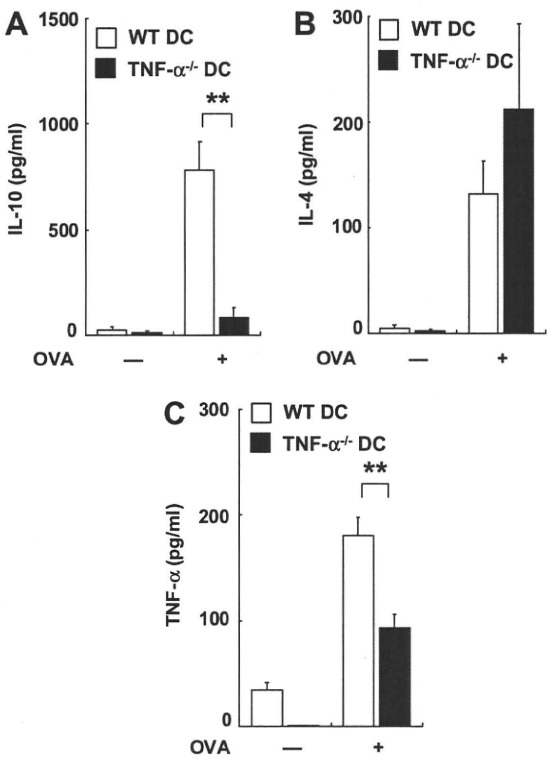

Fig. 2. Cytokine production in the culture of CD4$^+$ T cells and WT or TNF-$\alpha^{-/-}$ DCs. Purified OT-II CD4$^+$ T cells were cocultured with WT or TNF-$\alpha^{-/-}$ DCs in the absence or presence of $OVA_{323-339}$ peptide (OVA – or +). After 5 days, the culture supernatants were subjected to quantification of IL-10 (A), IL-4 (B) and TNF-α (C) by ELISA. Each column represents the means ± SE of four independent experiments. Statistical significance was calculated by Student's *t*-test (**: <0.01).

3.3. Development of IL-10-producing CD4$^+$ T cells with WT or TNF-$\alpha^{-/-}$ DCs

We next performed intracellular cytokine staining of OT-II CD4$^+$ T cells after the antigen presentation by WT or TNF-$\alpha^{-/-}$ immature DCs for 5 days (Fig. 3A – upper, B). Considerable proportions of the OT-II CD4$^+$ T cells were IL-10 and/or IL-4 positive after the antigen stimulation with WT DCs. In contrast, the proportions of IL-10$^+$IL-4$^-$ and IL-10$^+$IL-4$^+$ T cells in the culture with TNF-$\alpha^{-/-}$ DCs were significantly lower than those with WT DCs. Thus, the ability of DCs to induce IL-10-producing CD4$^+$ T cells was attenuated by the TNF-α deficiency in DCs. Nevertheless, the proportion of IL-4 single positive T cells was slightly increased by the TNF-α deficiency in DCs.

We also examined whether addition of exogenous TNF-α during the antigen presentation by TNF-$\alpha^{-/-}$ DCs recovered their impaired ability to develop IL-10-producing CD4$^+$ T cells (Fig. 3A – lower, B). Exogenous TNF-α significantly increased the proportions of IL-10$^+$IL-4$^-$ and IL-10$^+$IL-4$^+$ T cells in the culture with TNF-$\alpha^{-/-}$ DCs but not WT DCs. Thus, the TNF-α supply could recover the impaired ability of TNF-$\alpha^{-/-}$ DCs to induce IL-10-producing CD4$^+$ T cells. On the other hand, the TNF-α addition showed no significant effects on the proportion of IL-4 single positive cells in the culture with WT or TNF-$\alpha^{-/-}$ DCs.

We next examined the role of TNF-α in ability of mature DCs to induce IL-10 producing CD4$^+$ T cells. WT or TNF-$\alpha^{-/-}$ DCs were cultured with LPS for 24 h and used as mature DCs. Both types of DCs showed mature phenotype, high level expressions of CD86, CD40 and I-A^b. No significant difference in the expression level of these molecules was detected between WT and TNF-$\alpha^{-/-}$ DCs (data not shown). The WT or TNF-$\alpha^{-/-}$ mature DCs were cocultured with OT-II CD4$^+$ T cells for 5 days in the presence of $OVA_{323-339}$ peptide, and the proportion of IL-10 producing CD4$^+$ T cells was determined (Fig. 4A and B). The proportion of IL-10 producing CD4$^+$ T cells after the antigen presentation with WT mature DCs was lower than that with WT immature DCs compared Fig. 4 with Fig. 3. The TNF-α deficiency of mature DCs exerted no significant influence in the proportion of IL-10$^+$ and/or IL-4$^+$ T cells after the antigen presentation. Exogenous TNF-α addition showed no significant effects on the proportion of IL-10 producing CD4$^+$ T cells in the culture with WT or TNF-$\alpha^{-/-}$ DCs (data not shown).

4. Discussion

Increasing evidence indicates that DCs play pivotal roles not only in T cell immunity but also immune homeostasis [14,15]. Immunogenicity of DCs appears to be dependent on their maturational stage. It is generally considered that immature DCs are tolerogenic and involved in peripheral immune tolerance to self-antigens in the steady state, while mature DCs are immunogenic and initiate immune responses against harmful foreign antigens in the state of infection. Actually, it has been reported that immature, but not mature, DCs induce T cell anergy and IL-10 producing regulatory T cells in vitro and in vivo [5–8]. The tolerogenic properties of immature DCs may be attributed to the absence or low level expression of co-stimulatory molecules. However, the precise mechanism underlying induction of the IL-10 producing CD4$^+$ T cells by immature DCs is poorly understood.

Our present study also demonstrated that immature DCs induced development of antigen-specific IL-10-producing CD4$^+$ T cells. We then examined the role of DC-derived TNF-α in the induction of IL-10-producing CD4$^+$ T cells. The ability of immature DC to induce IL-10-producing CD4$^+$ T cells was significantly attenuated by their deficiency of TNF-α. It should be noted that the addition of exogenous TNF-α recovered the impaired ability of TNF-$\alpha^{-/-}$

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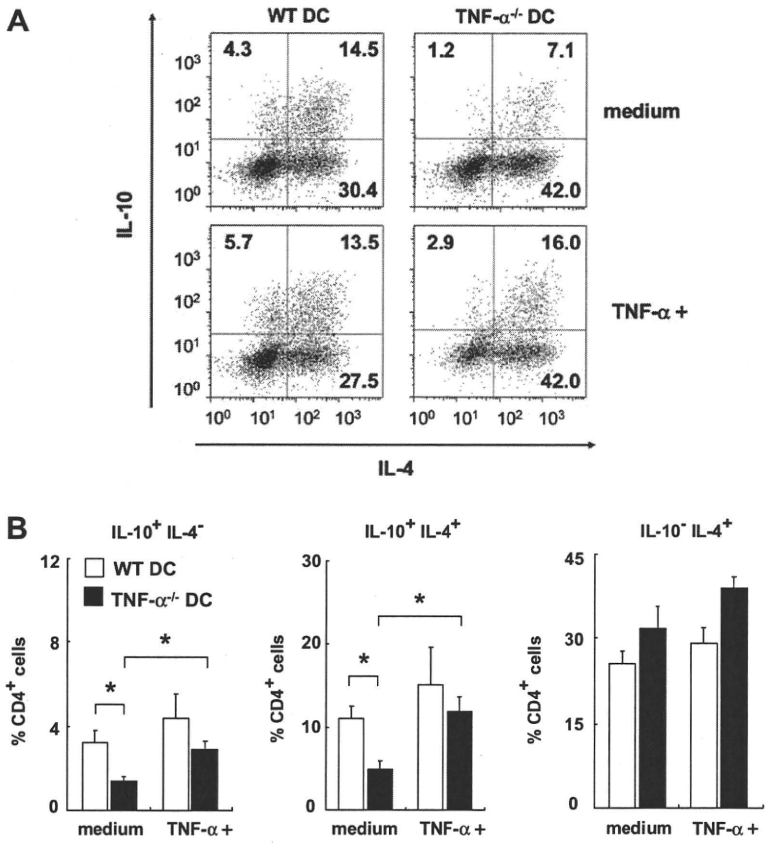

Fig. 3. The effect of TNF-α deficiency on DC ability to induce IL-10-producing CD4$^+$ T cells. Purified OT-II CD4$^+$ T cells and WT or TNF-α$^{-/-}$ DCs were cocultured with $OVA_{323-339}$ peptide for 5 days in the absence (medium) or presence (TNF-α$^+$) of exogenous TNF-α. The cells were restimulated with PMA and ionomycin for 5 h and the proportions of IL-4 and IL-10 positive cells in the CD4$^+$ T cells were determined by flow cytometry. (A) Dot plots of IL-4 and IL-10 positive cells in CD4$^+$ T cells. Data are representative of four independent experiments. (B) The proportion of IL-4 and IL-10 positive cells in CD4$^+$ T cells. Each column represents the means ± SE of four independent experiments. Statistical significance was calculated by Student's *t*-test (*: <0.05).

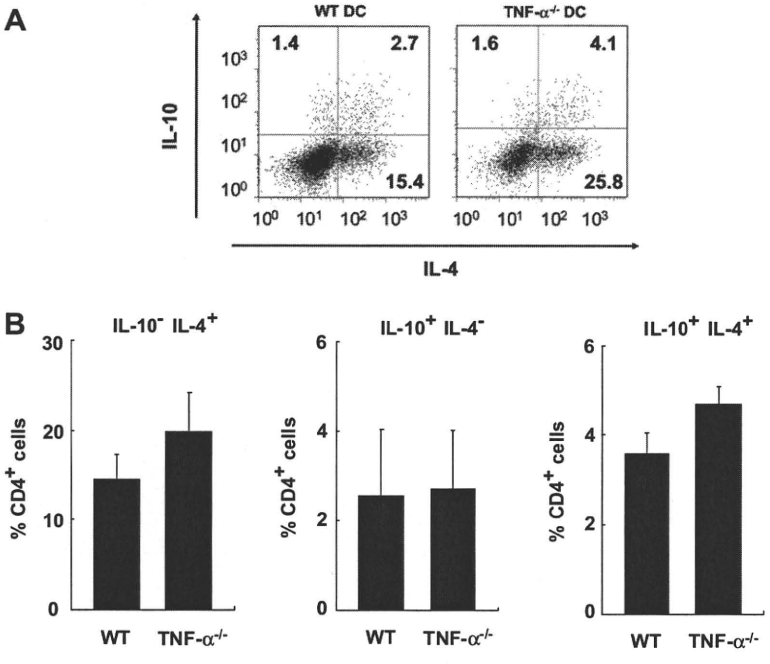

Fig. 4. The ability of mature DCs to induce IL-10-producing CD4$^+$ T cells. WT or TNF-α$^{-/-}$ DCs were stimulated with LPS for 24 h and used as mature DCs. Purified OT-II CD4$^+$ T cells were cocultured with each type of mature DCs in the presence of $OVA_{323-339}$ peptide for 5 days. The cells were restimulated with PMA and ionomycin for 5 h and the proportions of IL-4 and IL-10 positive cells in the CD4$^+$ T cells were determined by flow cytometry. (A) Dot plots of IL-4 and IL-10 positive cells in CD4$^+$ T cells. Data are representative of four independent experiments. (B) The proportion of IL-4 and IL-10 positive cells in CD4$^+$ T cells. Each column represents the means ± SE of four independent experiments.

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