resulting in MHC-negative tumor progression. This study clearly shows that tumor growth is suppressed in an MHC-negative/Rae-1-positive population of tumor cells by treatment of tumor-implant mice with Spirulina, which can induce NK activation to damage tumor cells via NKG2D receptors.

In human cancer patients receiving BCG–CWS therapy, tumor cells have not completely disappeared from the primary region,[41] although patients' quality of life (QOL) scores are increased in response to the BCG–CWS therapy. Similar observations were reported in patients with bladder cancer who selected BCG immunotherapy.[42] Growing the low-MHC tumor cells may account for the incomplete remission of tumors in patients with cancer.

We offer the possible immune therapy in combination with BCG–CWS and Spirulina in this communication. Additive tumor cytotoxicity based on BCG–CWS/Ag and Spirulina suggests that they elicit different effectors, putative CTL and NK. Their combinational function merely targets the MyD88 pathway and is clearly distinct from that of LPS that induces toxic shock. Although which constituents of the Spirulina extract are responsible for NK enhancement, and why Spirulina and BCG–CWS differentially activate the TLR2/4-mediated MyD88 pathway in DCs should be further investigated, this is the fist report predicting that the combination of BCG–CWS/Ag and Spirulina is applicable to immunotherapy for patients with tumor mass of variable MHC levels. We favor the premise that Spirulina is a candidate for NK activator applicable to cancer patients by oral usage.

Acknowledgments

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Oligomerized TICAM-1 (TRIF) in the cytoplasm recruits nuclear BS69 to enhance NF-κB activation and type I IFN induction

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Although adenovirus 5 E1A-binding protein (BS69) is a nuclear protein acting as a transcriptional repressor, we found by an yeast two-hybrid and human cell immunoprecipitation another cytoplasmic function for this protein. BS69 bound Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule-1 (TICAM-1) (also named TRIF), an adaptor protein that couples with TLR3 around the endosome. BS69 translocated from the nucleus to the cytoplasm when cells were stimulated with dsRNA or transfected with TICAM-1. Confocal analysis of cells with over-expressed TICAM-1 or those stimulated with dsRNA revealed the characteristic "TICAM-1 speckle", which reflects signalosome formation necessary for the activation of NF-κB and IFN-regulatory factor (IRF)-3. BS69 was involved in the TICAM-1 complex, and the activation of NF-κB/IRF-3 followed by cytokine production was augmented in the presence of BS69 overexpression. Knockdown of endogenous BS69 resulted in a decrease of IFN-β induction, suggesting that BS69 is a positive regulator for the TLR3-TICAM-1 pathway. These results, together with a recent report showing the negative regulatory properties of BS69 in NF-κB activation by EBV-derived latent membrane protein 1, suggest that BS69 harbors dual modes of cytoplasmic NF-κB regulation, positively in the TICAM-1 pathway and negatively in the latent membrane protein 1 pathway.

Key words: BS69 · IFN-β · NF-κB · TICAM-1/TRIF · TLR3

Supporting Information available online

Introduction

Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule-1 (TICAM-1) acts as an adaptor for TLR3 and activates both the IFN-regulatory factor (IRF)-3 and the IFN-β promoter [1]. TLR3 is localized to the endosome in immature myeloid dendritic cells (mDC) and resting macrophages [2]. Recent imaging analyses revealed that TICAM-1 merges with endosomal TLR3 within 20 min in response to dsRNA stimuli, and after 60 min translocates to form speckles in the cytoplasm which represent the TICAM-1 signalosome [3]. NAP1 and RIP1 are recruited to the TICAM-1 complex, both of which are known to be important factors for activating downstream elements of the TICAM-1 pathway [3, 4]. The forced expression of TICAM-1 leads to the formation of multimers in the signalosome complex [4]. To elucidate what molecules constitute the TICAM-1 complex, we screened TICAM-1-binding proteins by

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an yeast two-hybrid assay. We identified adenovirus 5 E1A-binding protein (BS69) as a member of the TICAM-1 signalosome, in addition to the TRAF family proteins previously noted [1].

BS69, a multidomain cellular protein containing PHD, Bromo, PWWP and MYND domains [5], was originally identified as an adenovirus E1A-binding protein that inhibits the transactivation function of E1A [6, 7]. The C-terminal MYND domain of BS69 was shown to bind to the PxLxP motif existing on E1A, EBV-encoded EBNA2 and a Myc-related cellular protein MGA [8]. Although BS69 is unequivocally a nuclear protein, it has been shown that BS69 interacts with EBV-encoded latent membrane protein 1 (LMP1) in the cytoplasm through its MYND domain and acts as a scaffold protein in the LMP1-mediated JNK pathway by interacting with TRAF6 [9]. Furthermore, a recent report speculated that nuclear BS69 colocalizes with LMP1 in the cytoplasm proximal to the nucleus [10]. The stimulus which induces BS69 protein trafficking, however, remains undetermined.

In this study, we identified BS69 as a TICAM-1-binding protein and demonstrated that the TLR3 agonist polyI:C facilitates the BS69 nucleus-to-cytoplasm trafficking. This property of BS69 further highlights the function of this protein in the cytoplasm: BS69 is involved in the TICAM-1 complex and participates in TICAM-1-mediated IRF-3 and NF-κB activation. Here, we clarified a trigger of BS69 movement and the function of BS69 in the TICAM-1 pathway.

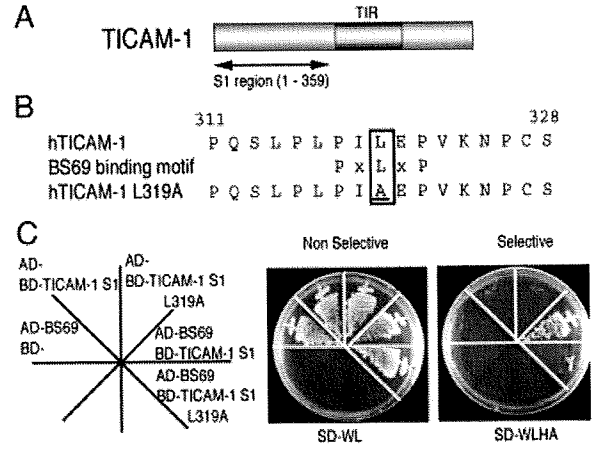

Figure 1. Yeast two-hybrid screening for the collection of TICAM-1-binding proteins. (A) The schema of human TICAM-1 protein. The S1 region (1–359 aa) of TICAM-1 was inserted into the pGBKT7 (bait) vector. From a total of 2.2 billion genes, six genes were obtained which encode for gene products capable of binding to the TICAM-1-S1 region. (B) Sequence alignment of human TICAM-1 and the BS69-binding motif (PxLxP). A point mutation (L319A) was introduced into the PxLxP motif. (C) Interaction between TICAM-1 S1 and BS69 in the yeast two-hybrid system. A strong association was observed between TICAM-1 S1 and BS69 (SD-WLHA plate), whereas the TICAM-1 S1 L319A-BS69 association was barely observable in the SD-WLHA plate.

Results

Yeast two-hybrid screening for collection of TICAM-1-binding proteins

TICAM-1 is a 712 aa protein (Fig. 1A). Since high background expression disturbed screening with the full-length protein, two segments consisting of the N-terminal S1 (1–359 aa) and C-terminal S2 (368-712 aa) regions were separately expressed in yeast (Fig. 1A). No growth was observed in yeast expressing solely S1 (Fig. 1C). The S1 and S2 fragments were ligated into pGBD-C1 and pGBKT7, respectively, to act as bait plasmids. The yeast cells containing bait plasmids were cultured on SD medium lacking Trp, Leu and His, while those cells harboring prey plasmids containing a human lung cDNA library were cultured on SD medium without Trp, Leu, His and Ade. Positive colonies were harvested and retested in the same growth medium (Fig. 1C). Six genes were finally obtained which encoded for gene products responsible for the S1 binding (data not shown). BS69 as well as TRAF-1, TRAF-2 and TRAF-6 were identified as TICAM-1-binding molecules. A reported BS69-binding motif, PxLxP, was identified in the 317–321 aa portion of TICAM-1 (Fig. 1B).

BS69 as a TICAM-1 N-terminal-binding protein

The direct binding of BS69 to the N-terminal of TICAM-1 was confirmed by retesting in yeast. We found the PxLxP motif at 317–321 aa in the TICAM-1 S1 fragment was crucial for BS69 binding, since a TICAM-1 S1 mutant (mt) containing a single point mutation (L319A) resulting in PxAxP, failed to bind BS69 (Fig. 1C). Next, plasmids with the BS69 cDNA and TICAM-1 cDNA were transfected into HEK293 cells and immunoprecipitation was performed. As observed in the yeast cells, WT TICAM-1 coprecipitated with BS69 (Fig. 2A). When the PxLxP motif in the N-terminal region of TICAM-1 was mutated to PxAxP, no BS69 binding was observed (data not shown). Hence, the mt lost the ability to bind BS69 in human cells as well as yeast, indicating that BS69 directly binds the PxLxP motif in the TICAM-1 molecule.

The interaction between TICAM-1 and BS69 was further examined in human cells by molecular imaging. When WT TICAM-1 was co-expressed with BS69 in HeLa cells, the majority of cells showed typical speckle-like TICAM-1 expression (Fig. 2B). This is consistent with a previous report [4], although ~30% of the cells displayed a diffuse expression profile of TICAM-1 (Fig. 2C). BS69 was exclusively stained in the nucleus in cells with diffuse TICAM-1 expression. Surprisingly, in cells with speckled TICAM-1, the cytoplasmic TICAM-1 merged with BS69 by FLAG tag staining. The results indicate that BS69 translocates from the nucleus to the cytoplasm by TICAM-1 over-expression and binds speckled TICAM-1 in the cytoplasm.

The TICAM-1 RHIM mt is efficiently expressed in cells without the induction of apoptosis [3], whereas TICAM-1 N+TIR P434H lacks the two sites essential for self-oligomerization [4]. We found that BS69 recruitment by TICAM-1 occurs in parallel with TICAM-1 oligomerization (speckle formation), since the RHIM mt

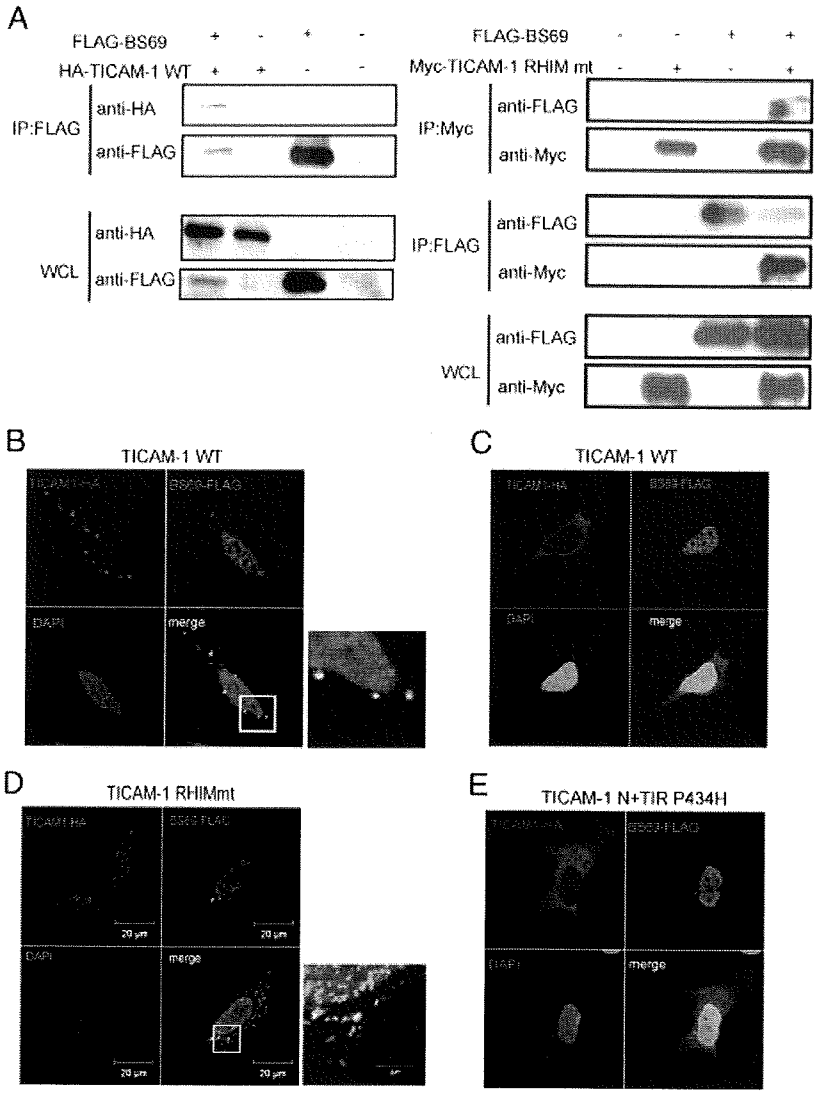

Figure 2. BS69 co-localizes with activated TICAM-1. (A) *Left panel*, HEK 293T cells were transfected with pEF-BOS HA-TICAM-1 WT and pEF-BOS FLAG-BS69. After 24 h, the cells were lysed, immunoprecipitated with anti-FLAG Ab and immunoblotted with anti-HA or anti-FLAG Ab. An aliquot of each whole cell lysate (WCL) was immunoblotted with either anti-HA or anti-FLAG Ab. A typical speckle pattern of TICAM-1 was observed. *Right panel*, HEK 293T cells were transfected with pEF-BOS Myc-TICAM-1 RHIM mt and pEF-BOS FLAG BS69. After 24 h, the cells were lysed, immunoprecipitated with anti-FLAG or anti-Myc Ab and immunoblotted with anti-Myc or anti-FLAG Ab. An aliquot of each whole cell lysate (WCL) was immunoblotted with either anti-Myc or anti-FLAG Ab. (B and C) HeLa cells were transfected with 1 ng of pEF-BOS HA-human TICAM-1 WT and 400 ng of pEF-BOS FLAG-human BS69. After 24 h, the cells were fixed and stained with anti-HA and anti-FLAG Ab, and visualized with either Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary Ab. The same slide was also treated with DAPI for the staining of nuclei. (B) The transfected HeLa cell with activated TICAM-1, whereas (C) shows a cell with inactive TICAM-1. (D) TICAM-1 RHIM mt was transfected into HeLa cells instead of TICAM-1 WT. (E) TICAM-1 N+TIR P434H was transfected into HeLa instead of TICAM-1 WT. The transfection and staining conditions were identical to those in (B). An enlarged scale of the area within the white square in the merged image in (B) and (D) is shown to the right of the image.

recruited BS69 (Fig. 2D), whereas TICAM-1 N+TIR P434H failed to recruit BS69 in the cytoplasm (Fig. 2E).

Translocation of BS69 in response to TICAM-1 signaling

To observe the nucleus-to-cytoplasm shuttling of BS69, HEK293T cells were transfected with the FLAG-BS69 and HA-TICAM-1 plasmids, and 24 h later the cells were solubilized to separate the nuclei and cytoplasm. Each fraction was further solubilized and immunoprecipitated with anti-FLAG and anti-HA Ab (Fig. 3A). The cytoplasmic fraction did not contain any detectable lamin A, suggesting that nuclear contamination in the cytoplasmic fraction was negligible (Fig. 3A center panel). TICAM-1 overexpression clearly allowed some BS69 to move to the cytoplasm (Fig. 3A). The dynamics of BS69 translocation in response to TICAM-1 stimulation was then examined using polyI:C as a TLR3/TICAM-1 stimulator [1]. Cytoplasmic BS69 was detected 3 h after

polyI:C stimulation in both HeLa (Fig. 3B) and HEK293T cells (Fig. 3C). Imaging analysis using the polyI:C-stimulated cells indicated that cytoplasmic speckle formation of BS69 and TICAM-1 also appeared 3 h after polyI:C stimulation (Fig. 3D). PolyI:C stimulation barely altered the BS69 mRNA levels (data not shown). Hence, BS69 moves from the nucleus to the cytoplasm in association with the activation and oligomerization of TICAM-1.

BS69 is a positive regulator of the TICAM-1 pathway

We next examined if the TICAM-1 signal was enhanced by transfected BS69. NF-κB activation was up-regulated by the over-expression of BS69 (Fig. 4A). PolyI:C-dependent induction of IFN-β luciferase was also enhanced by the transfection and expression of BS69 in HEK293T and HeLa cells (Fig. 4B and C). IFN-β mRNA levels

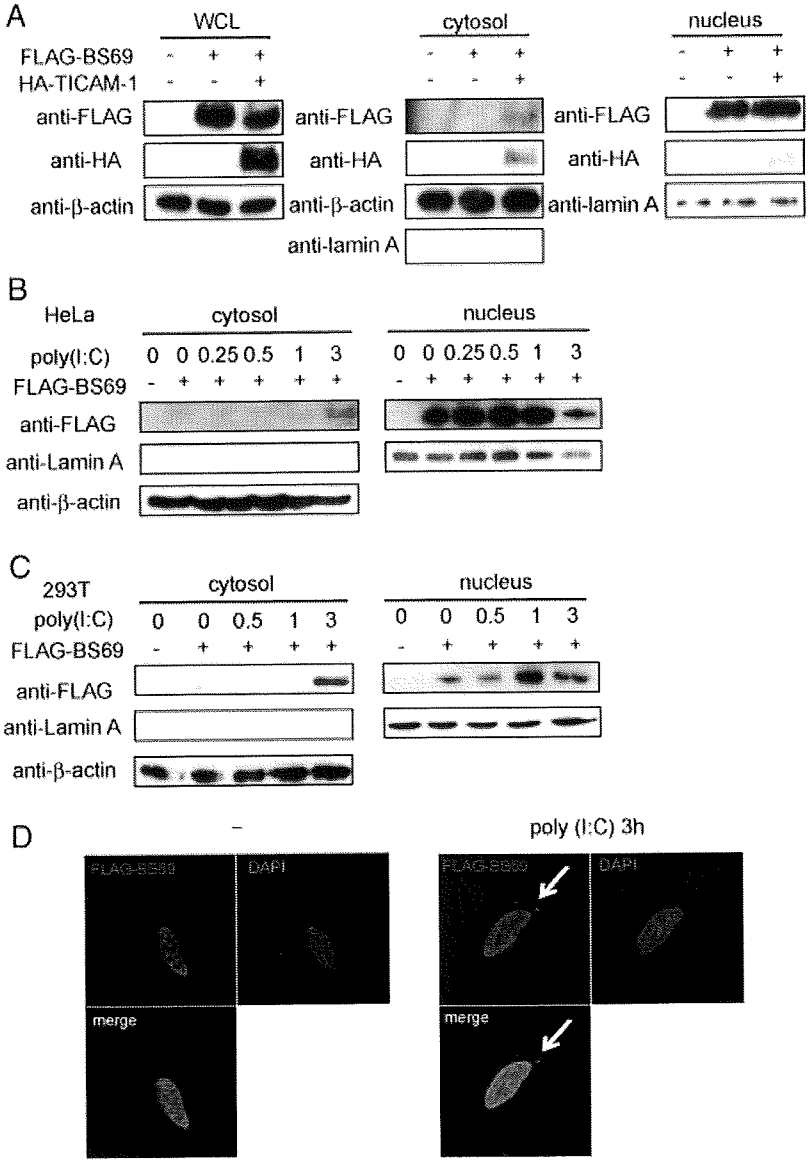

Figure 3. TICAM-1 over-expression induces cytoplasmic translocation of BS69. (A) HEK 293T cells were transfected with 1 ng of pEF-BOS HA-TICAM-1 WT and 100 ng of pEF-BOS FLAG BS69. After 24 h, the cells were lysed and cytoplasmic and nuclear extractions were prepared. Each extraction was resolved by SDS-PAGE and immunoblotted with either anti-HA, anti-FLAG anti-β-actin or anti-Lamin A (as a nuclear marker) Ab. (B) HeLa cells were transfected with 1 μg of pEF-BOS FLAG-BS69. After 24 h, the cells were stimulated with 10 μg/mL of polyI:C for either 0, 0.25, 0.5, 1 or 3 h. The cytoplasmic and nuclear extractions were then prepared, run on SDS-PAGE gels and immunoblotted with either anti-FLAG, anti-β-actin or anti-Lamin A (as a nuclear marker) Ab. (C) HEK293T cells were transfected with 100 ng of pEF-BOS FLAG-BS69 and 100 ng of pEF-BOS human TLR3. After 24 h, the cells were stimulated with 50 μg/mL of polyI:C for the indicated periods. The cytosolic and the nuclear extractions were analyzed as shown in (B). (D) HeLa cells were transfected with 400 ng of pEF-BOS FLAG-BS69. After 24 h, the cells were stimulated with 10 μg/mL of polyI:C for 3 h. Thereafter, the cells were fixed and stained with anti-FLAG Ab and visualized with Alexa Fluor 594-conjugated secondary Ab. The same slide was also treated with DAPI for the staining of nuclei. The white arrows indicate BS69 cytoplasmic speckles.

were quantitatively measured in cells expressing BS69 after polyI:C stimulation (Fig. 4D). The levels of mRNA significantly increased at 6 and 12 h after polyI:C stimulation in the BS69-transfected cells in comparison with cells containing the control vector. We next introduced an siRNA of BS69 into HeLa cells and examined polyI:C-mediated IFN-β induction. The IFN-β mRNA level induced by polyI:C dropped down by the presence of the siRNA (Supporting Information Fig. S1). The data suggest that BS69 acts as a positive regulator of the TICAM-1 pathway in both NF-κB activation and IFN-β induction through its trafficking from the nucleus to the cytoplasm.

Discussion

We demonstrated in this study that BS69 binds TICAM-1 and positively modulates the function of TICAM-1 in terms of NF-κB and IRF-3 activation. BS69 is essentially a nuclear protein that can be displaced from the nucleus to the cytoplasm to regulate TICAM-1 signaling. Either low doses of polyI:C stimulation or TICAM-1 expression induces BS69 translocation, whereas high TICAM-1 expression leads to the disappearance of the nuclear and cytosolic BS69, presumably due to apoptosis (data not shown). BS69 not only augments the TICAM-1 pathway *via* its binding to TCAM-1, but also participates in BS69 nucleus-to-cytoplasm displacement.

BS69 is a 74-kDa protein with three truncated isoforms that are formed through alternative splicing [5]. All four forms are unstable as they can be easily degraded by post-translational modification through the proteasome pathway [5]. Our preliminary data suggest that protein modification, particularly one other than ubiquitination, participates in the degradation of BS69 (data not shown). This is consistent with the finding that high doses of TICAM-1 induce the activation of TRAF E3 ligases [11] and protein modification [12], though the mechanisms have yet to be determined.

The previous reports have demonstrated that BS69 physically binds EBV-derived LMP1 and negatively regulates the canonical NF-κB activation by LMP1 [10, 13]. Although the regulatory mode of LMP1 is reciprocal to that of TICAM-1, the extranuclear displacement of BS69 commonly occurs in polyI:C- and LMP1-activating pathways. Thus, BS69 exerts a functional modulation of NF-κB in at least in two cytoplasmic pathways: positive regulation in the TICAM-1 pathway and negative regulation in the LMP1 pathway.

TICAM-1 recruits TRAF1, TRAF2 and TRAF6 to sites within its N-terminal region [11], and TRAF3 indirectly couples with the

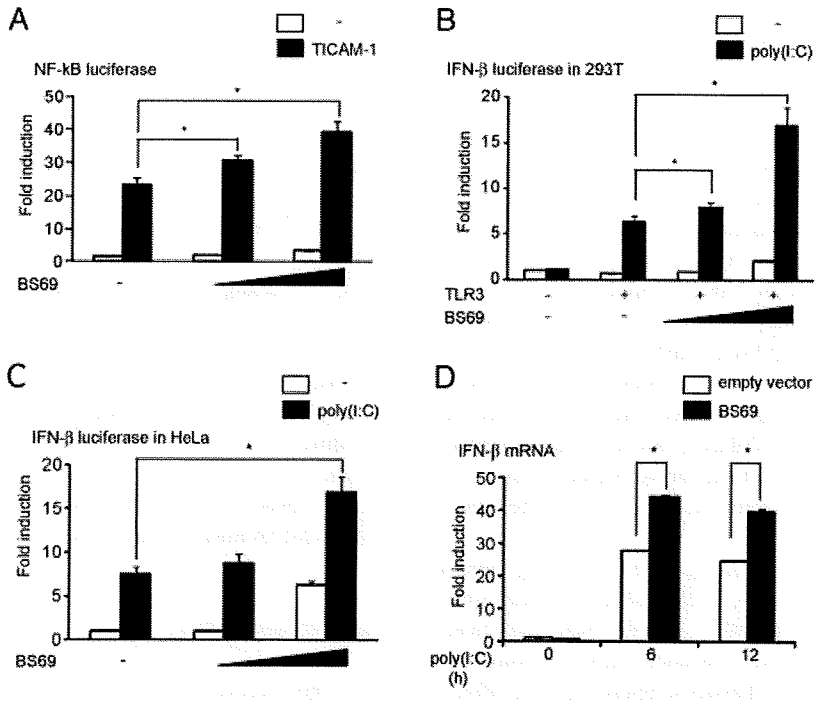

Figure 4. BS69 is a positive regulator of the TICAM-1 pathway. (A) HEK 293T cells in 24-well plates were transfected with pEF-BOS FLAG-BS69 (10, 100 ng) together with pEF-BOS HA-TICAM-1 WT (100 ng), the NF-κB reporter (100 ng) and phRL-TK (5 ng). Twenty-four hours after transfection, the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. (B) HEK 293T cells were transfected with pEF-BOS FLAG-BS69 (10, 100 ng) together with pEF-BOS TLR3 (10 ng), the IFN-β promoter reporter (100 ng) and phRL-TK (5 ng). After 24 h, the cells were stimulated with 10 μg/mL polyI:C for 6 h and the luciferase reporter activity was then measured. The average activities from three independent assays are shown as fold induction. (C) HeLa cells in 24-well plates were transfected with pEF-BOS FLAG-BS69 (10,100 ng) together with the IFN-β promoter reporter (100 ng) and phRL-TK (5 ng). Twenty-four hours after transfection, the cells were stimulated with 10 μg/mL polyI:C for 6 h, and then the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. (D) HeLa cells in 12-well plates were transfected with either pEF-BOS FLAG-BS69 (1 μg) or empty vector (1 μg). After 24 h, the cells were stimulated with 10 μg/mL polyI:C for the indicated time periods. The IFN-β mRNA levels were determined by real-time PCR. *$p<0.05$.

molecular complex of these proteins [14, 15]. NAP1 also bind indirectly to the N-terminus of TICAM-1 [3]. Thus, the visible TICAM-1 multimer observed by confocal analysis is likely a signal platform directing the activation of IRF-3-activating kinases and IκB degradation kinases [4]. From our data, we can infer that when an optimal stimulus, such as an RNA viral infection, is present in the target cells, two molecular events are independently triggered: BS69 translocation to the cytoplasm and TICAM-1 signalosome formation. These two events could be simultaneously reproduced in our system, resulting in the up-regulation of the TICAM-1 inflammatory pathway.

Upon infection of a cell by EBV, the EBV product LMP1 induces NF-κB and JNK activation. This LMP1-derived NF-κB activation is negatively regulated by BS69 [10] coping with internal TLR3 signaling. Radical activation of the TICAM-1 pathway, however, is not supported by BS69-mediated NF-κB up-regulation as BS69 is degraded *via* post-translational modification. This is in accordance with the fact that full length TICAM-1 occasionally induces apoptotic cell death, which reflects a natural feature of the antiviral response.

The previous studies have demonstrated that BS69 acts as a transcriptional repressor in association with a variety of transcription factors such as c-Myb, B-Myb, Ets2 and MGA [7, 16, 17]. BS69 has also been shown to repress transcription by recruiting N-CoR [6]. A recent study suggested that BS69 has another role in gene repression since it co-precipitates with a set of chromatin remodeling factors and interacts with the transcription factor ZHX1 [18]. Furthermore, BS69 has been shown to associate with mitotic chromosomes and to interact with Brg1 (the catalytic subunit of the mammalian SWI/SNF complex), indicating an additional role of BS69 in chromatin remodeling [19]. In either case, it is clear that BS69 functions in the nucleus. As a sensitive Ab against BS69 is not available, it is extremely difficult to detect endogenous BS69 protein in the cytoplasm. However, our studies, together with a report on BRAM1, a truncated form of BS69 which displaces TRADD from LMP1 to inhibit LMP1-mediated NF-κB activation [10], indicate that BS69 plays a role in the cytoplasm to modulate inflammation secondary to viral infection. In keeping with its NF-κB modulating function and chromatin-associated properties, BS69 is a bifunctional protein acting in the nucleus and cytoplasm to maintain the homeostasis of the cellular environment.

In mDC, TICAM-1 has a unique role in driving cellular immunity as $CD8^+$ T, $CD4^+$ Treg, Th1, Th17 and NK cells are all activated in response to TICAM-1-mediated mDC maturation [1, 20]. We found the TICAM-1 pathway in mDC is activated *via* endosomal TLR3 through the phagocytic uptake of viral-infected cell debris [21]. Our data suggest the possibility that BS69 is an agent used to regulate the induction of TICAM-1-mediated cellular immunity in addition to the NF-κB- and IFN-activating pathways. More detailed analysis of endogenous TLR-associated proteins and BS69/BRAM1, including *in vivo* functional analysis, will be needed in order to highlight the precise cytoplasmic function(s) of BS69.

Materials and methods

Cell culture and reagents

HEK293 T cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. HeLa cells were cultured in Eagle's MEM with 10% heat-inactivated FBS and L-glutamine. The following Ab were obtained commercially: anti-FLAG, anti-HA and anti-β-actin (Sigma-Aldrich); anti-Myc (Santa Cruz); anti-Lamin A (Cell Signaling Technology). Alexa Fluor 488- and Alexa Fluor 568-conjugtaed secondary Ab were from Invitorgen Life Technologies. polyI:C was from Amersham Biosciences.

Plasmids

Complementary DNA from human TLR3, TICAM-1WT, TICAM-1 N+TIR P434H and RHIM mt were cloned in our laboratory by RT-PCR and ligated into the cloning site of the expression vector, pEF-BOS and pcDNA4 Myc-HisA [4]. BS69 cDNA was cloned as described previously [11]. Mutations were introduced by site-directed mutagenesis using PCR. [3]. All constructs were confirmed by sequencing.

Confocal microscopy

HeLa cells (2.5×10^4 cells/well) were plated on a micro cover glass (Matsunami Glass) in 12-well plate. The following day, cells were transfected with the indicated plasmids using FuGENE HD (Roche). The total amounts of DNA were kept constant by adding empty vector. After 24 h, cells were fixed in acetone and blocked in PBS containing 1% BSA and then labeled with the indicated primary Ab for 1 h at room temperature. Alexa Fluor 488- or Alexa Fluor 594-conjugtaed secondary Ab were used for the visualizing proteins detected by the primary Ab. For nucleus staining, cells were treated with DAPI in PBS. After all staining procedures were finished, micro cover glasses were mounted onto a slide glass using PBS containing 2.3% DABCO and 50% glycerol. Cells were visualized at ×63 magnification under an LSM510 META microscope (Zeiss).

Reporter gene assay

Cells were seeded onto 24-well plates and transfected with various amounts of expression vectors, the reporter gene and the phRL-TK control plasmid using FuGene HD (Roche) according to the manufacturer's instructions. After 24 h, the cells were harvested in 50 μL lysis buffer. The luciferase activity was measured using Dual-Luciferase Reporter assay systems (Promega) and was shown as the means ± SD of three experiments.

Western blotting and immunoprecipitation assay

For whole cell lysis, cells were solubilized in the SDS sampling buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol, 32% Urea) and then sonicated for 5 min. For cytosol extraction, cells were solubilized in the lysis buffer A (10 mM HEPES-KOH, pH 7.9, 150 mM NaCl, 15 mM $MgCl_2$, 10 mM KCl, 40 mg/mL digitonin, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF and 1 mM Na_3VO_4) on ice for 30 min and then centrifuged at 10 000 × *g* for 1 min at 4°C. The supernatant was collected as a cytosol extraction. After centrifugation, the nuclei-containing pellet was resuspended in the buffer C (50 mM HEPES-KOH (pH 7.9), 420 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF and 1 mM Na_3VO_4) at 4°C for 30 min. The suspension was pelleted by centrifugation and the supernatants were collected as a nuclear extraction. The supernatants were separated by SDS-PAGE, and the gel was transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with TBS, pH 8.0, containing 5% skim milk, immunoblotted with specific Ab and visualized with the appropriate horseradish peroxidase-conjugated secondary Ab using the ELC plus Western Blotting Detection System (Amersham Pharmaica). For immunoprecipitation, cells were lysed in the TritonX-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% TritonX-100, 10% glycerol, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF and 1 mM Na_3VO_4) and then centrifuged at 12 000 × *g* for 10 min at 4°C. The supernatants were incubated with anti-FLAG or anti-Myc Ab and protein G-Sepharose (Amersham Pharmacia) for overnight at 4°C. The immunoprecipitates were collected by centrifugation, washed four times in the lysis buffer and then analyzed by SDS-PAGE.

RNA purification and real-time PCR

Total RNA was prepared using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. RT-PCR was carried out using the High Capacity cDNA Reverse Transcripition kit (Applied Biosystems) according to the manufacturer's instructions. The following oligonucleotides were used for human β-actin: 5′-CCT GGC ACC CAG CAC AAT-3′ and 5′-GCC GAT CCA CAC ACG GAG TAC T-3′; and for human IFN-β: 5′-TGG GAG GAT TCT GCA TTA CC-3′ and 5′-CAG CAT CTG CTG GTT GAA GA-3′; and for human BS69: 5′-GTC CAC GGT ATG CAC CCT AAA GAG and 5′-AAC ACC TCT CCA GGC AAA TGG. IFN-β mRNA were normalized to β-actin and fold inductions of transcripts were calculated using the δδCT method relative to unstimulated HeLa cells.

Yeast two-hybrid screening

The yeast two-hybrid assay was performed as described previously [12]. The yeast AH109 strain (Clontech, Palo Alto, CA, USA) was transformed using bait (pGBKT7) and prey (pGADT7) plasmids. The transformants were streaked onto plates and incubated for 3–5 days. and in the figures represent the bait and prey plasmid, respectively. The various BD-TICAM-1 and AD-BS69 were constructed by inserting each cDNA fragment into the pGBKT7 (bait) or pGADT7 (prey) plasmids (Clontech). SD-WLH is a yeast synthetic dextrose medium that lacks Trp, Leu and His aa. SD-WLHA lacks adenine in addition to Trp, Leu and His.

Gene silencing

Knockdown of BS69 was carried out using siRNA, BS69 siRNA-1: 5′-GGA UAU UGG CCA GGA GTT-3′, BS69 siRNA-2: 5′-CGG UAU GCA CCC UAA AGA GTT-3′ and control siRNA: 5′-GGG AAG AUC GGG UUA GAC UUC-3′. In total, 20 pmol of each siRNA was transfected into HeLa cells in 24-well plate with Lipofectamin 2000 according to the manufacture's protocol. Knockdown of BS69 was confirmed 48 h after siRNA transfection. Experiments were repeated twice for confirmation of the results. One of the two siRNA, BS69 siRNA-1 was effective in BS69 gene silencing. Typically, 6 h after polyI:C (10 μg/mL) stimulation, the level of the BS69 mRNA was determined by real-time PCR as described in RNA purification and real-time PCR section.

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Abbreviations: **BS69:** adenovirus 5 E1A-binding protein · **IRF:** IFN-regulatory factor · **LMP1:** latent membrane protein 1 · **mDC:** myeloid dendritic cells · **mt:** mutant · **TICAM-1:** Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule-1

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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 PeproTech (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

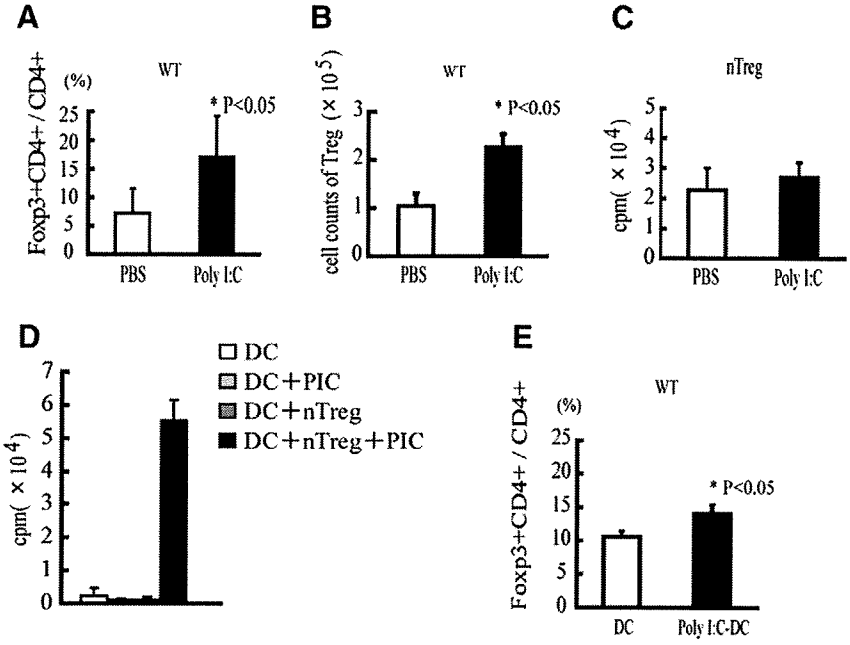

Fig. 1. PolyI:C induces the proliferation of Treg *in vivo* and *in vitro*. (A,B) C57BL/6J wild-type (WT) mice were intraperitoneally injected with polyI:C (1.25 mg/ml:200 μl) or PBS twice every 3 days throughout the experiments. Inguinal lymph nodes were excised, and the ratio of $CD4^+Foxp3^+$ /$CD4^+$ 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 $[^3H]$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.

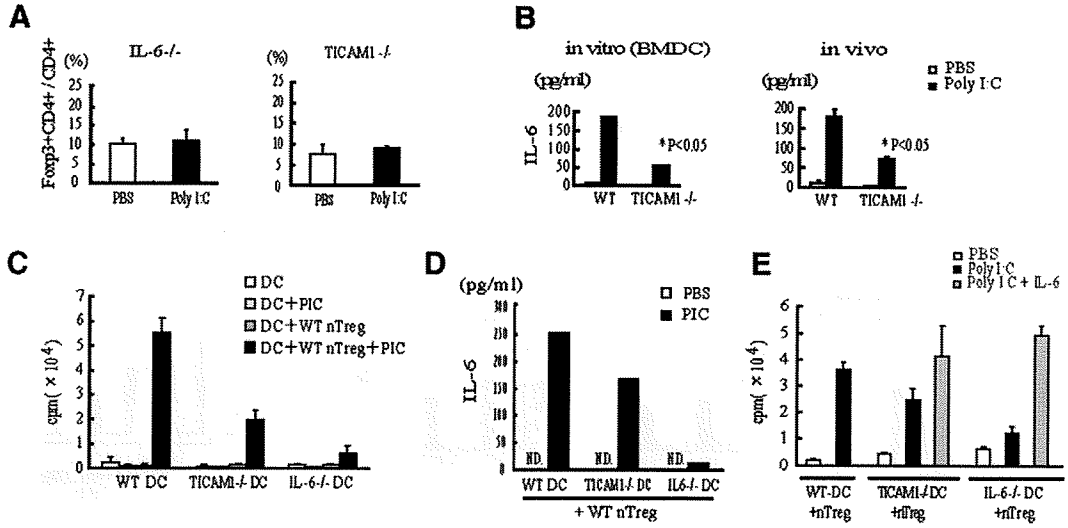

Fig. 2. The Treg proliferation by polyI:C plus BMDCs requires TICAM-1 signaling and IL-6. (A) TICAM-$1^{-/-}$ mice and IL-$6^{-/-}$ mice were intraperitoneally injected with polyI:C or PBS as in Fig. 1A and the ratio of $Foxp3^+CD4^+$ Treg/$CD4^+$ T cells was determined. There was no statistical difference 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 $[^3H]$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 $[^3H]$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

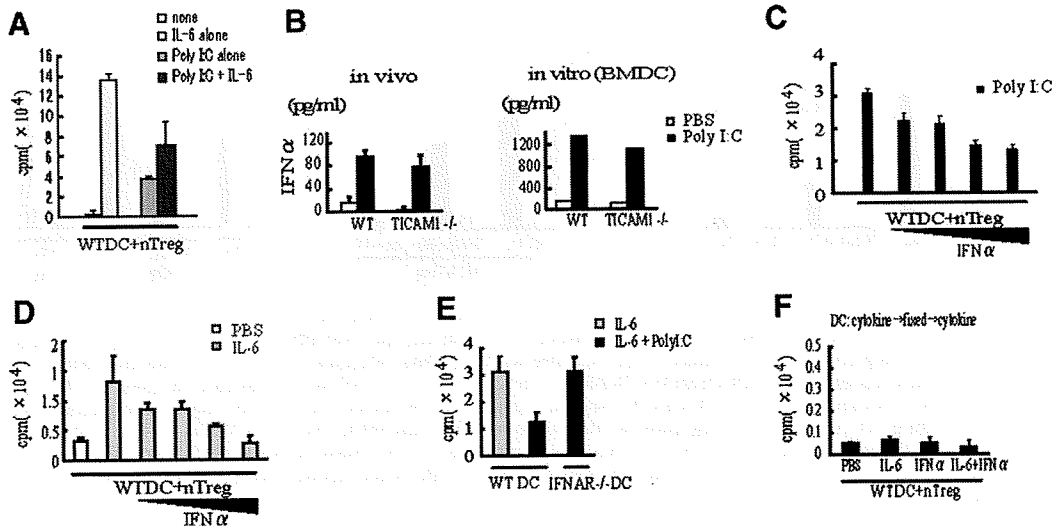

Fig. 3. Effect of IFN-α and IL-6 on Treg expansion. (A) As in Fig. 2C, but Treg from WT mice were cultured with WT BMDCs with or without 50 μg/ml polyI:C or 10 ng/ml IL-6. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (B) As in Fig. 2B, but the supernatants and sera 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.

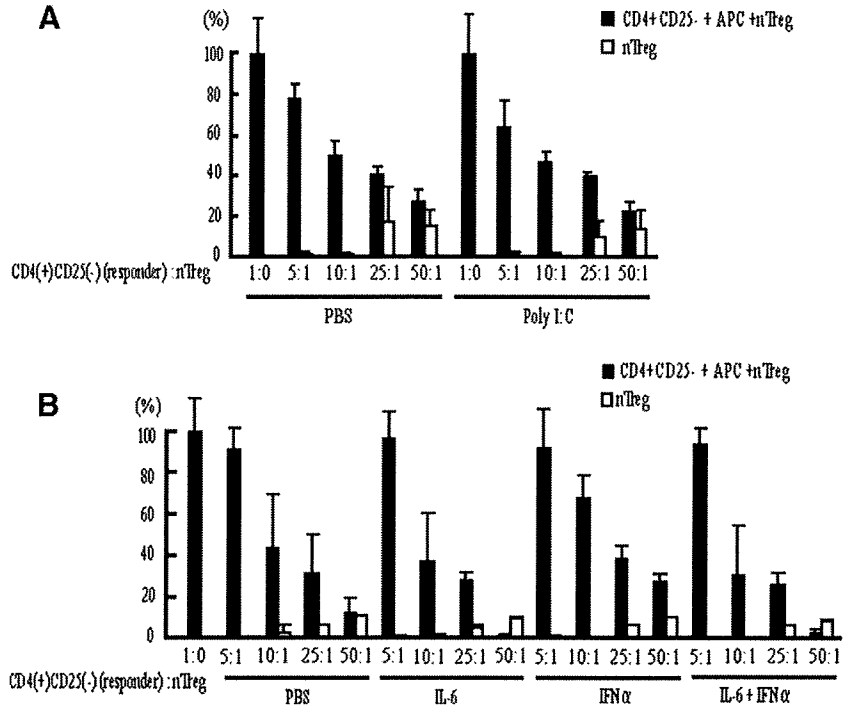

Fig. 4. Treg expanded by polyI:C plus BMDCs are suppressive *in vitro*. (A) Treg were isolated after 2-day culture with BMDCs in the absence (PBS) or presence of 50 μg/ml polyI:C (polyI:C). Then, these nTreg (suppressor) were cultured with freshly isolated $CD4^+CD25^-$ T cells (responder, 2.5×10^4), 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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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

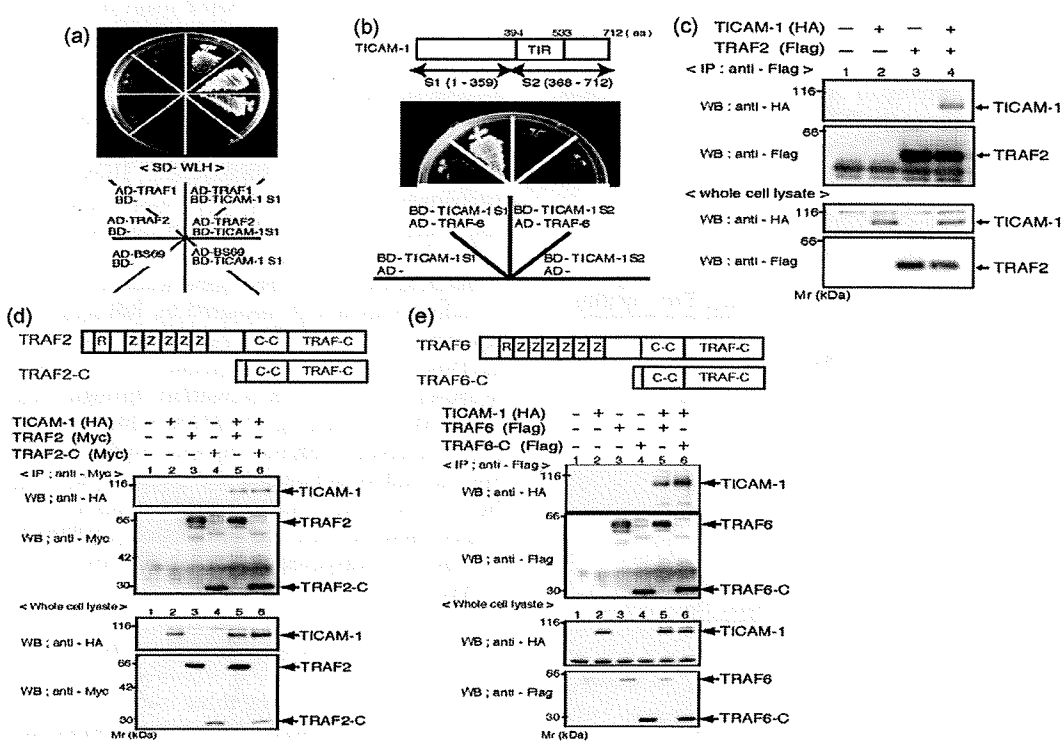

Fig. 1. Molecular interaction of TICAM-1 with TRAF2 and 6. (a) Direct interaction between the N-terminal region of TICAM-1 and either TRAF2 or TRAF6 as identified by yeast two-hybrid screening. Full-length TRAF2 and TRAF6 were cloned into pGADT7, transformed into yeast strain AH109, and then cultured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

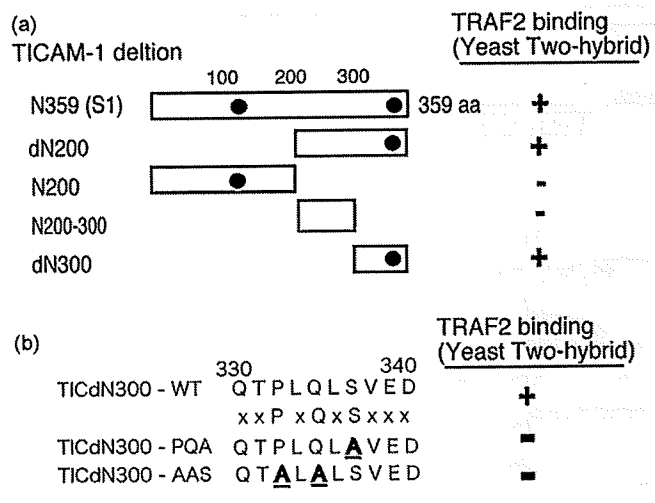

Fig. 2. Identification of the TRAF2-binding site in TICAM-1. (a) Scheme of TICAM-1 truncated mutants and location of the TRAF2 binding motif (black dot). TRAF2 in pGADT7 and each TICAM-1 construct in pGBKT7 were transformed into yeast. TRAF2 binding was assessed on SD-WLH plates as described in Fig.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