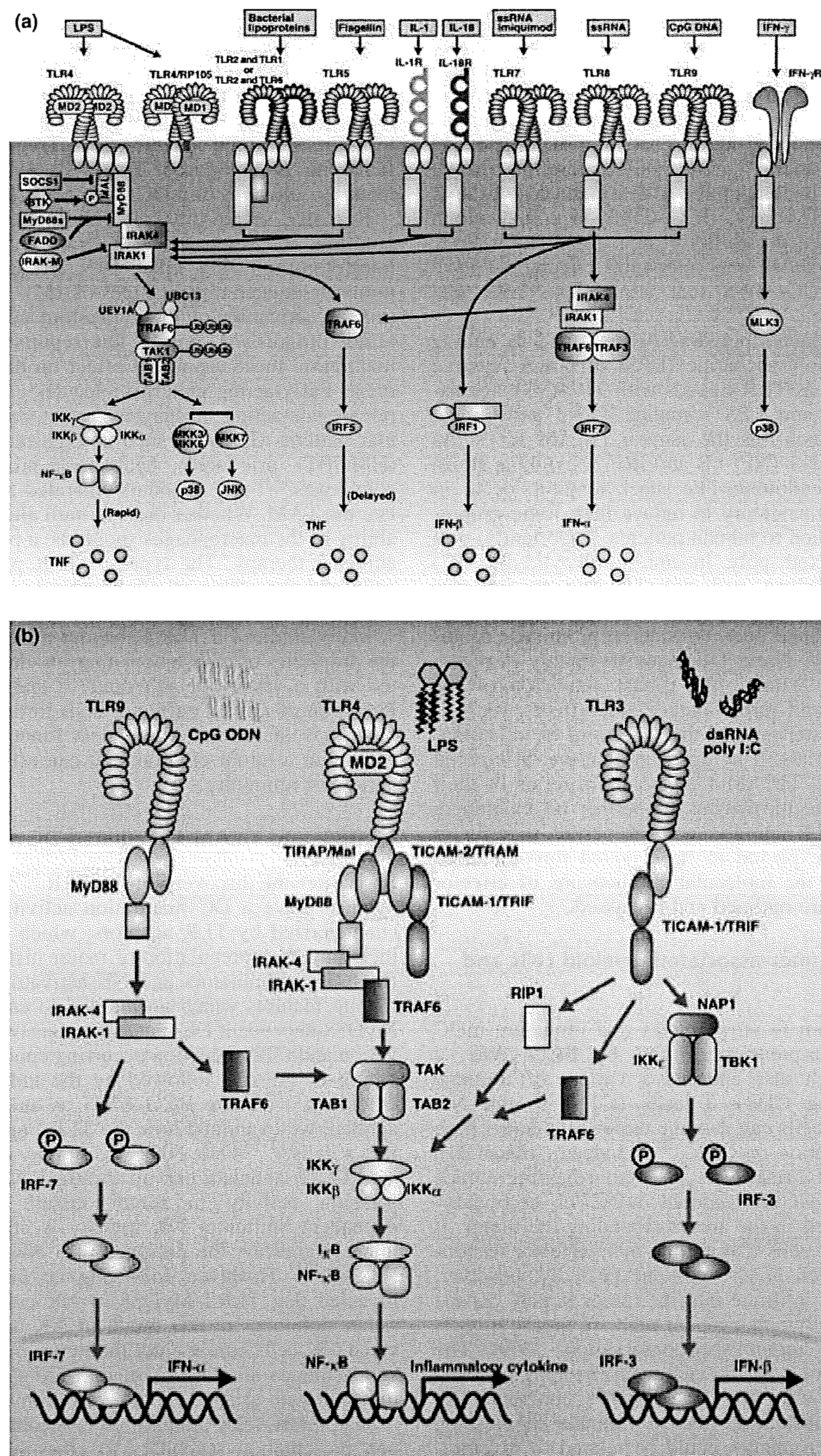

Fig. 1. MyD88 and Toll-interleukin I receptor-domain (TIR)-containing adoptor molecule (TICAM-1) pathways. MyD88 is an adaptor for all Toll-like receptors (TLR), except TLR3 (a). TLR2 and TLR4 recruit MyD88 via the bridging adaptor Toll-interleukin I receptor-domain (TIR)-containing adoptor protein (TIRAP)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

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

Human TLR		Ligands
TLR1		Pam3
TLR2		Pam2, Pam3, PGN
TLR3		dsRNA
TLR4		LPS, virus fusion units
TLR5		Flagellin
TLR6		Pam2
TLR7		ssRNA
TLR8		ssRNA
TLR9		CpG DNA
TLR10		–
MyD88 activators (Lipoproteins, PGN)		**Reference**
M161Ag (MALP-2)		(62)
TAN33		(63)
OM-174		(64)
BCG-CWS (Azuma lot)		(22)
SMP105		(65)
TICAM-1 activators (RNA, lipid A)		
DI RNA (stem loop)		(66)
Poly(A:U)		(67)
Poly(I:C_{12}U)		(68)
PolyI:C(LC)		(20)
MPLA		(21)
Anti-human TLR monoclonal antibodies		
TLR1	TLR1.136	(58)
TLR2	TLR2.45	(59)
TLR3	TLR3.7	(60)
TLR4	HTA125	(61)
TLR6	TLR6.127	(58)

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

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

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

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

DC subsets and TLR expression

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

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

Table 2. TLR expression profiles in human DC subsets

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

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

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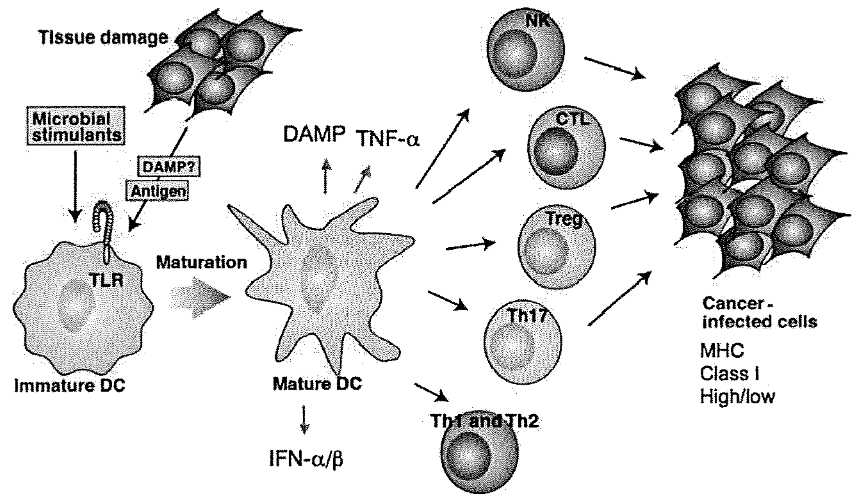

Fig. 2. Selective induction of immune effector lymphocytes by different agonistic stimuli. Each pattern molecule (PAMP) has its own uniqueness in myeloid dendritic cell (mDC) maturation. Differential maturation of mDC results in different effector driving as shown. CD8 T, various CD4 T, and B cells are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

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

DC subsets and effector induction

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

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

Mechanism of DC-mediated antitumor NK activation

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

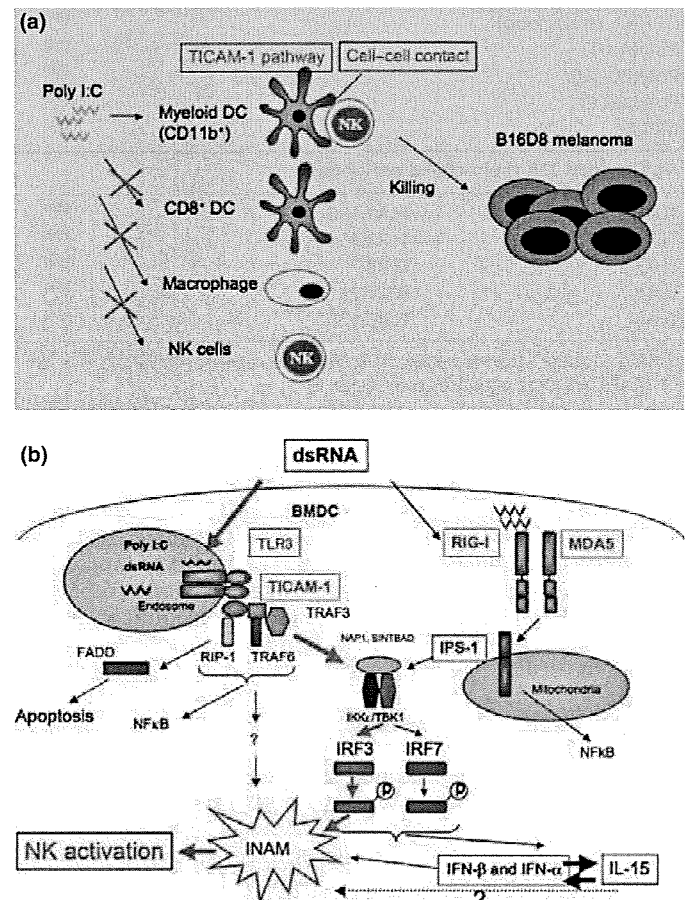

Fig. 3. A molecular mechanism of myeloid dendritic cell (mDC)-mediated natural killer (NK) activation. (a) CD11b+ bone marrow-derived dendritic cells (BMDC) act for natural killer (NK) activation by double-stranded (ds) RNA. NK cells express tumoricidal activity against major histocompatibility complex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

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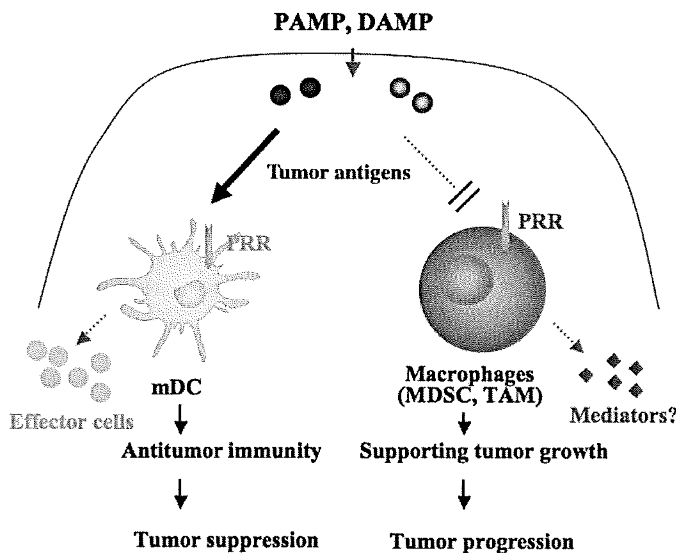

Fig. 4. Diverged functions of myeloid cells in tumor mass. A variety of myeloid subsets reside in tumor masses. Some of the subsets exhibit an immune suppressive feature that facilitates escape of tumor cells from immune effectors. Since pattern molecules (PAMP) act on both myeloid dendritic cells (mDC)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

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

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

Points to trigger antitumor immune potential

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

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

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

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

Acknowledgments

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References

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

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Mycobacterium bovis bacillus Calmette-Guérin (BCG)-peptidoglycan (PGN). *Infect Immun* 2003; **71**: 4238–49.

60 Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun* 2002; **293**: 1364–9.

61 Bosisio D, Polentarutti N, Sironi M *et al.* Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. *Blood* 2002; **99**: 3427–31.

62 Nishiguchi M, Matsumoto M, Takao T *et al.* Mycoplasma fermentans lipoprotein M161Ag-induced cell activation is mediated by Toll-like receptor 2: role of N-terminal hydrophobic portion in its multiple functions. *J Immunol* 2001; **166**: 2610–6.

63 Matsuo-Tanabe M, Kawamoto T, Tanida S, Matsumoto M, Seya T Toll-like receptor 2 agonists with unique properties synthesized with reference to a product of *Streptosporangium*. *Immunology* 2004: 235–41.

64 Brandenburg K, Lindner B, Schromm A *et al.* Physicochemical characteristics of triacyl lipid A partial structure OM-174 in relation to biological activity. *Eur J Biochem* 2000; **267**: 3370–7.

65 Murata M. Activation of Toll-like receptor 2 by a novel preparation of cell wall skeleton from Mycobacterium bovis BCG Tokyo (SMP-105) sufficiently enhances immune responses against tumors. *Cancer Sci* 2008; **99**: 1435–40.

66 Shingai M, Ebihara T, Begum NA *et al.* Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J Immunol* 2007; **179**: 6123–33.

67 Sugiyama T, Hoshino K, Saito M *et al.* Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7. *Int Immunol* 2008; **20**: 1–9.

68 Navabi H, Jasani B, Reece A *et al.* A clinical grade poly I:C-analogue (Ampligen) promotes optimal DC maturation and Th1-type T cell responses of healthy donors and cancer patients in vitro. *Vaccine* 2009; **27**: 107–15.

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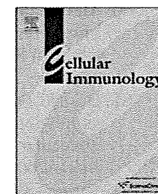

Dendritic cell-derived TNF-α is responsible for development of IL-10-producing CD4$^+$ T cells

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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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ DCs to induce these CD4$^+$ T cells was considerably depressed. Addition of exogenous TNF-α recovered the impaired ability of the TNF-$\alpha^{-/-}$ DCs to induce IL-10-producing T cells. However, no difference in this ability was observed between TNF-$\alpha^{-/-}$ 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.

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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-$\alpha^{-/-}$) 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 $I\text{-}A^b$, 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 $TNF\text{-}\alpha^{-/-}$ 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 $TNF\text{-}\alpha^{-/-}$ 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 $TNF\text{-}\alpha^{-/-}$ 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 $TNF\text{-}\alpha^{-/-}$ DCs

In this study, we used BMDCs (>95% $CD11c^+$) from WT and $TNF\text{-}\alpha^{-/-}$ 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 $I\text{-}A^b$, on these DCs (Fig. 1). Both types of DCs from WT and $TNF\text{-}\alpha^{-/-}$ mice showed immature phenotype, low expression of CD86 and CD40 and moderate expression of $I\text{-}A^b$. No significant difference was detected in CD86 and $I\text{-}A^b$ expressions between WT and $TNF\text{-}\alpha^{-/-}$ DCs. CD40 expression on $TNF\text{-}\alpha^{-/-}$ DCs was slightly but significantly lower than that of WT DCs.

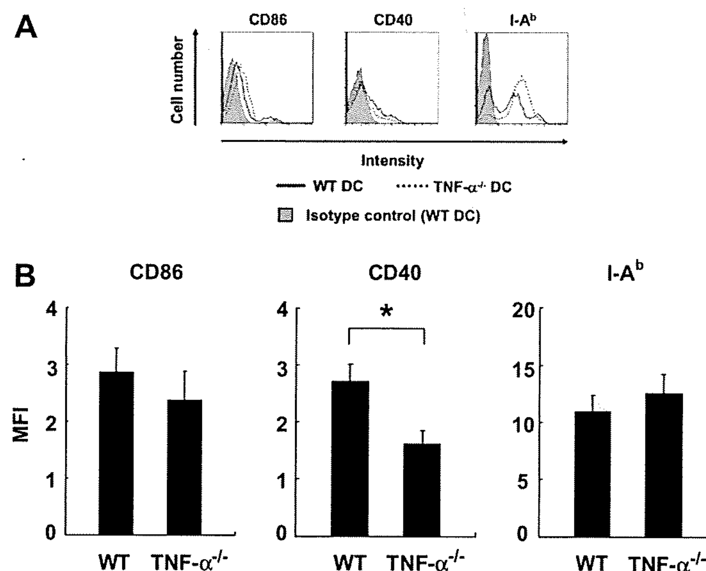

Fig. 1. Expressions of surface molecules on WT and $TNF\text{-}\alpha^{-/-}$ DCs. BMDCs were generated from WT or $TNF\text{-}\alpha^{-/-}$ mice. Expressions of CD86, CD40 and $I\text{-}A^b$ were analyzed by flow cytometry. (A) Representative histogram of each molecule on DCs. (B) Each column represents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

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-α−/− 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-α−/− 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-α−/− 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-α−/− 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-α−/− 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-α−/− 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-α−/− DCs.

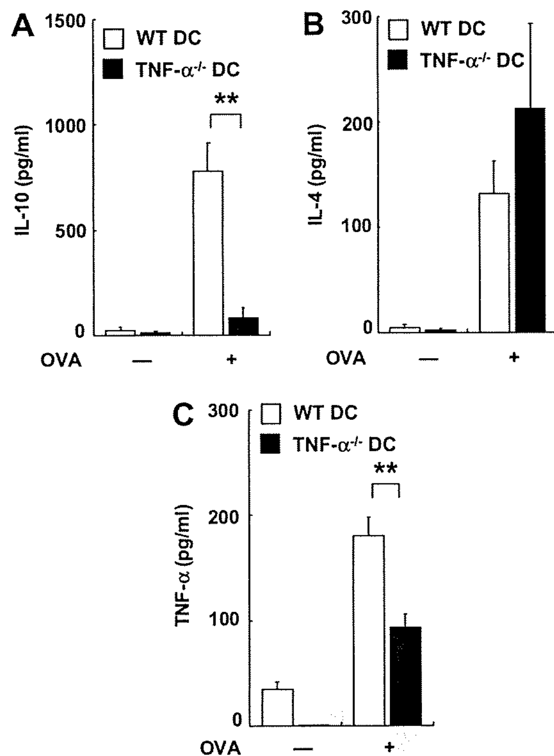

Fig. 2. Cytokine production in the culture of CD4+ T cells and WT or TNF-α−/− DCs. Purified OT-II CD4+ T cells were cocultured with WT or TNF-α−/− DCs in the absence or presence of $OVA_{323-339}$ peptide (OVA – or +). After 5 days, the culture supernatants were subjected to quantification of IL-10 (A), 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-α−/− DCs

We next performed intracellular cytokine staining of OT-II CD4+ T cells after the antigen presentation by WT or TNF-α−/− 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-α−/− 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-α−/− 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-α−/− DCs but not WT DCs. Thus, the TNF-α supply could recover the impaired ability of TNF-α−/− 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-α−/− 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-α−/− 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-α−/− DCs (data not shown). The WT or TNF-α−/− 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-α−/− 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-α−/−

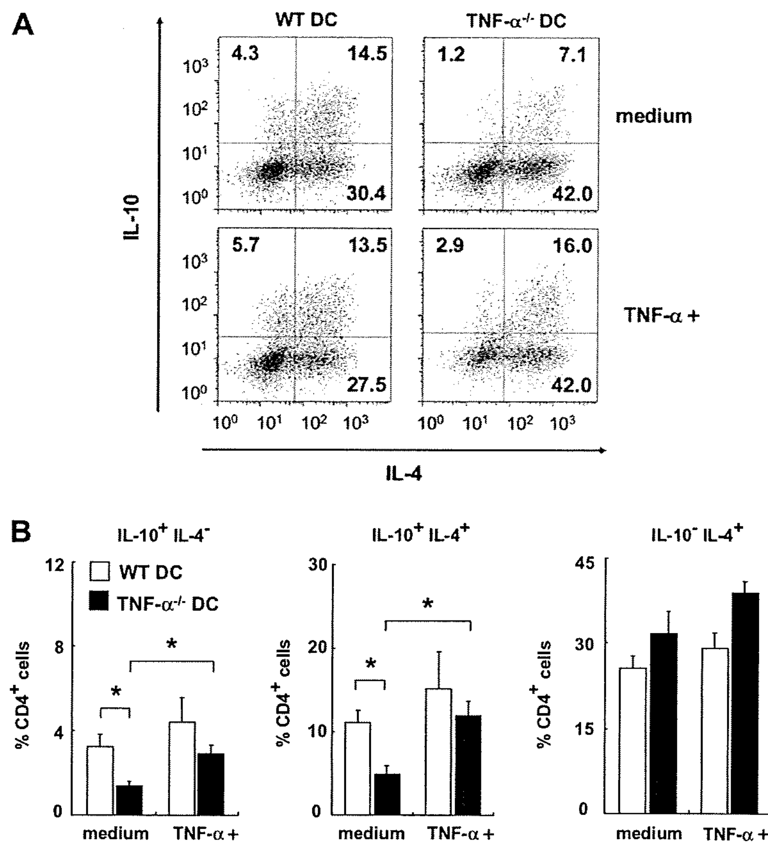

Fig. 3. The effect of TNF-α deficiency on DC ability to induce IL-10-producing CD4+ T cells. Purified OT-II CD4+ T cells and WT or TNF-$\alpha^{-/-}$ DCs were cocultured with $OVA_{323-339}$ peptide for 5 days in the absence (medium) or presence (TNF-α^{+}) of exogenous TNF-α. The cells were restimulated 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).

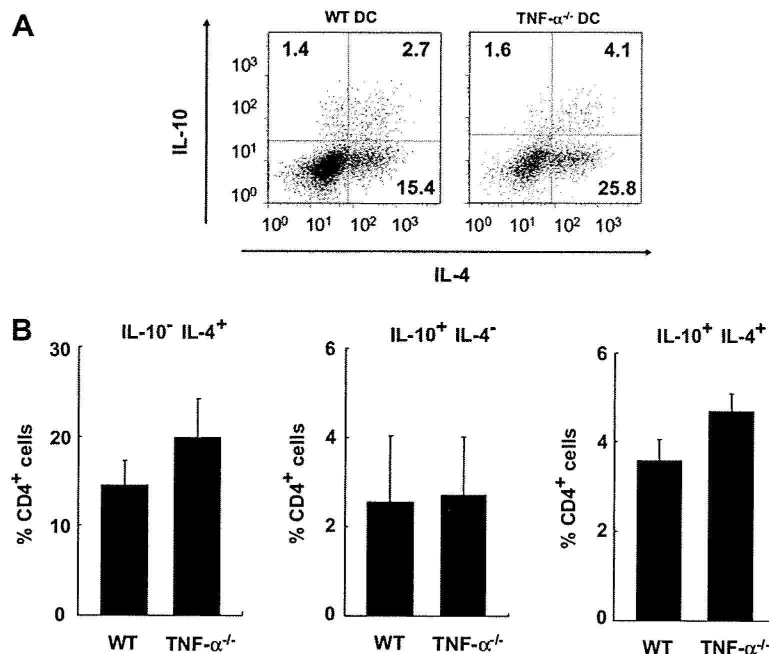

Fig. 4. The ability of mature DCs to induce IL-10-producing CD4+ T cells. WT or TNF-$\alpha^{-/-}$ DCs were stimulated with LPS for 24 h and used as mature DCs. Purified OT-II CD4+ T cells were cocultured with each type of mature DCs in the presence of $OVA_{323-339}$ peptide for 5 days. The cells were 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.

DCs to induce IL-10-producing CD4+ T cells. Thus, we conclude that TNF-α is a critical factor for the generation of IL-10-producing CD4+ T cells during the antigen presentation by immature DCs.

TNF-α promotes inflammatory responses in infected sites and contributes to elimination of foreign pathogens. However, several studies have indicated that TNF-α also exhibits immune regulatory nature. It has been reported that TNF-α-pretreated DCs prevent experimental autoimmune encephalomyelitis [9]. In tumor immunity, TNF-α induces apoptosis of tumor-infiltrating T cells and may blunt the immune surveillance against tumor cells within the tumor site [16]. Thus, we consider that TNF-α not only promotes inflammatory responses but also regulates immune responses. In this study, we found a novel role of DC-derived TNF-α in the immature DCs-mediated development of IL-10 producing CD4+ T cells, which appear to be involved in the immune regulations.

The ability of DCs to induce IL-10-producing CD4+ T cells was decreased after the maturation by LPS. TNF-α deficiency of the mature DCs did not affect their ability to induce IL-10-producing CD4+ T cells. Thus, the role of TNF-α in the DC ability to induce IL-10-producing CD4+ T cells appeared to be restricted in the immature state. It seems to us that TNF-α promotes the development of IL-10 producing CD4+ T cells for the immune homeostasis in the steady state. After the continuous infection in which DCs are maturated, TNF-α appears to contribute no longer to the development of the IL-10 producing CD4+ T cells.

At least three distinct types of regulatory T cells have been identified; T regulatory (Treg) cells, T helper type 3 (Th3) cells and T regulatory type 1 (Tr1) cells [17]. Tr1 cells mainly produce IL-10 and thereby suppress inflammatory responses [17,18]. It has been suggested that IL-10-producing T cells induced by immature DCs are Tr1-like cells [7]. Tr1 cells are Foxp3 negative different from Treg cells. We could not detect Foxp3 expression in the IL-10 producing CD4+ T cells induced by immature DCs in this study (data not shown). Thus, it seems that these IL-10 producing CD4+ T cells are Tr1-like cells as was shown in the previous study [7].

We described a novel immune regulation system involving TNF-α and immature DCs for the development of IL-10-producing CD4+ T cells. As the IL-10 producing T cells play a critical role in immune homeostasis, elucidation of the precise mechanism underlying this regulation system may lead to the development of new treatments for various immune disorders.

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References

[1] R.M. Steinman, The dendritic cell system and its role in immunogenicity, Annu. Rev. Immunol. 9 (1991) 271–296.
[2] D.N.J. Hart, Dendritic cells: unique leukocyte populations which control the primary immune response, Blood 90 (1997) 3245–3287.
[3] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, Nature 392 (1998) 245–251.
[4] S. Akira, K. Takeda, T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, Nat. Immunol. 2 (2001) 675–680.
[5] M.B. Lutz, N.A. Kukutsch, M. Menges, S. Röbner, G. Schuler, Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce allo antigen-specific CD4 T cell anergy in vitro, Eur. J. Immunol. 30 (2000) 1048–1052.
[6] M.B. Lutz, R.M. Suri, M. Niimi, A.L.J. Ogilvie, N.A. Kukutsch, S. Röbner, G. Schuler, J.M. Austyn, Immature dendritic cells generated with low doses of GMCSF in the absence of IL-4 are maturation-resistant and prolong allograft survival in vivo, Eur. J. Immunol. 30 (2000) 1813–1822.
[7] H. Jonuleit, E. Schmitt, G. Schuler, J. Knop, A.H. Enk, Induction of interleukin 10-producing, non-proliferating CD4+ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells, J. Exp. Med. 192 (2000) 1213–1222.
[8] M.V. Dhodapkar, R.M. Steinman, J. Krasovsky, C. Munz, N. Bhardwaj, Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells, J. Exp. Med. 193 (2001) 233–238.
[9] M. Menges, S. Rössner, C. Voigtländer, H. Schindler, N.A. Kukutsch, C. Bogdan, K. Erb, G. Schuler, M.B. Lutz, Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity, J. Exp. Med. 195 (2002) 15–21.
[10] K. Inaba, M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, R.M. Steinman, Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor, J. Exp. Med. 176 (1992) 1693–1702.
[11] S. Yamazaki, A.J. Bonito, R. Spisek, M. Dhodapkar, K. Inaba, R.M. Steinman, Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors, Blood 110 (2007) 4293–4302.
[12] S. Yamazaki, M. Patel, A. Harper, A. Bonito, H. Fukuyama, M. Pack, K.V. Tarbell, M. Talmor, J.V. Ravetch, K. Inaba, R.M. Steinman, Effective expansion of alloantigen-specific Foxp3+ CD25+ CD4+ regulatory T cells by dendritic cells during the mixed leukocyte reaction, Proc. Natl. Acad. Sci. USA 103 (2006) 2758–2763.
[13] N. Hirata, Y. Yanagawa, T. Ebihara, T. Seya, S. Uematsu, S. Akira, F. Hayashi, K. Iwabuchi, K. Onoé, Selective synergy in anti-inflammatory cytokine production upon cooperated signaling via TLR4 and TLR2 in murine conventional dendritic cells, Mol. Immunol. 45 (2008) 2734–2742.
[14] Y. Belkaid, G. Oldenhove, Tuning microenvironments: induction of regulatory T cells by dendritic cells, Immunity 29 (2008) 362–371.
[15] J.L. Coombes, F. Powrie, Dendritic cells in intestinal immune regulation, Nat. Rev. Immunol. 8 (2008) 435–446.
[16] J.B. Mumm, M. Oft, Cytokine-based transformation of immune surveillance into tumor-promoting inflammation, Oncogene 27 (2008) 5913–5919.
[17] E. Bettelli, M. Oukka, V.K. Kuchroo, T_H-17 cells in the circle of immunity and autoimmunity, Nat. Immunol. 8 (2007) 345–350.
[18] A. Awasthi, Y. Carrier, J.P. Peron, E. Bettelli, M. Kamanaka, R.A. Flavell, V.K. Kuchroo, M. Oukka, H.L. Weiner, A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells, Nat. Immunol. 8 (2007) 1380–1389.

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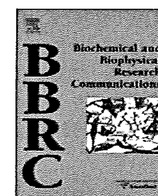

IL-6 and IFN-α from dsRNA-stimulated dendritic cells control expansion of regulatory T cells

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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.

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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

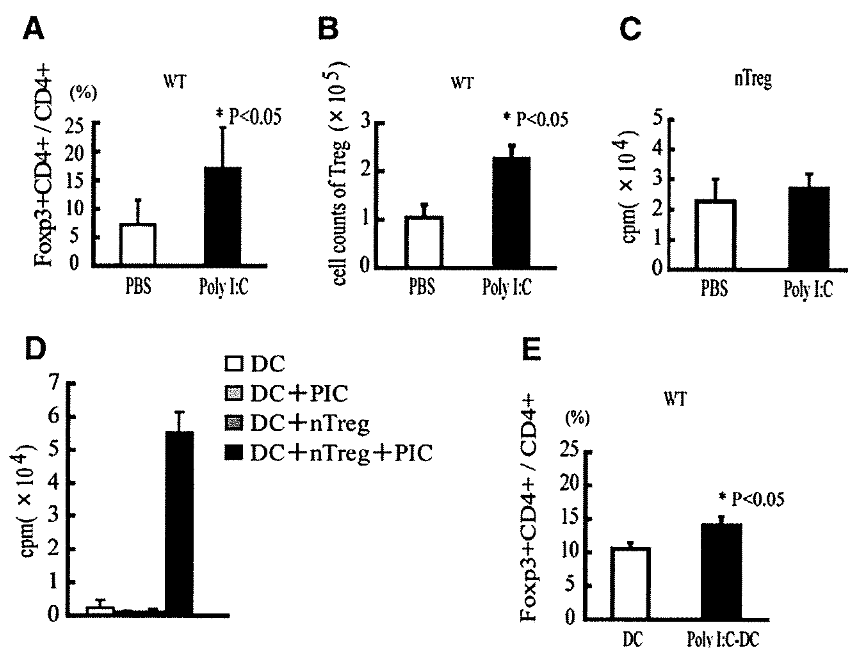

Fig. 1. PolyI:C induces the proliferation of Treg *in vivo* and *in vitro*. (A,B) C57BL/6J wild-type (WT) mice were intraperitoneally injected with polyI:C (1.25 mg/ml:200 μl) or PBS twice every 3 days throughout the experiments. Inguinal lymph nodes were excised, and the ratio of $CD4^+Foxp3^+$ /$CD4^+$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

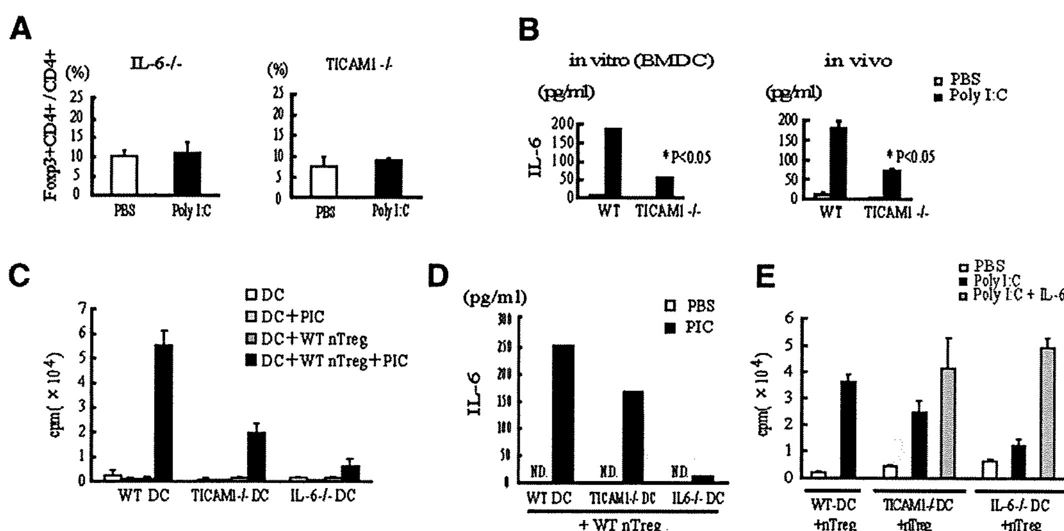

Fig. 2. The Treg proliferation by polyI:C plus BMDCs requires TICAM-1 signaling and IL-6. (A) TICAM-1$^{-/-}$ mice and IL-6$^{-/-}$ mice were intraperitoneally injected with polyI:C or PBS as in Fig. 1A and the ratio of $Foxp3^+CD4^+$ Treg/$CD4^+$ T cells was determined. There was no statistical difference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

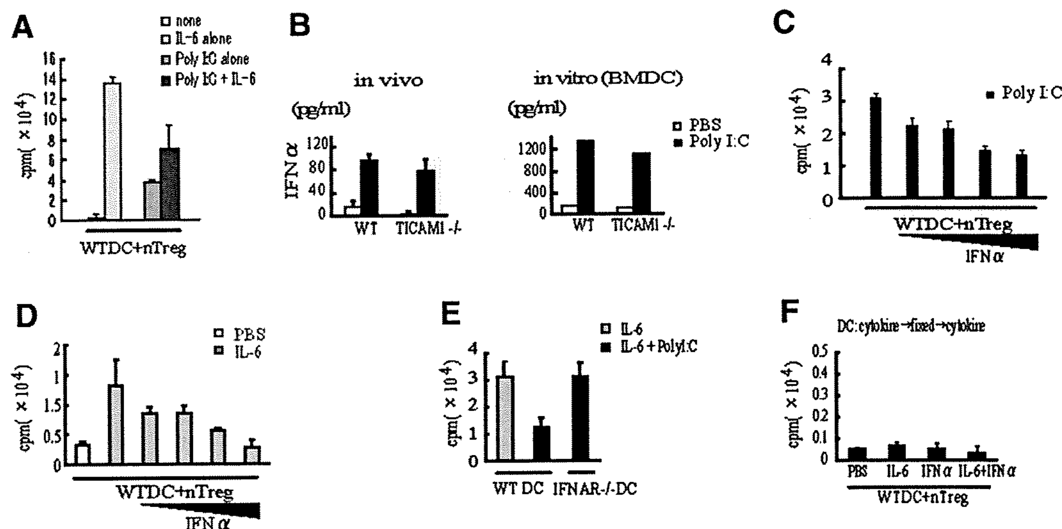

Fig. 3. Effect of IFN-α and IL-6 on Treg expansion. (A) As in Fig. 2C, but Treg from WT mice were cultured with WT BMDCs with or without 50 μg/ml polyI:C or 10 ng/ml IL-6. The proliferation was determined by [^{3}H]thymidine uptake after 2 day culture. (B) As in Fig. 2B, but the supernatants and sera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

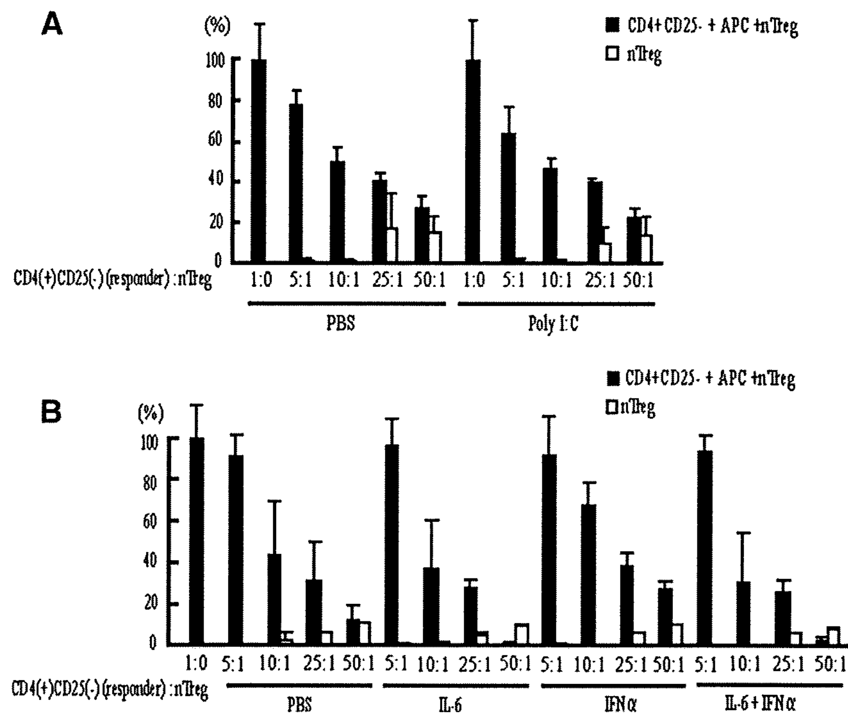

Fig. 4. Treg expanded by polyI:C plus BMDCs are suppressive *in vitro*. (A) Treg were isolated after 2-day culture with BMDCs in the absence (PBS) or presence of 50 μg/ml polyI:C (polyI:C). Then, these nTreg (suppressor) were cultured with freshly isolated $CD4^+CD25^-$ T cells (responder, 2.5×10^4), mitomycin C-treated splenocytes (1×10^5) and anti-CD3 Ab for 2 days. The proliferation was determined by $[^3H]$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.

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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.

References

[1] J.M. Kim, J.P. Rasmussen, A.Y. Rudensky, Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice, Nat. Immunol. 8 (2007) 191–197.
[2] S. Sakaguchi, T. Yamaguchi, T. Nomura, M. Ono, Regulatory T cells and immune tolerance, Cell 133 (2008) 775–787.
[3] W. Chen, W. Jin, N. Hardegen, K.J. Lei, L. Li, N. Marinos, G. McGrady, S.M. Wahl, Conversion of peripheral CD4+CD25− naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3, J. Exp. Med. 198 (2003) 1875–1886.

[4] S. Sakaguchi, Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses, Annu. Rev. Immunol. 22 (2004) 531–562.
[5] Y. Belkaid, B.T. Rouse, Natural regulatory T cells in infectious disease, Nat. Immunol. 6 (2005) 353–360.
[6] A. Dolganiuc, G. Szabo, T cells with regulatory activity in hepatitis C virus infection: what we know and what we don't, J. Leukoc. Biol. 84 (2008) 614–622.
[7] M.P. Longhi, C. Trumpfheller, J. Idoyaga, M. Caskey, I. Matos, C. Kluger, A.M. Salazar, M. Colonna, R.M. Steinman, Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant, J. Exp. Med. 206 (2009) 1589–1602.
[8] T. Kawai, S. Akira, Innate immune recognition of viral infection, Nat. Immunol. 7 (2006) 131–137.
[9] M. Yoneyama, K. Onomoto, T. Fujita, Cytoplasmic recognition of RNA, Adv. Drug Deliv. Rev. 60 (2008) 841–846.
[10] N. Kadowaki, S. Ho, S. Antonenko, R.W. Malefyt, R.A. Kastelein, F. Bazan, Y.J. Liu, Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens, J. Exp. Med. 194 (2001) 863–869.
[11] M. Matsumoto, T. Seya, TLR3: interferon induction by double-stranded RNA including poly(I:C), Adv. Drug Deliv. Rev. 60 (2008) 805–812.
[12] I. Caramalho, T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, J. Demengeot, Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide, J. Exp. Med. 197 (2003) 403–411.
[13] R.P. Sutmuller, M.E. Morgan, M.G. Netea, O. Grauer, G.J. Adema, Toll-like receptors on regulatory T cells: expanding immune regulation, Trends Immunol. 27 (2006) 387–393.
[14] G. Peng, Z. Guo, Y. Kiniwa, K.S. Voo, W. Peng, T. Fu, D.Y. Wang, Y. Li, H.Y. Wang, R.F. Wang, Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function, Science 309 (2005) 1380–1384.
[15] T. Akazawa, T. Ebihara, M. Okuno, Y. Okuda, M. Shingai, K. Tsujimura, T. Takahashi, M. Ikawa, M. Okabe, N. Inoue, et al., Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells, Proc. Natl. Acad. Sci. USA 104 (2007) 252–257.
[16] S. Yamazaki, T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, R.M. Steinman, Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells, J. Exp. Med. 198 (2003) 235–247.
[17] T. Kubo, R.D. Hatton, J. Oliver, X. Liu, C.O. Elson, C.T. Weaver, Regulatory T cell suppression and anergy are differentially regulated by proinflammatory cytokines produced by TLR-activated dendritic cells, J. Immunol. 173 (2004) 7249–7258.
[18] Z. Fehervari, S. Sakaguchi, Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells, Int. Immunol. 16 (2004) 1769–1780.
[19] M. Matsumoto, K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, T. Seya, Subcellular localization of Toll-like receptor 3 in human dendritic cells, J. Immunol. 171 (2003) 3154–3162.
[20] D.L. Brassard, M.J. Grace, R.W. Bordens, Interferon-alpha as an immunotherapeutic protein, J. Leukoc. Biol. 71 (2002) 565–581.
[21] S. Yamazaki, D. Dudziak, G.F. Heidkamp, C. Fiorese, A.J. Bonito, K. Inaba, M.C. Nussenzweig, R.M. Steinman, CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells, J. Immunol. 181 (2008) 6923–6933.
[22] C. Pasare, R. Medzhitov, Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells, Science 299 (2003) 1033–1036.
[23] S. Wan, C. Xia, L. Morel, IL-6 produced by dendritic cells from lupus-prone mice inhibits CD4+CD25+ T cell regulatory functions, J. Immunol. 178 (2007) 271–279.
[24] E. Bettelli, Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, V.K. Kuchroo, Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, Nature 441 (2006) 235–238.
[25] P.R. Mangan, L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, R.D. Hatton, S.M. Wahl, T.R. Schoeb, C.T. Weaver, Transforming growth factor-beta induces development of the T(H)17 lineage, Nature 441 (2006) 231–234.
[26] S.G. Zheng, J. Wang, D.A. Horwitz, Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6, J. Immunol. 180 (2008) 7112–7116.

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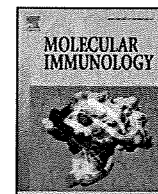

Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the Toll-like receptor 3/4 pathway

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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.

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1. Introduction

Tumor necrosis factor receptor-associated factor (TRAF) family proteins are frequently involved in signaling of Toll-like receptors (TLRs) to evoke immune responses (Chung et al., 2002; Kawai and Akira, 2007). Of the TRAF family members, TRAF6 plays a significant role in signal transduction by both the TNF receptor (TNFR) and interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) 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

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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

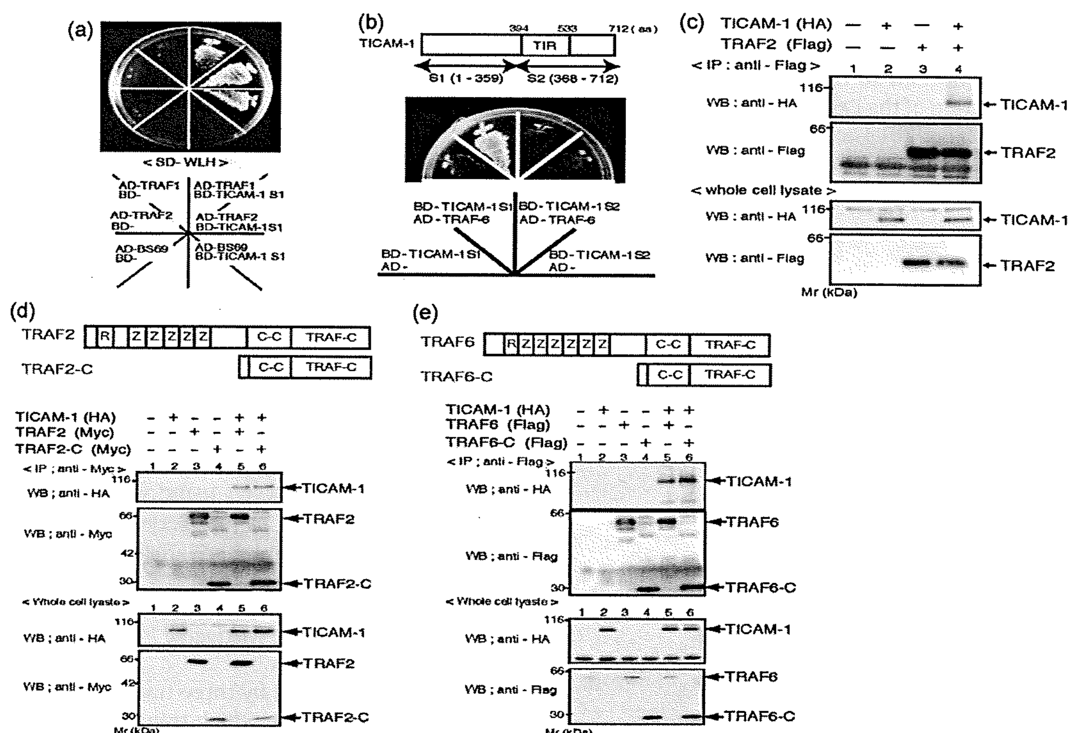

Fig. 1. Molecular interaction of TICAM-1 with TRAF2 and 6. (a) Direct interaction between the N-terminal region of TICAM-1 and either TRAF2 or TRAF6 as identified by yeast two-hybrid screening. Full-length TRAF2 and TRAF6 were cloned into pGADT7, transformed into yeast strain AH109, and then cultured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