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**IL-6 and IFN- $\alpha$  from dsRNA-stimulated dendritic cells control expansion of regulatory T cells**

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Running title: **Treg regulation by IL-6 and IFN- $\alpha$**

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## **Abstract**

Foxp3<sup>+</sup> CD4<sup>+</sup> 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- $\alpha$  from polyI:C-stimulated DCs. These data suggest that the balance of IL-6 and IFN- $\alpha$  in the region of RNA virus infection may determine the number of peripheral Treg, which affects the effective immune responses against viruses.

## Introduction

CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>-</sup> T cells [3, 4]. Both Treg constitute 5-15% of peripheral CD4<sup>+</sup> 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- $\alpha$ , etc.) by NF- $\kappa$ B 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- $\beta$  promoter stimulator 1 (IPS-1, also called MAVS/VISA/CARDIF), which activates IRF-3 (interferon-regulatory factor 3), NF- $\kappa$ B (nuclear factor-kappaB), and AP-1 (activator protein 1) and induces IFN- $\beta$  and inflammatory cytokines [9].

TLRs are also known to be expressed on CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg and directly modulate the proliferation and suppressive functions [12,13]. CD4<sup>+</sup>CD25<sup>+</sup> Treg selectively expresses TLR4, TLR5, TLR7 and TLR8 [12]. In contrast, TLR1, TLR2, TLR3 and TLR6 are more widely expressed on CD4<sup>+</sup> 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*. The polyI:C plus BMDCs expanded Treg in a TICAM-1-

and IL-6-dependent manner. We also found that IFN- $\alpha$  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- $\alpha$  upon polyI:C stimulation.

## Materials and Methods

### *Mice and reagents*

C57BL/6J mice and IL6<sup>-/-</sup> mice were purchased from Charles River (Yokohama, Japan). TICAM-1<sup>-/-</sup> mice were generated in our laboratory [15]. IFNAR<sup>-/-</sup> 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- $\alpha$  (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<sup>+</sup>CD25<sup>+</sup> (Treg) cells and CD4<sup>+</sup>CD25<sup>-</sup> cells were purified from mouse splenocytes using a MACS CD4<sup>+</sup>CD25<sup>+</sup> 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 ( $1 \times 10^6$ /ml) were incubated with or without 50  $\mu$ g/ml PolyI:C for 24 hrs and the supernatants were collected for ELISA. The concentrations of cytokines (IL-6 and IFN- $\alpha$ ) were measured by commercial ELISA kits (Invitrogen, Carlsbad, CA, USA; PBL Biomedical Laboratories, Piscataway, NJ, USA). PolyI:C (1.25 mg/ml: 200  $\mu$ L) was injected intraperitoneally and inguinal lymph nodes were excised for FACS analysis. The ratio of Treg cells (CD4<sup>+</sup>Foxp3<sup>+</sup>/CD4<sup>+</sup>) was determined by analysis from FlowJo (Tree Star Inc. OR, USA).

### *In vivo polyI:C administration*

PolyI:C (250 µg/200 µl) or control phosphate-buffered saline (PBS) was intraperitoneally administered into mice twice at three days interval. 24 hrs after the last injection, the spleen and lymph nodes were extracted and total cell numbers were counted. Then, the numbers of the CD4<sup>+</sup> and CD4<sup>+</sup>Foxp3 populations were assessed by FACS as described [16] and the scales of the CD4<sup>+</sup> and CD4<sup>+</sup>Foxp3 fractions were evaluated.

### *Treg proliferation assay*

$5 \times 10^4$  Treg cells 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,  $1 \times 10^6$  BMDCs were added to the well. Occasionally, IL-6 (10 ng/ml) and/or IFN-α (10-10<sup>4</sup> IU/ml) were added to the culture. During the last 6 hrs of culturing, [<sup>3</sup>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 ( $1 \times 10^5$ ) were treated with mytomycin C (20 µg/ml, 45 min) and cultured with freshly-isolated CD4<sup>+</sup>CD25<sup>-</sup> T cells (responder,  $2.5 \times 10^4$ ) for 2 days. The ratio of CD4<sup>+</sup>CD25<sup>-</sup>/CD4<sup>+</sup>CD25<sup>+</sup> was indicated in the figure. The proliferation of responder cells was measured by [<sup>3</sup>H] thymidine uptake assay.

## **Results**

### *PolyI:C induces the proliferation of Treg in vivo and 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<sup>+</sup>Foxp3<sup>+</sup>) compared to CD4<sup>+</sup> T cells in the inguinal lymph nodes (LN) and spleen. Treg numbers were increased after polyI:C administration in LN (Fig. 1A, 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<sup>+</sup>CD25<sup>+</sup> T cell) directly as a proliferation stimulator or whether polyI:C converts CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> T cells from CD4<sup>+</sup>CD25<sup>-</sup> 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<sup>+</sup> 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<sup>-/-</sup> and TICAM-1<sup>-/-</sup> mice. When we injected polyI:C into IL-6<sup>-/-</sup> mice or TICAM-1<sup>-/-</sup> mice, there was no significant increase of Treg in LN (Fig.2A). Consistent with our previous report [15], we found that TICAM-1<sup>-/-</sup> 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<sup>-/-</sup>, IL-6<sup>-/-</sup> or wild-type mice with or without polyI:C. The Treg expansion by polyI:C was largely suppressed with TICAM-1<sup>-/-</sup> BMDCs and more severely abrogated in IL-6<sup>-/-</sup> 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<sup>-/-</sup> or IL-6<sup>-/-</sup> 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<sup>-/-</sup> and IL-6<sup>-/-</sup> 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- $\alpha$ 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 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- $\alpha$  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- $\alpha$  production in serum from polyI:C-injected wild-type and TICAM-1<sup>-/-</sup> mice. As shown in Fig3B left, IFN- $\alpha$  production was intact in TICAM-1<sup>-/-</sup> mice after the polyI:C injection. IFN- $\alpha$  production in culture supernatants was also similar between BMDCs from wild-type mice stimulated with polyI:C and those from TICAM-1<sup>-/-</sup> mice (Fig3B right). The results infer that cytoplasmic MDA5 rather than TLR3 preferentially induces IFN- $\alpha$  in response to polyI:C in our setting *in vivo* and *in vitro*.

Next, we checked if exogenous IFN- $\alpha$  could inhibit the Treg proliferation. When Treg were cultured with BMDCs in the presence of polyI:C and graded doses of IFN- $\alpha$ , IFN- $\alpha$  actually inhibited the Treg proliferation in a dose-dependent manner (Fig. 3C). IFN- $\alpha$  also abolished the proliferation of Treg induced by BMDCs plus IL-6 in a dose-dependent manner (Fig. 3D). To see if IFN- $\alpha$  derived from BMDCs is responsible for the suppression of the Treg-proliferation induced by IL-6 from polyI:C-stimulated BMDCs, we used IFNAR<sup>-/-</sup> BMDCs which barely amplify type I IFN production but can activate the MDA5/IPS-1 pathway [15]. We found that IFNAR<sup>-/-</sup> BMDCs did not suppress IL-6-mediated Treg expansion induced by polyI:C-stimulated BMDCs (Fig 3E). These indicate that IFN- $\alpha$  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- $\alpha$  and co-cultured with Treg cells in the presence of IL-6 and/or IFN- $\alpha$ . In this series of experiments, we could not observe any effects of IL-6 and

IFN- $\alpha$  on direct Treg expansion (Fig. 3F), suggesting that IL-6 and IFN- $\alpha$  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- $\alpha$  (Fig. 4A and 4B). 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- $\alpha$  after sensing dsRNA. Although IFN- $\alpha$  negatively acts on Treg expansion, IL-6 overwhelmed the inhibitory effects of IFN- $\alpha$  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- $\alpha$  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 antiviral function of IFN- $\alpha$ . IFN- $\alpha$  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- $\alpha$  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- $\alpha$  is a determinant factor of Treg expansion.

IFN- $\alpha$  and IL-6 are known to up-regulate co-stimulatory molecules such as CD80 and CD86 on DCs. We have shown that CD8<sup>+</sup> CD205<sup>+</sup> splenic DCs in the steady state induce antigen-specific Foxp3<sup>+</sup> Treg from Foxp3<sup>-</sup> CD25<sup>-</sup> CD4<sup>+</sup> T cells using endogenous TGF- $\beta$  [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- $\alpha$ -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 that IL-6 and IFN- $\alpha$  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<sup>+</sup> CD25<sup>-</sup> 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- $\beta$  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, proinflammatory effects of IL-6 promote differentiation of Th17, but not that of Treg.

In our experiments, CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cells were not induced from CD4<sup>+</sup> CD25<sup>-</sup> 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- $\beta$  is a key cytokine for differentiation of iTreg and Th17, serum level of TGF- $\beta$  did not increase after i.p. polyI:C administration, and BMDC did not produce TGF- $\beta$  (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- $\alpha$ -treated mDCs actually suppress Treg growth, whereas signaling of IL-6 on mDCs overcomes the

IFN- $\alpha$ -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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## **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- $\beta$  promoter stimulator 1; RLRs , RIG-I-like Receptors.

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## Figure Legends

**FIGURE 1.** PolyI:C induces the proliferation of Treg in vivo and vitro.

(A, B) C57BL/6J wild-type (WT) mice were intraperitoneally injected with polyI:C (1.25mg/ml: 200uL) or PBS twice every 3 days throughout the experiments. Inguinal lymph nodes were excised, and the ratio of CD4<sup>+</sup> Foxp3<sup>+</sup> /CD4<sup>+</sup> T cells (A) and the absolute number of CD4<sup>+</sup> Foxp3<sup>+</sup> (B) cells were determined by FACS at 1 day after the final administration.

(C) Freshly isolated CD4<sup>+</sup> CD25<sup>+</sup> Treg ( $5 \times 10^4$ ) from WT mice were cultured in the presence of 1  $\mu$ g/ml anti-CD3 antibody and 100 U/ml recombinant IL-2 with or without 50  $\mu$ g/ml polyI:C. The proliferation was determined by [<sup>3</sup>H] thymidine uptake after 2 day culture. There was no statistical difference between them.

(D) As in (C), but  $1 \times 10^6$  WT BMDCs were added to each well.

(E) The ratio of CD4<sup>+</sup> Foxp3<sup>+</sup> /CD4<sup>+</sup> T cells in LN was analyzed at 24 hrs after injection of non-treated BMDCs (DC) or BMDCs incubated with 50  $\mu$ g/ml polyI:C (polyI:C DC) for 24 hrs.

Data represented the mean  $\pm$  SD of three independent experiments.

**FIGURE 2.** The Treg proliferation by polyI:C plus BMDCs requires TICAM-1 signaling and IL-6.

(A) TICAM1<sup>-/-</sup> mice and IL-6<sup>-/-</sup> mice were intraperitoneally injected with polyI:C or PBS as in Fig.1A and the ratio of Foxp3<sup>+</sup> CD4<sup>+</sup> Treg /CD4<sup>+</sup> 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  $\mu$ g/ml polyI:C for 24 hrs, and the supernatants were collected. The sera were collected at 24 hrs after injection of polyI:C.

(C) BMDCs from WT, TICAM-1<sup>-/-</sup> or IL-6<sup>-/-</sup> mice ( $1 \times 10^6$ ) were cultured in the presence of 1  $\mu$ g/ml anti-CD3 antibody and 100 U/ml recombinant IL-2 with or without Treg ( $5 \times 10^4$ ) from WT mice in the presence or absence of 50  $\mu$ g/ml polyI:C. The proliferation was determined by [<sup>3</sup>H] thymidine uptake after 2 day culture.

(D) As in Fig.2C, but Treg from WT mice were cultured with BMDCs from WT, TICAM1<sup>-/-</sup> or IL-6<sup>-/-</sup> mice. After 24 h culture, supernatants were collected and measured for IL-6 production.

(E) As in Fig.2C, but Treg from WT mice were cultured with BMDCs from WT, TICAM1<sup>-/-</sup> or IL-6<sup>-/-</sup> mice with or without 50  $\mu$ g/ml polyI:C or polyI:C plus 10 ng/ml IL-6. The proliferation was determined by [<sup>3</sup>H] thymidine uptake after 2 day culture.

Data represented the mean  $\pm$  SD of three independent experiments.

**FIGURE 3.** Effect of IFN- $\alpha$  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  $\mu$ g/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- $\alpha$

(C) As in Fig.3A, but graded doses of IFN- $\alpha$  ( $10$ - $10^4$  IU/ml) was added to the culture with 50  $\mu$ g/ml polyI:C. The proliferation was determined by [ $^3$ H] thymidine uptake after 2 day culture.

(D) As in Fig.3C, but graded doses of IFN- $\alpha$  ( $10$ - $10^4$  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  $\mu$ g/ml polyI:C. The proliferation was determined by [ $^3$ H] thymidine uptake after 2 day culture.

(F) WT BMDCs were incubated with IFN- $\alpha$  ( $10^3$  IU/ml) and/or IL-6 (10 ng/ml) for 24 hrs 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  $\pm$  SD of three independent experiments.

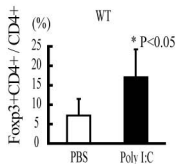
**FIGURE 4.** Treg expanded by polyI:C plus BMDCs are suppressive in vitro

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

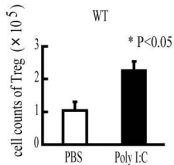
(B) As in Fig.4A, but Treg were cultured with BMDCs with or without IL-6 (10 ng/ml) or IFN- $\alpha$  ( $10^3$  IU/ml), and used for the suppression assay.

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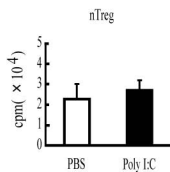
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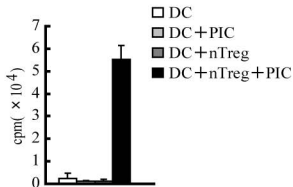
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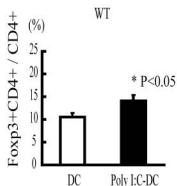
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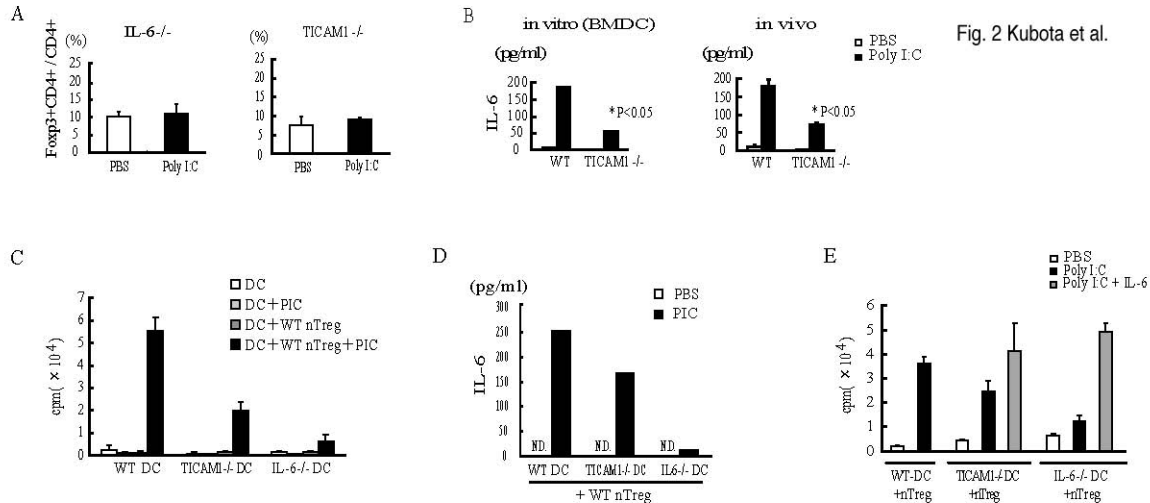


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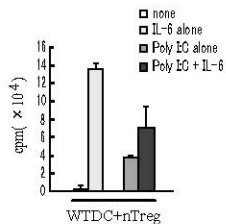


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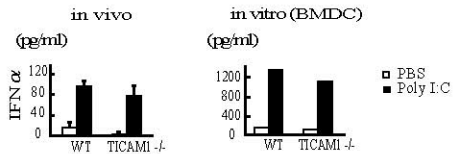




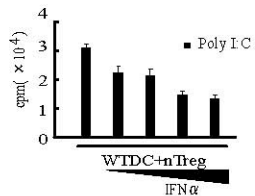
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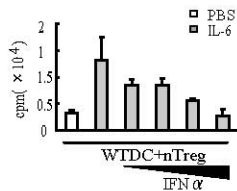
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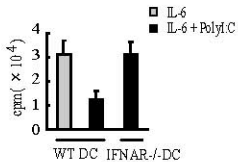
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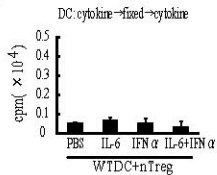
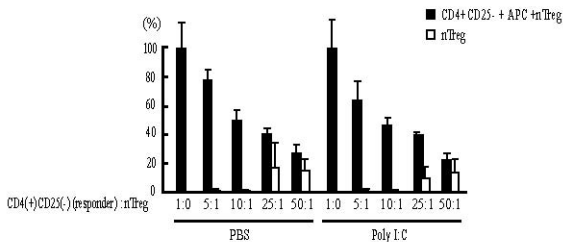


Fig. 3

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Fig. 4 Kubota et al.

A



B

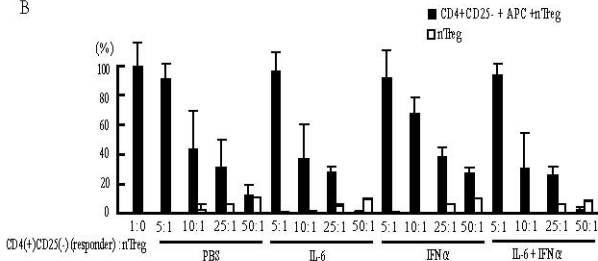
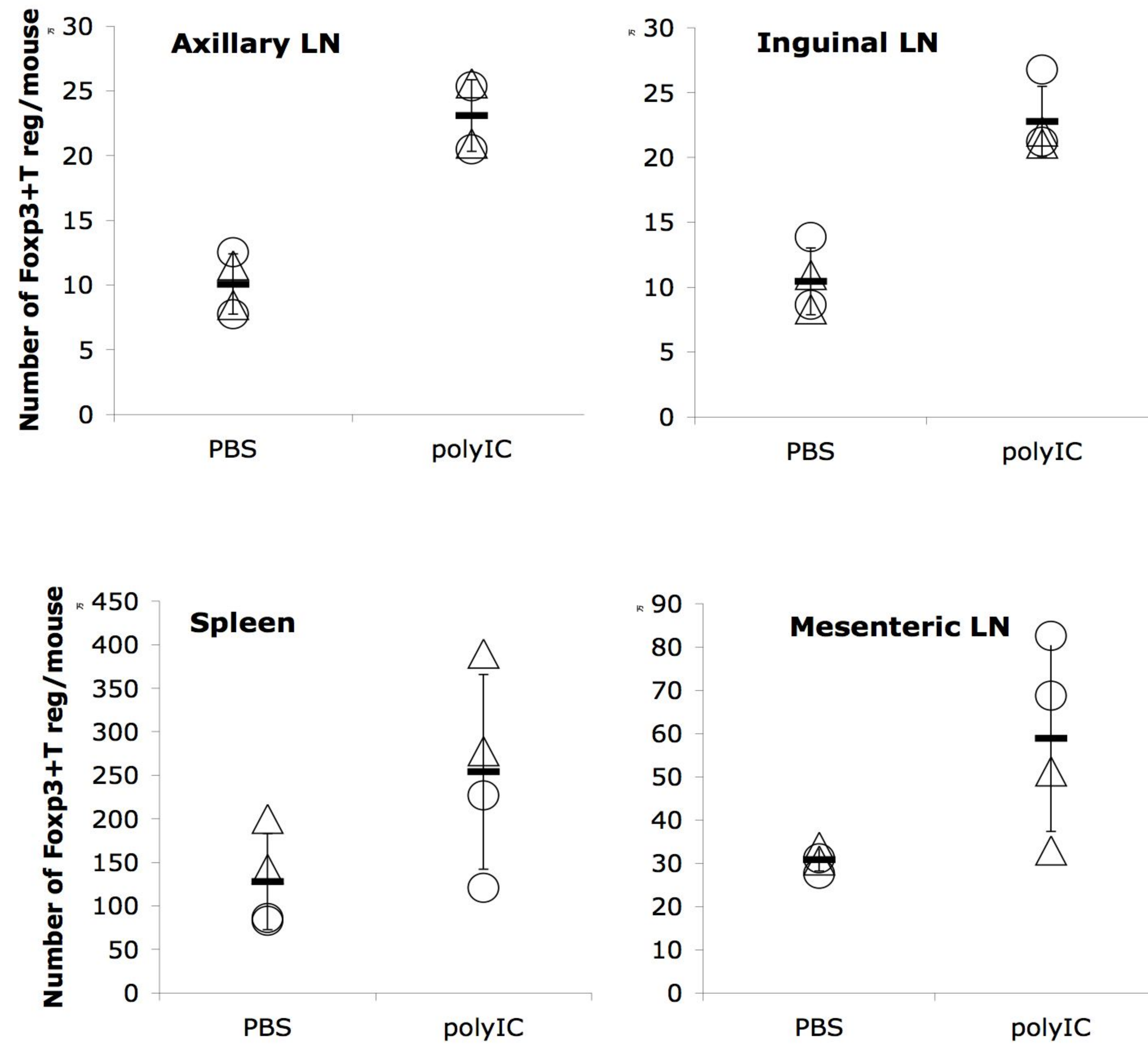


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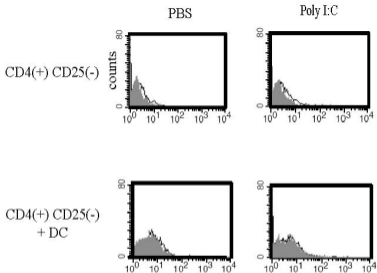




Fig. S2

→  
 Foxp3

 isotype control  
 FITC-Foxp3