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Toll-like receptor 2-mediated modulation of growth and functions of regulatory T cells by oral streptococci

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SUMMARY

This study was designed to determine whether oral streptococci modulate the growth and functions of regulatory T cells. Heat-killed cells of wild-type strains of *Streptococcus gordonii* and *Streptococcus mutans* induced the Toll-like receptor 2 (TLR2)-mediated nuclear factor-κB (NF-κB) activation, but their lipoprotein-deficient strains did not. Stimulation with these streptococci resulted in a significant increase in the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells in splenocytes derived from both $TLR2^{+/+}$ and $TLR2^{-/-}$ mice, but the level of increase in $TLR2^{+/+}$ splenocytes was stronger than that in $TLR2^{-/-}$ splenocytes. Both strains of *S. gordonii* enhanced the proliferation of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells isolated from $TLR2^{+/+}$ mice at the same level as those from $TLR2^{-/-}$ mice in an interleukin-2-independent manner. However, wild-type and lipoprotein-deficient strains of both streptococci did not enhance the suppressive activity of the isolated regulatory T cells *in vitro*, but rather inhibited it. TLR ligands also inhibited the suppressive activity of the regulatory T cells. Inhibition of the suppressive activity was recovered by the addition of anti-IL-6 antibody. Pretreatment of antigen-presenting cells with the NF-κB inhibitor BAY11-7082 enhanced the suppressive activity of the regulatory T cells. These results suggested that interleukin-6 produced by antigen-presenting cells inhibits the suppressive activity of the regulatory T cells. Wild-type strain, but not lipoprotein-deficient strain, of *S. gordonii* reduced the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells in the acute infection model, whereas both strains of *S. gordonii* increased it in the chronic infection model mice. Hence, this study suggests that oral streptococci are capable of modulating the growth and functions of regulatory T cells *in vitro* and *in vivo*.

INTRODUCTION

In the human oral cavity, over 700 bacterial species live in homeostasis with the host immune system (Jenkinson & Lamont, 2005). Commensal oral

bacteria can, in the case of immune dysregulation, trigger the onset of harmful inflammatory diseases such as gingivitis and periodontitis. Oral streptococci, major members of the oral microflora, occasionally cause bacteremia and infective endocarditis (Douglas *et al.*, 1993). *Streptococcus mutans* and *Streptococcus gordonii* are gram-positive commensal bacteria and components of the normal microbial flora of the human oral cavity. The former is generally known to be a major pathogen of dental caries (Loesche, 1986) and the latter has been developed as a vaccine vector (Oggioni *et al.*, 1999). Of the oral streptococci, *S. mutans* and *S. gordonii* have been isolated from the blood of patients with infective endocarditis (Douglas *et al.*, 1993; Gauduchon *et al.*, 2001).

Recent studies have shown that many pathogens, to extend their survival, exploit regulatory T (Treg) cells that are normally associated with the termination of effector immune responses of the host by suppressing excess and unnecessary immune responses (Mills, 2004; Belkaid, 2007). Certain gut bacteria can promote the induction of Treg cells, which are key players in gut homeostasis or oral tolerance, by inducing the production of anti-inflammatory cytokines, transforming growth factor-β (TGF-β) or interleukin-10 (IL-10) (Faria & Weiner, 2005; Tsuji & Kosaka, 2008; Round & Mazmanian, 2010;; Atarashi *et al.*, 2011; Weiner *et al.*, 2011). There are multiple subpopulations of Treg cells, including $CD4^+$ $CD25^+$ $Foxp3^+$ T (nTreg) cells, IL-10-producing Tr1 cells, TGF-β-producing T helper type 3 cells, $CD8^+$ T suppressor cells, natural killer T cells, $CD4^-$ $CD8^-$ T cells and γδ T cells (Tang & Bluestone, 2008). Some of these Treg cells, such as $CD4^+$ $CD25^+$ $Foxp3^+$ cells, originate in the thymus during ontogeny and are referred to as natural Treg (nTreg) cells (Mills, 2004). Treg cells can also be induced from naive T cells in the periphery (Mills, 2004).

A large number of bacteria inhabit the oral cavity. For example, 10^8–10^{10} colony-forming units (CFU) $ml^{-1}$of bacteria are present in whole saliva, approximately 1 l of which we swallow in a day. In other words, we ingest $10^{11–13}$ CFU of bacteria in a day. It has been reported that Treg cells specific for orally administered proteins, such as collagen, bovine serum albumin and bovine β-lactoglobulin, regulate oral tolerance against these proteins (Tsuji *et al.*, 2001; Hauet-Broere *et al.*, 2003; Park *et al.*, 2008; Tsuji & Kosaka, 2008). Therefore, we hypothesize that oral tolerance against oral bacteria should be established in the same way as that against foods that we eat every day and that oral bacteria also use Treg cells to inhabit in the oral cavity. In addition, it has recently been reported that Treg cells exist in salivary glands and are involved in the maintenance of peripheral tolerance for the protection of autoimmunity (Ishimaru *et al.*, 2010).

Recently, we have made mutants of *S. mutans* and *S. gordonii* (Arimoto & Igarashi, 2008) that are not able to synthesize lipoproteins (LP), which are well known to be recognized by Toll-like receptor 2 (TLR2) and TLR1 (Akira & Takeda, 2004; Takeuchi & Akira, 2010). In addition, it has been reported that Treg cells express TLR2 and TLR2 ligands regulate the functions of Treg cells (Sutmuller *et al.*, 2006).

As a first step to clarify oral tolerance against oral bacteria or use of Treg cells allowing them to inhabit the oral cavity, therefore, this study was designed to investigate whether wild-type and LP-deficient strains of *S. mutans* and *S. gordonii* interact with Treg cells, mainly nTreg cells, because most Treg cells (approximately 90%) in mice are thymus-derived nTreg cells (Cheng *et al.*, 2011).

METHODS

Reagents and antibodies

CD16/CD32 antibody (clone 93), allophycocyanin-conjugated monoclonal antibody (mAb) against CD4 (clone RM4-5), phycoerythrin-conjugated mAb against CD25 (clone PC61), Alexa Fluor 488-anti-Foxp3 mAb (clone 150D), Fix/Perm Buffer Set, anti-CD3ε mAb, anti-IL6 antibody (blocking antibody) (clone MP5-20F3) and its appropriate isotype control were purchased from BioLegend (San Diego, CA). A $CD4^+$ $CD25^+$ Treg cell isolation kit was purchased from Miltenyi Biotec (GmbH, Bergisch Gladbach, Germany). Anti-IL-2 antibody (blocking antibody) was purchased from R&D Systems (Minneapolis, MN). Mitomycin C and *Escherichia coli* lipopolysaccharide (LPS) were purchased from Sigma (St Louis, MO). The TLR2 ligand FSL-1, a diacylated lipopeptide from *Mycoplasma salivarium*, was synthesized according to the method described previously (Shibata *et al.*, 2000). The TLR3 ligand Poly I:C was purchased from InvivoGen (San Diego, CA). Phosphorothioate-stabilized CpG-oligonucleotide 1826 (5′-TCCATGACGTTCCT-

GACGTT-3′) (hereafter abbreviated as CpG) was purchased from Hokkaido System Science (Sapporo, Japan). The nuclear factor-κB (NF-κB) inhibitor BAY11-7082 [E-3-(4-methylphenylsulfonyl)-2-propenenitrile; BAY11] was purchased from Calbiochem (Darmstadt, Germany) and reconstituted in dimethyl sulfoxide (DMSO).

All other reagents were purchased from commercial sources and were of analytical or reagent grade.

Bacteria

Oral streptococci used were wild-type *S. mutans* 109c (Sm-wt) and *S. gordonii* Challis (Sg-wt) and their LP-deficient mutants (Sm-dLP and Sg-dLP) lacking prolipoprotein diacylglyceryl transferase, a key enzyme for lipoprotein synthesis in bacteria. Sm-dLP and Sg-dLP were generated by a double-crossover recombination as described previously (Arimoto & Igarashi, 2008). These streptococci were grown in Trypton-based broth consisting of 2% (wt/vol) trypton, 0.5% (wt/vol) K_2HPO_4, 0.2% (wt/vol) glucose and 0.05% (vol/vol) Tween-80 until late logarithmic phase was reached and the cultures were centrifuged at 8000 ***g*** for 10 min to collect the cells. A part of the cultures was used to determine viable counts. The cells collected were washed three times with PBS, treated for 10 min in boiling water, and stored at −20°C until used as heat-killed cells.

Mice

Sex-matched 8-week-old C57BL/6 mice ($TLR2^{+/+}$) were purchased from Japan Clea (Tokyo, Japan). TLR2-deficient mice ($TLR2^{-/-}$) on the same genetic background were kindly provied by Dr. Shizuo Akira, Osaka University. All mice were maintained in specific pathogen-free conditions at our animal facility at Hokkaido University, and all experiments were carried out in accordance with the regulations of the Hokkaido University Animal Care and Use Committee.

Nuclear factor-κB reporter assay

Human embryonic kidney (HEK) 293 cells obtained from the Americal Type Culture Collection (Manassas, VA; CRL-1573) were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin G (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) (Sigma). Activation of NF-κB was measured as described previously (Fujita *et al.*, 2003). Briefly, HEK293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) with 50 ng of an NF-κB luciferase reporter plasmid (Stratagene, Santa Clara, CA) and 2.5 ng of a construct directing expression of *Renilla* luciferase under the control of the constitutively active thymidine kinase promoter (Promega Co., Madison, WI) together with 150 ng of TLR2 gene, which was cloned into a pEF6/V5-His TOPO vector (Invitrogen) from THP-1 cells. The HEK293 transfectant was referred to as HEK293/TLR2 cells. Twenty-four hours after transfection, the cells were stimulated for 6 h with the mycoplasmal diacylated lipopeptide FSL-1 (0.1, 1, 10 nM), heat-killed cells of streptococci at multiplicity of infection (MOI) of 1, 10 or 100 (1×10^5, 1×10^6, 1×10^7 CFU $1^{-1} \times 10^5$ HEK293 cells) of Sm-wt, Sg-wt, Sm-dLP and Sg-dLP. The luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions.

Detection of nTreg cells in splenocytes by flow cytometry

Splenocytes (1×10^6) from $TLR2^{+/+}$ or $TLR2^{-/-}$ mice were incubated at 37°C for 48 h in a 24-well flat-bottomed plate (0.5 ml) with the mycoplasmal diacylated lipopeptide FSL-1 (10 nM) synthesized as described previously (Shibata *et al.*, 2000) or heat-killed bacterial cells at an MOI of 70 (7.0×10^7 CFU $1^{-1} \times 10^6$ splenocytes) of Sm-wt, Sg-wt, Sm-dLP or Sg-dLP. The cell suspensions were washed three times with PBS containing 0.5% BSA (PBS/BSA) and resuspended in 0.2 ml of PBS/BSA. The resulting cell suspensions were incubated for 10 min on ice with anti-mouse CD16/CD32 antibody to block the Fc receptor and then incubated with an allophycocyanin-conjugated mAb against CD4 (clone RM4-5) and PE-conjugated mAb against CD25 (clone PC61). The cells were fixed and permeabilized using a Fix/Perm Buffer. After washing, the cells were incubated with an Alexa Fluor 488-anti-Foxp3 mAb (clone 150D) and an appropriate

isotype control. Data for 50,000 cells falling within appropriate forward and side scatter gates were collected from each sample with a FACS Calibur. Data were analysed using FLOW JO software (Tree Star Inc., Ashland, OR). The culture medium used for all culture conditions was RPMI-1640 complete medium (Sigma-Aldrich, St Louis, MO) containing 10% fetal bovine serum (Invitrogen), penicillin G (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) (Sigma) and 28.6 μM 2-mercaptoethamol (Sigma) (complete medium) was used for all culture conditions.

Isolation of nTreg cells

Natural Treg cells were isolated from spleen cells of C57BL/6 mice by a two-step procedure using a Treg cell isolation kit (Miltenyi Biotec GmbH) according to the manufacturer's instructions. Briefly, for the isolation of $CD4^+$ T cells, non-$CD4^+$ T cells were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against CD8a, CD11b, CD45R, CD49b and Ter-119 and anti-biotin microbeads. In parallel, the cells were labeled with phycoerythrin-conjugated antibody against CD25. The cell suspension was loaded onto a column placed in the magnetic field of a Magnetic Cell Separator (MACS) (Miltenyi Biotec GmbH). The magnetically labeled non-$CD4^+$ T cells were retained in the column, whereas the $CD4^+$ T cells passed through it. The retained non-$CD4^+$ T cells were eluted after removal of the column from the magnetic field and used as antigen-presenting cells (APCs) without further purification. For the isolation of $CD4^+$ $CD25^+$ cells, $CD25^+$ phycoerythrin-labeled cells in the enriched $CD4^+$ T-cell fraction were magnetically labeled with anti-phycoerythrin microbeads. The cell suspension was again loaded onto a column placed in the magnetic field of a MACS Separator. The magnetically labeled $CD4^+$ $CD25^+$ cells were retained in the column, whereas the unlabeled cells passed through it. After removal of the column from the magnetic field, the retained $CD4^+$ $CD25^+$ cells were eluted and used as Treg cells. Most of the Treg cells eluted are considered to be nTreg cells, because most Treg cells (approximately 90%) in mice are thymus-derived nTreg cells (Cheng *et al.*, 2011). The $CD4^+$ $CD25^-$ cells that passed through the column were used as $CD4^+$ helper T cells (Th cells). The culture medium used was RPMI-1640 complete medium.

Proliferation of nTreg cells or Th cells in response to streptococci

Natural Treg cells (1×10^5 cells) or Th cells (1×10^6 cells) isolated from splenocytes from $TLR2^{+/+}$ or $TLR2^{-/-}$ mice by the method described above were incubated for 48 h in a 96-well U-bottomed plate (0.2 ml) with heat-killed cells at an MOI of 120 or 12 (1.2×10^7 CFU/1×10^5 nTreg or 1.2×10^7 CFU/1×10^6 Th cells) of Sg-wt or Sg-dLP. In some experiments, neutralizing anti-IL-2 antibody (10 $\mu g\ ml^{-1}$) was added to the cultures. Cultures were pulsed with 0.2 μCi per well of [^{3}H]thymidine for the last 16 h of culture. The cultures were harvested with a Labo Mash cell harvester. The radioactivity of the cells deposited on filters was measured in a liquid scintillation counter.

Assay for immunosuppressive activity of nTreg cells

T helper cells (2×10^4 cells per 0.2 ml) were cultured for 48 h in a 96-well flat-bottomed plate in the presence of anti-CD3ε mAb (1 $\mu g\ ml^{-1}$) and APCs (2.0×10^4 cells) that had been treated with 50 $\mu g\ ml^{-1}$ mitomycin C in RPMI-1640 complete medium. The nTreg cells (2.0×10^4 cells) were also added to selected wells. In some experiments, the cells were stimulated with heat-killed cells (1×10^6, 1×10^7 CFU) of Sm-wt, Sg-wt, Sm-dLP or Sg-dLP or with various amounts (10, 100, 1000 ng ml^{-1}) of the TLR2 ligand FSL-1, the TLR3 ligand Poly I:C, the TLR4 ligand *Escherichia coli* LPS and the TLR9 ligand CpG. Cultures were pulsed with 0.2 μCi per well of [^{3}H]thymidine for the last 16 h of culture. The cultures were harvested with a Labo Mash cell harvester (Futaba Medical Inc., Tokyo, Japan). The radioactivity of the cells deposited on filters was measured in a liquid scintillation counter. In some experiments, neutralizing anti-IL-6 antibody (10 $\mu g\ ml^{-1}$) (clone MP5-20F3) and IgG1 isotype control (clone RTK2071) were added to the cultures. In some experiments to investigate the effects of the NF-κB inhibitor on immunosuppressive activity of nTreg cells, nTreg cells, Th cells or APCs were preincubated for 1 h with BAY11-7082 (10 μM) or DMSO (control) and used for Treg cell suppression assays after washing.

Average suppression (%) were calculated using the formula $(S - C)/S \times 100\%$, where S or C is

[^{3}H]thymidine uptake (counts per minute) obtained by culture of Th cells and APCs in the absence or presence of nTreg cells, respectively.

Mouse infection model with streptococci

Acute phase infection model

Sg-wt and Sg-LP cells (2.6×10^8 CFU per mouse) were injected intravenously six times at a 3-day interval into C57BL/6 female mice (8–10 weeks old) from their tails. Splenocytes were prepared from spleens that were taken out 3 days after final injection and examined for the frequency of nTreg cells.

Chronic infection model

We used a modified subcutaneous chamber model described previously (Genco & Arko, 1994). A chamber (length 10 mm, diameter 5 mm), which was constructed from coils of stainless-steel wire, was implanted subcutaneously into the back of each mouse. After the healing period, the chamber was used as a biological compartment for inducing inflammation. Sg-wt cells (1.5×10^8 CFU per mouse) were injected into the chamber four times at 1-week intervals. Single cell suspensions were prepared from the inguinal lymph node, axillary lymph node and spleen, which were taken out 7 days after the final injection and examined for the frequency of nTreg cells.

RESULTS

Recognition of heat-killed cells of oral streptococci by HEK293/TLR2 transfectant

Triacylated and diacylated LP are recognized by TLR2 in cooperation with TLR1 and TLR6, respectively (Akira & Takeda, 2004). To confirm the differences in the recognition of the wild-type and LP-deficient strains of these streptococci by TLR2, first, we examined whether heat-killed cells of Sm-wt, Sm-dLP, Sg-wt and Sg-dLP induce activation of NF-κB in HEK293/TLR2 cells by using the TLR2 ligand FSL-1 as a positive control. Heat-killed cells of wild-type, but not LP-deficient, strains of these streptococci induced NF-κB activation in a dose-dependent manner (Fig. 1), suggesting that LP released from whole cells or cell debris containing LP are included in heat-killed cell fractions.

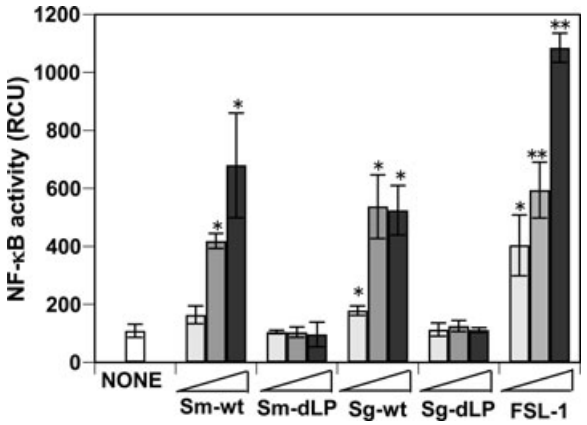

Figure 1 Recognition of heat-killed cells of wild-type and lipoprotein (LP) -deficient streptococci by HEK293/TLR2 cells. HEK293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected with genes of Toll-like receptor 2 and nuclear factor-κB (NF-κB) reporter. After a 24-h incubation, the cells were stimulated for 6 h with heat-killed cells of wild-type *Streptococcus mutans* 109c (Sm-wt) and *Streptococcus gordonii* Challis (Sg-wt) and their LP-deficient mutants (Sm-dLP and Sg-dLP) at different multiplicities of infection (1, 10 and 100) or FSL-1 (0.1, 1, 10 nM) in a serum-free condition. The NF-κB reporter activity was measured using the Dual-Luciferase reporter assay system. The mean values and SD of triplicate wells of a representative experiment obtained by three separate experiments are shown. See text for details. Statistically significant difference from control was assessed by Student's *t*-test; $*P < 0.05$, $**P < 0.01$.

These results strongly suggest that something containing LP released from whole cells of these streptococci is recognized by TLR2 and that a key ligand for TLR2 in these streptococcal cells is LP.

Increase in the frequency of nTreg cells

We investigated whether whole cells of Sm-wt, Sm-dLP, Sg-wt and Sg-dLP affected the frequency of Treg cells in mouse splenocytes. Splenocytes from TLR2$^{+/+}$ and TLR2$^{-/-}$ mice were incubated with heat-killed cells of these streptococci or with the TLR2 ligand FSL-1 *in vitro*. The frequency of nTreg cells was analysed by flow cytometry after staining with anti-CD4, anti-CD25 and anti-Foxp3 antibodies. That is, cells falling within the CD4$^+$ gate were analysed with the expression of Foxp3 and/or CD25 (Fig. 2A,B). CD4$^+$ CD25$^+$ Foxp3$^+$ cells were referred to as nTreg cells. Stimulation with all of these streptococcal cells resulted in significant increases in the frequencies of nTreg cells in both TLR2$^{+/+}$ and TLR2$^{-/-}$ splenocytes, and the magnitudes of increases (approximately

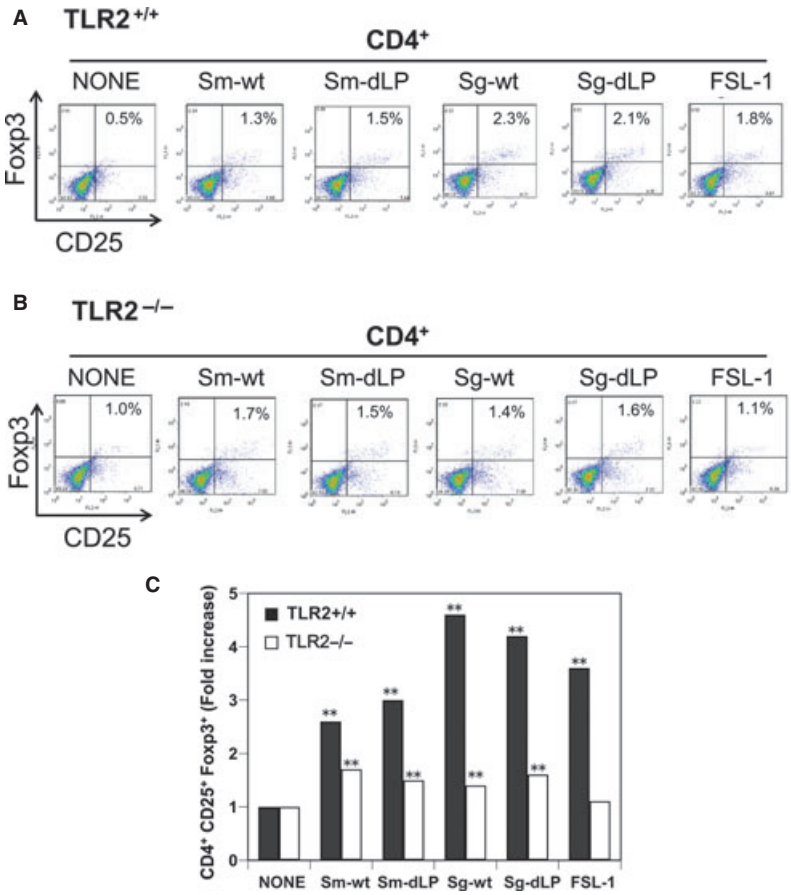

Figure 2 Upregulation of the frequency of natural regulatory T (nTreg) cells in splenocytes by stimulation with streptococci. Splenocytes from TLR2$^{+/+}$ or TLR2$^{-/-}$ mice were incubated at 37°C for 48 h with the mycoplasmal diacylated lipopeptide FSL-1 (10 nM) or heat-killed bacterial cells at a multiplicity of infection of 70 of wild-type *Streptococcus mutans* 109c (Sm-wt) and *Streptococcus gordonii* Challis (Sg-wt) and their LP-deficient mutants (Sm-dLP and Sg-dLP). The cell suspensions were incubated with anti-mouse CD16/CD32 antibody to block the Fc receptor and then incubated with an allophycocyanin-conjugated monoclonal antibody (mAb) against CD4 and a phycoerythrin-conjugated mAb against CD25. The cells were fixed and permeabilized using Fix/Perm Buffer. After washing, the cells were incubated with an Alexa Fluor 488-conjugated anti-Foxp3 mAb and an appropriate isotype control. Data for 50,000 cells falling within appropriate forward and side light scatter gates were collected from each sample with a FACSCalibur. Data were analysed using FlowJo software. The cells falling within the CD4^{+} gate were analysed with the expression of Foxp3 and/or CD25. See text for details. Statistically significant difference from control was assessed by 2 × 2 chi-squared test; $^{**}P < 0.01$.

2.6-fold to 4.6-fold) in TLR2$^{+/+}$ splenocytes (Fig. 2A,C) was higher than those (1.4-fold to 1.7-fold) in TLR2$^{-/-}$ splenocytes (Fig. 2B,C). In TLR2$^{+/+}$ splenocytes, Sg-wt and Sg-dLP increased the frequency of Treg cells more than did Sm-wt and Sm-dLP, but no difference as such in the magnitudes of increase were observed in TLR2$^{-/-}$ mice (Fig. 2A–C). Expectedly, however, the TLR2 ligand FSL-1 increased the frequency of nTreg cells in TLR2$^{+/+}$ mice but not in TLR2$^{-/-}$ mice (Fig. 2A–C). No big difference in the activity to increase the frequency of nTreg cells was observed between wild-type and LP-deficient strains of both species in TLR2$^{+/+}$ as well as TLR2$^{-/-}$ mice.

These results suggest that whole cells of these streptococci upregulate the growth of murine nTreg cells in a TLR2-dependent manner, although the frequencies of nTreg cells in TLR2$^{-/-}$ splenocytes were slightly upregulated.

Enhancement of the proliferation of nTreg cells by heat-killed cells of Sg-wt and Sg-dLP

We have previously shown that the TLR2 ligand FSL-1, but not the TLR9 ligand CpG-DNA, enhanced the growth of nTreg cells isolated from murine spleens (Kiura *et al.*, 2011). Therefore, an experiment was

carried out to investigate whether heat-killed cells of Sg-wt and Sg-dLP enhanced the proliferation of nTreg cells isolated from splenocytes of TLR2$^{+/+}$ or TLR2$^{-/-}$ mice and the proliferation of CD4^{+} CD25^{-} T cells (Th cells) prepared during the isolation process of nTreg cells. Approximately 91% of the nTreg cells isolated were CD4^{+} CD25^{+} cells (data not shown). Sg-dLP as well as Sg-wt enhanced the proliferation of nTreg cells from both TLR2$^{+/+}$ and TLR2$^{-/-}$ mice, but no significant differences in the upregulation were observed between TLR2$^{+/+}$ and TLR2$^{-/-}$ nTreg cells and also between Sg-wt and Sg-dLP (Fig. 3A). The proliferation of Th cells was also enhanced by these bacterial cells in the same way as nTreg cells (Fig. 3B).

The IL-2 receptor α-chain CD25 is expressed in activated T cells as well as nTreg cells. The nTreg cells contained approximately 20% CD4^{+} CD25^{+} Foxp3^{-} cells, which are presumed to be activated T cells. This indicated the possibility that the enhancement of proliferation of nTreg cells by bacterial cells is a result of IL-2, a T-cell growth factor, produced by the activated T cells contaminated in nTreg cell preparation. To investigate this possibility, the proliferation of nTreg cells induced by bacterial cells was measured in the presence of anti-IL-2 antibody, which is known to block the IL-2 function (Fig. 3A). It was found that the proliferation of nTreg cells was not affected by the addition of anti-IL-2 antibody (Fig. 3A), suggesting that IL-2 produced by activated T cells did not contribute to nTreg cell proliferation. However, the proliferation of Th cells induced by bacterial cells was downregulated in the presence of anti-IL-2 antibody (Fig. 3B), suggesting that IL-2 secreted in response to bacterial stimulation is partially involved in the enhancement of Th cell proliferation.

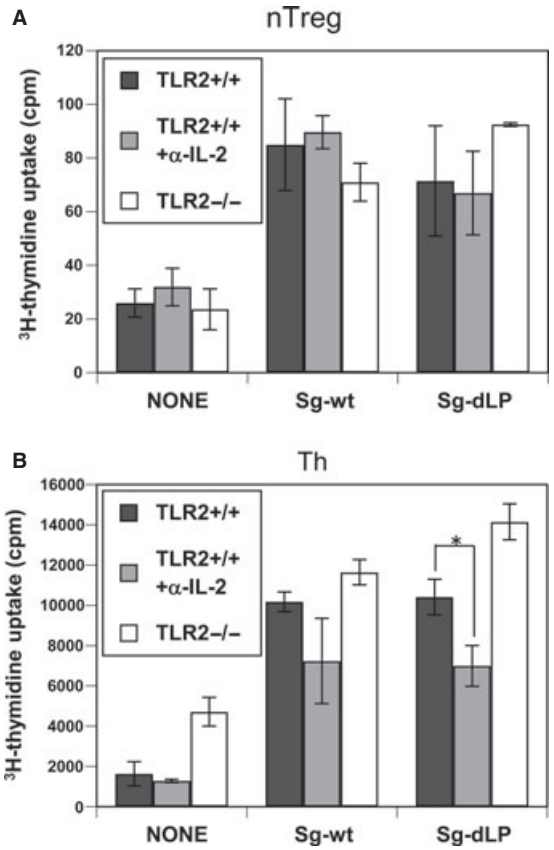

Figure 3 Proliferation of natural regulatory T (nTreg) cells or T helper (Th) cells in response to *Streptococcus gordonii*. The nTreg cells (1×10^5 cells) (A) isolated from splenocytes from TLR2$^{+/+}$ or TLR2$^{-/-}$ mice were incubated for 48 h with heat-killed bacterial cells at a multiplicity of infection (MOI) of 120 of wild-type *S. gordonii* Challis (Sg-wt) and its LP-deficient mutant (Sg-dLP). Th cells (1×10^6 cells) (B) isolated from splenocytes from TLR2$^{+/+}$ or TLR2$^{-/-}$ mice were incubated for 48 h with heat-killed bacterial cells at an MOI of 12 of Sg-wt or Sg-dLP. In some experiments, neutralizing α-interleukin-2 antibody (10 μg ml^{-1}) was added to the cultures. Cultures were pulsed with 0.2 μCi per well of [^{3}H]thymidine for the last 16 h of culture. The radioactivity of the cells deposited on filters was measured in a liquid scintillation counter. Results are expressed as mean ± SD of three separate experiments. Statistically significant difference was assessed by Student's *t*-test; $^*P < 0.05$.

Inhibition of the immunosuppressive activity of nTreg cells

We have previously demonstrated that the TLR2 ligand FSL-1 reduced the activity of nTreg cells to suppress the proliferation of Th cells triggered by anti-CD3ε (Kiura *et al.*, 2011). Therefore, an experiment was carried out to determine whether Sm-wt, Sm-dLP, Sg-wt and Sg-dLP affect the suppressive activity of nTreg cells. When nTreg cells, Th cells and APCs prepared from TLR2$^{+/+}$ mice were used, nTreg cells suppressed the proliferation of Th cells by approximately 50% in the absence of bacterial stimuli (Fig. 4A,A'). However, the suppressive level was significantly reduced in the presence of bacterial stimuli (Fig. 4A,A'). Reduction of the suppressive activity as such was also observed when these cell populations prepared from TLR2$^{-/-}$ mice were used (Fig. 4B,B'). However, these bacterial cells stimulated the growth of Th cells from TLR2$^{+/+}$ mice, but not from TLR2$^{-/-}$ mice, in the absence of Treg cells (Fig. 4A,B). This

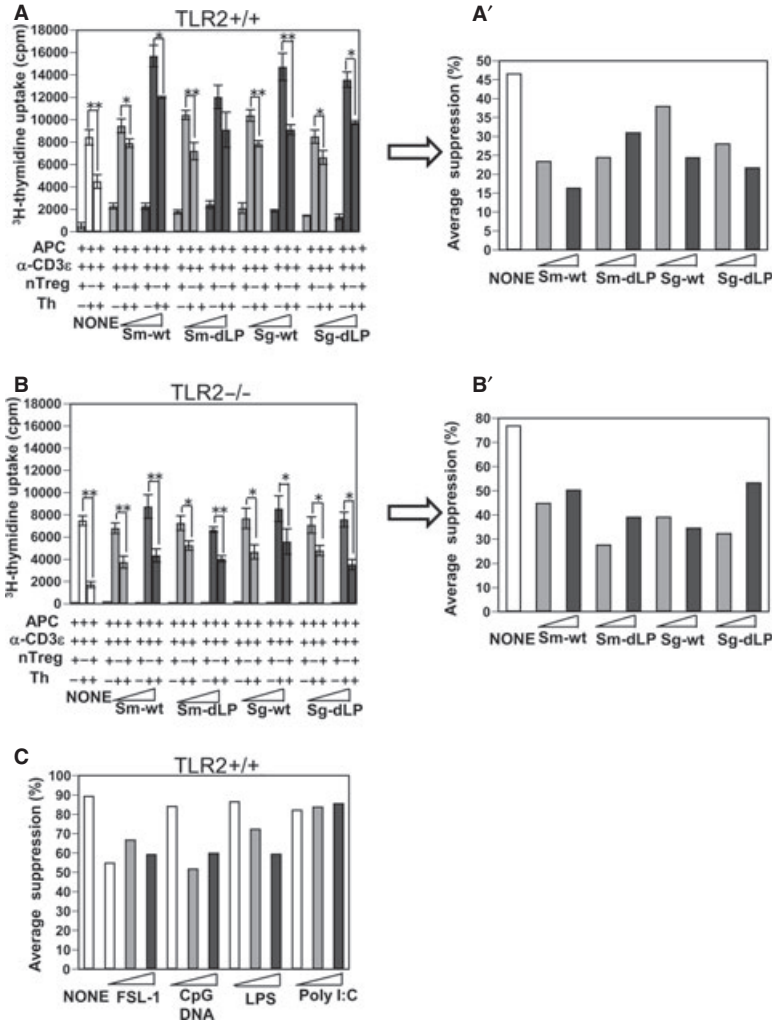

Figure 4 Effects of streptococcal cells or Toll-like receptor (TLR) ligands on the immunosuppressive activity of natural regulatory T (nTreg) cells. Helper T (Th) cells (2×10^4 cells/0.2 ml) were cultured for 48 h in a 96-well flat-bottomed plate in the presence of anti-CD3ε monoclonal antibody (1 μg ml^{-1}) and antigen-presenting cells (APCs; 2×10^4 cells) that had been treated with 50 μg ml^{-1} of mitomycin C in RPMI-1640 complete medium. The nTreg cells (2×10^4 cells) were also added to selected wells. The cells were stimulated with heat-killed cells (1×10^6, 1×10^7 colony-forming units) of wild-type *Streptococcus mutans* 109c (Sm-wt) and *Streptococcus gordonii* Challis (Sg-wt) and their LP-deficient mutants (Sm-dLP and Sg-dLP) (A, A', B, B') or various amounts (10, 100, 1000 ng ml^{-1}) of the TLR2 ligand FSL-1, the TLR3 ligand Poly I:C, the TLR4 ligand *Escherichia coli* lipopolysaccharide and the TLR9 ligand CpG (C). Cultures were pulsed with 0.2 μCi per well of [^{3}H]thymidine for the last 16 h of culture. The radioactivity of the cells deposited on filters was measured in a liquid scintillation counter. Results are expressed as mean ± SD of three separate experiments. Results are expressed as mean ± SD of three separate experiments. Statistically significant difference was assessed by Student's *t*-test; $*P < 0.05$, $**P < 0.01$. Average suppression (A', B' and C) was calculated using the following formula: (S − C) / S × 100 (%), where S or C is the average of triplicate samples of [^{3}H]thymidine uptake (counts per minute; cpm) obtained by culture of T h cells and APCs in the absence or presence of nTreg cells, respectively.

difference may be explained by TLR2 expression in Th cells or APCs.

Hence, bacterial cells inhibited the suppressive activity of nTreg cells. These results are contradictory to our expectation that the suppressive activity of nTreg cells should be enhanced because of the finding that these bacterial cells enhanced the growth of nTreg cells (Fig. 3A). Therefore, we investigated whether various TLR ligands also have the activity to inhibit the suppressive activity of nTreg cells. As a result, it was found that FSL-1 (TLR2/6 ligand), CpG-DNA (TLR9 ligand) and LPS (TLR4 ligand), but not Poly I:C (TLR3 ligand), inhibited the suppressive activity (Fig. 4C). These results suggest that TLR

signals lead to inhibition of the suppressive activity of Treg cells.

We next tried to clarify the mechanism by which oral streptococci inhibited the suppressive activity of nTreg cells. It has been reported that IL-6 inhibits the suppressive activity of nTreg cells (Pasare & Medzhitov, 2003). Interleukin-6 is a multi-faceted cytokine with important functions in the immune system and is produced by a variety of cells including macrophages, dendritic cells, endothelial cells, B cells and T-cell subsets. To investigate the role of IL-6 induced by these bacterial stimuli in the suppressive activity of nTreg cells, anti-IL-6 antibody was added to the assay medium. The inhibition of suppressive activity of nTreg cells by Sg-wt, Sg-dLP and FSL-1 recovered by the addition of anti-IL-6 antibody (Fig. 5A,A'), suggesting that IL-6 is produced in the assay medium and plays some role in the inhibition of suppressive activity of nTreg cells by bacterial cells.

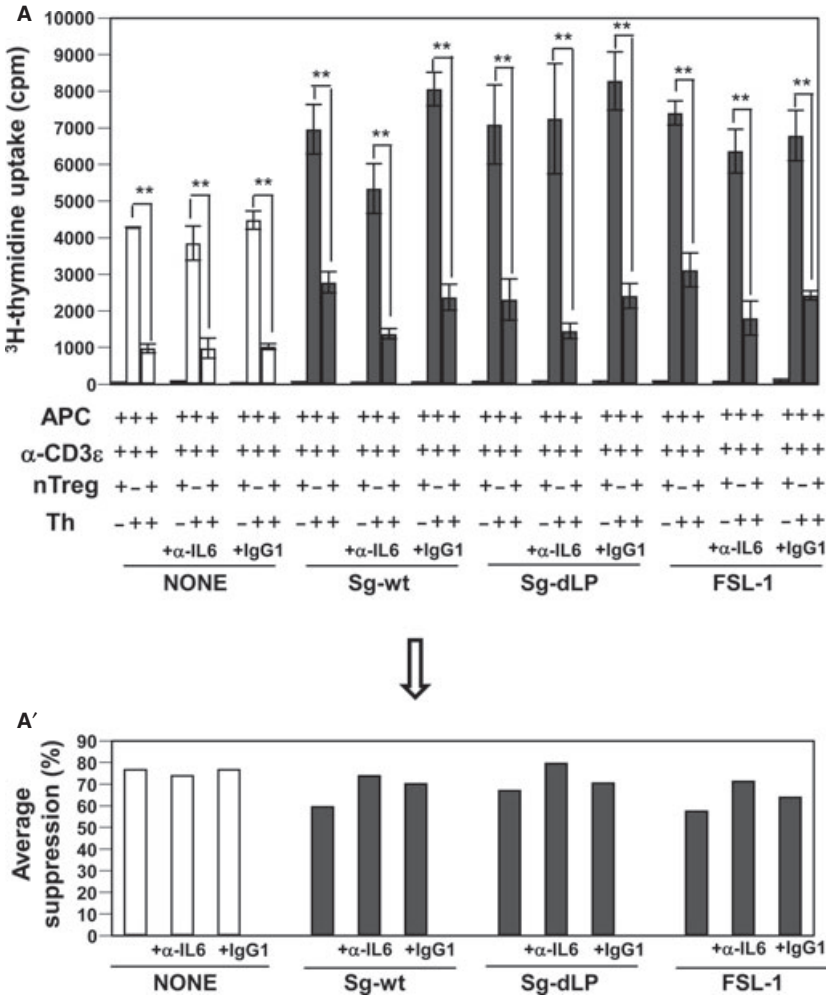

Figure 5 Inhibition of the suppressive activity of natural regulatory T (nTreg) cells by interleukin-6 (IL-6). T helper (Th) cells (2×10^4 cells per 0.2 ml) were cultured for 48 h in a 96-well flat-bottomed plate in the presence of anti-CD3ε monoclonal antibody (1 μg ml^{-1}) and antigen-presenting cells (2×10^4 cells) that had been treated with 50 μg ml^{-1} of mitomycin C in RPMI-1640 complete medium. The nTreg cells (2×10^4 cells) were also added to selected wells. The cells were stimulated with heat-killed cells (1×10^7 cells) of wild-type *Streptococcus gordonii* Challis (Sg-wt) and its LP-deficient mutant (Sg-dLP) or FSL-1 (10 nM) in the absence or presence of anti-IL-6 antibody (a blocking antibody, α-IL-6) (10 μg ml^{-1}) or IgG1 isotype control. Results (A) are expressed as mean ± SD of three separate experiments. Statistically significant difference was assessed by Student's *t*-test; $^{**}P < 0.01$. Average suppression (A') was calculated using the following formula: (S − C) / S × 100 (%), where S or C is the average of triplicate samples of [^{3}H]thymidine uptake (counts per minute; cpm) obtained by culture of Th cells and antigen-presenting cells in the absence or presence of Treg cells, respectively.

Involvement of NF-κB in inhibition of the suppressive activity of nTreg cells

NF-κB is a transcription factor that regulates the production of various inflammatory cytokines (Vallabhapurapu & Karin, 2009). As most TLR-mediated signals result in activation of NF-κB (Takeda *et al.*, 2003), we further investigated to what extent the NF-κB-mediated signal is involved in inhibition of the suppressive activity of nTreg cells by Sg-wt. The nTreg cells, Th cells or APCs were treated with the NF-κB inhibitor BAY11-7082 and examined for suppressive activity. nTreg cells pretreated with the inhibitor completely lost suppressive activity regardless of the presence of Sg-wt (Fig. 6A,A'). Pretreatment of APCs with the inhibitor reduced the proliferation of Th cells but enhanced the suppressive activity of nTreg cells (Fig. 6B,B'). In addition, Th cells treated with the inhibitor could not respond to anti-CD3ε stimulation, regardless of stimulation with Sg-wt (Fig. 6C,C').

In vivo effects of streptococci on the frequency of nTreg cells

Splenocytes prepared from the spleens of mice from an acute infection model, the size of which was more than twice that of control mice (Fig. 7A), were stained as described above to detect the frequency of nTreg cells. It was found that injection of live cells of Sg-wt but not Sg-dLP reduced the frequency of nTreg cells in $TLR2^{+/+}$ mice (Fig. 7B,C). In contrast, in the mice from the chronic infection model, with a subcutaneously buried chamber, the frequency of nTreg cells in the axillary nodes and spleen but not inguinal lymph nodes was increased (Fig. 8A,B). In addition, the size of spleens in the chronic infection model mice was similar to that in control mice (data not shown).

DISCUSSION

Considering that there is a large number of bacteria that inhabit the oral cavity and that we swallow 10^{11}–10^{13} CFU bacteria per day, we speculate that oral tolerance against oral bacteria is established in the same way as that against the foods that we eat every day. In addition, it has recently been reported that Treg cells play important roles in the establishment of oral tolerance (Weiner *et al.*, 2011). Treg cells consist of two populations, nTreg cells that originate in the thymus and inducible Treg cells induced in the periphery (Tang & Bluestone, 2008).

In this study, we tried to determine how oral streptococci interact with Treg cells as a first step to verify the establishment of oral tolerance against oral bacteria.

First, we investigated whether whole cells of wild-type and LP-deficient strains of *S. mutans* and *S. gordonii* are recognized by HEK293/TLR2 cells and found that wild-type strains of both species activated NF-κB, whereas LP-deficient strains did not (Fig. 1). It appeared that whole cells of these streptococci are recognized by TLR2. However, it is impossible for whole cells of these streptococci to have direct contact with the TLR2 molecule. Therefore, we consider three possibilities: (i) the whole cells used contain LP released from dead or destructive cells; (ii) LP are released from whole cells by heating; and (iii) whole cells are incorporated into HEK293 cells by endocytosis and LP are released in the endosome after degradation. HEK293 cell is not a phagocyte, we think it is more likely that whole cells of these streptococci that we used contain LP released from dead or destructive cells.

Then, we investigated whether whole cells of these streptococci affected the frequency of nTreg cells in mouse splenocytes. Stimulation with these bacterial cells resulted in increases in the frequency of nTreg cells in both $TLR2^{+/+}$ and $TLR2^{-/-}$ splenocytes, and the magnitudes of increase in $TLR2^{+/+}$ splenocytes were higher than those in $TLR2^{-/-}$ splenocytes (Fig. 2). No difference in the activity to increase the frequency of nTreg cells was observed between wild-type and LP-deficient strains of both species in $TLR2^{+/+}$ as well as $TLR2^{-/-}$ splenocytes (Fig. 2). Hence, whole cells of these streptococci upregulate the growth of nTreg cells in a TLR2-dependent manner. Streptococci possess some cell wall components such as peptidoglycan and lipoteichoic acid other than LP that are recognized by TLR2. nTreg cells are known to express TLR2 (Sutmuller *et al.*, 2006). Therefore, these results indicate the possibility that upregulation of the proliferation of nTreg cells is mediated mainly through the recognition of peptidoglycan or lipoteichoic acid other than LP by TLR2. The finding that these bacterial cells slightly upregulated the frequency of nTreg cells in $TLR2^{-/-}$ splenocytes (Fig. 2) suggests that they possess some components that upregulate the growth of nTreg cells

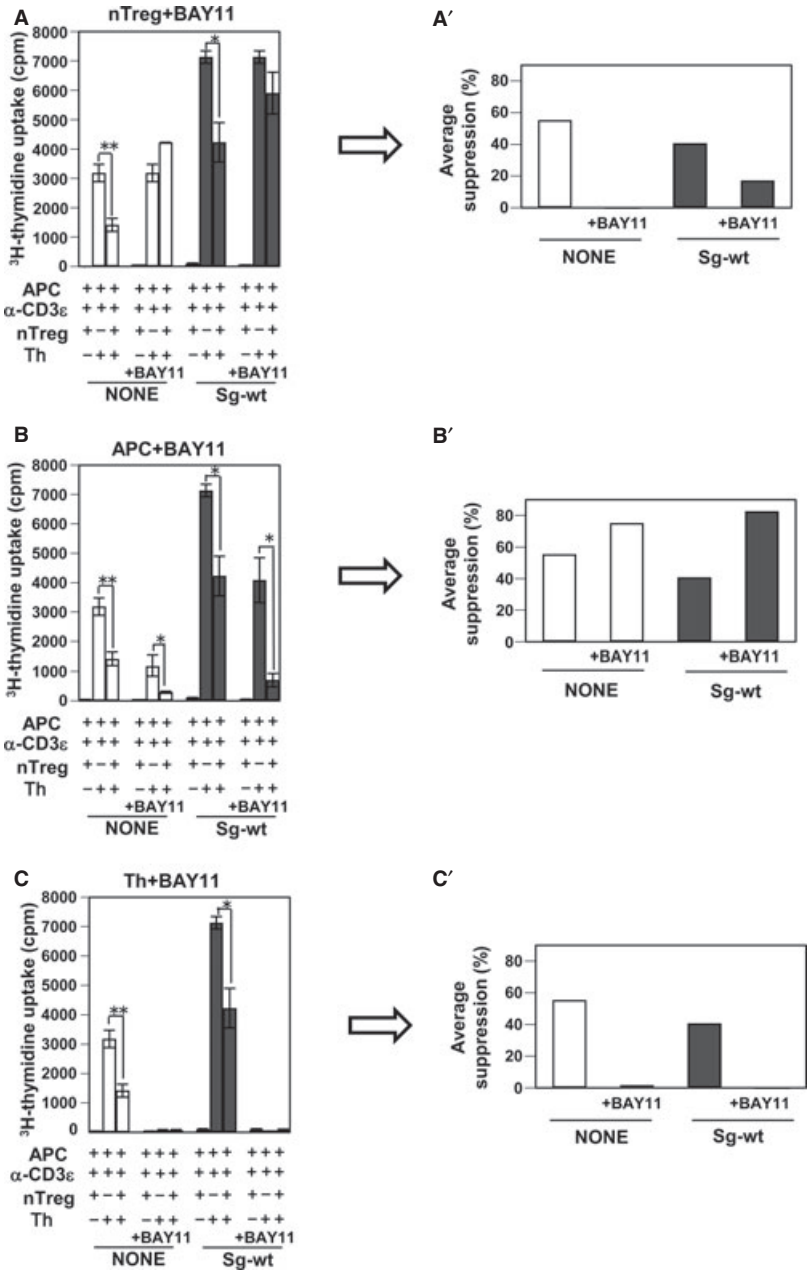

Figure 6 Effects of the nuclear factor-κB inhibitor BAY11-7082 on the suppressive activity of natural regulatory T (nTreg) cells. The nTreg cells (A), antigen-presenting cells (APCs; B) or T helper (Th) cells (C) were preincubated for 1 h with BAY11-7082 (10 μM) or dimethylsulfoxide (control), respectively, and used for Treg suppression assays after washing. Th cells (2×10^4 cells/0.2 ml) were cultured for 48 h in a 96-well flat-bottomed plate in the presence of anti-CD3ε monoclonal antibody (1 μg ml^{-1}) and APCs (2×10^4 cells) that had been treated with 50 μg ml^{-1} of mitomycin C in RPMI-1640 complete medium. nTreg cells (2×10^4 cells) were also added to selected wells. The cells were stimulated with heat-killed cells (1×10^7 cells) of wild-type *Streptococcus gordonii* (Sg-wt). Results are expressed as mean ± SD of three separate experiments. Statistically significant difference was assessed by Student's *t*-test; $*P < 0.05$, $**P < 0.01$. Average suppression (A', B', C') was calculated using the following formula: $(S - C)/S \times 100$ (%), where S or C is the average of triplicate samples of [^{3}H]thymidine uptake (counts per minute; cpm) obtained by culture of Th cells and APCs in the absence or presence of Treg cells, respectively.

through signals triggered by pattern recognition receptors other than TLR2. This is supported by our recent finding that IL-2, a T-cell growth factor, was not produced in the reaction mixtures of these bacterial cells and splenocytes even after 24 and 48 h of incubation (data not shown).

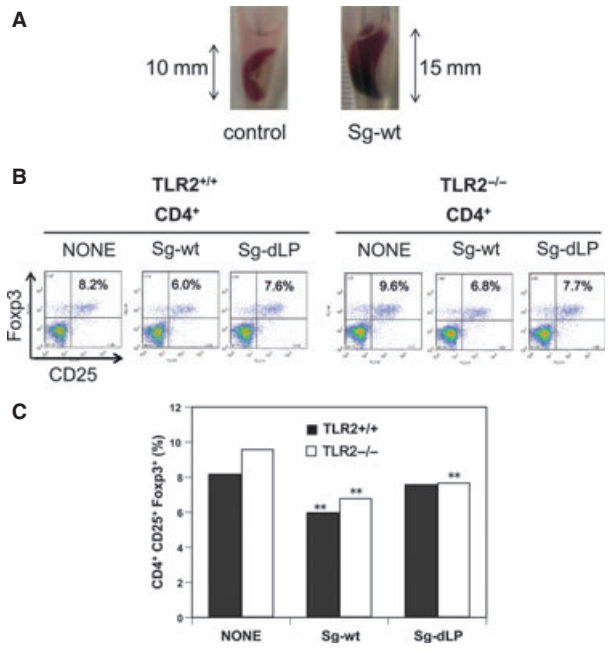

Figure 7 The frequency of regulatory T (Treg) cells in spleens of the acute infection model mice. Wild-type *Streptococcus gordonii* Challis (Sg-wt) and its LP-deficient mutants (Sg-dLP) cells (2.6×10^8 colony-forming units/mouse) were injected intravenously six times at 3-day intervals into three C57BL/6 female mice (8–10 weeks old) into their tails. Splenocytes were prepared from spleens (A), which were removed 3 days after the final injection and examined for the frequency of $CD4^+$ $Foxp3^+$ Treg cells in $CD4^+$ cells by being stained with anti-Foxp3 and anti-CD4 antibodies (B, C). The cells falling within the $CD4^+$ gate were analysed with the expression of Foxp3 and/or CD25. See text for details. Statistically significant difference from control was assessed by 2×2 chi-squared test; $^{**}P < 0.01$.

Although streptococcal cells stimulate the growth of nTreg cells in splenocytes in LP-independent and TLR2-dependent manners as described above, they stimulated the growth of nTreg cells isolated from splenocytes in LP-independent and TLR2-independent manners (Fig. 3A). Splenocytes include several types of cells such as monocytes and dendritic cells that express TLR2 on the cell surface. The nTreg cells that we isolated contained approximately 9% other types of cell. Judging from the isolation protocol (Miltenyi Biotec GmbH), it is considered that other types of cell do not contain APCs such as monocytes, dendritic cells and B cells and natural killer cells, which are well known to express TLR2. This may explain the finding that these streptococcal cells stimulated the growth of isolated nTreg cells in a TLR2-independent manner. However, it remains unknown what types of substances in these bacterial cells stimulated the proliferation of nTreg cells. In addition, anti-IL-2 blocking antibody had no effects on the proliferation of nTreg cells, suggesting that the proliferation was not mediated by IL-2. On the contrary, proliferation of Th cells prepared in the process of nTreg cell isolation was upregulated by bacterial cells and the upregulation was significantly inhibited by anti-IL-2 antibody (Fig. 3B). Judging from the fact that T cells are not activated by a free antigen, the Th cell fraction is considered to contain a small number of APCs such as dendritic cells or macrophages. The upregulation of proliferation of nTreg cells isolated was not inhibited by anti-IL-2 antibody (Fig. 3A), indicating that it is unlikely that cell populations contaminating nTreg cells produce IL-2 as a T-cell growth factor.

We expected that these streptococcal cells would stimulate the suppressive activity of nTreg cells because they upregulated the proliferation of nTreg cells as described above. However, they reduced the suppressive activity of nTreg cells from $TLR2^{+/+}$ and $TLR2^{-/-}$ mice (Fig. 4). Previous reports have shown temporary loss of suppressive function of Treg cells during acute infection (Liu *et al.*, 2006; Sutmuller *et al.*, 2006), which may explain our observations. That is, they have reported that in the early phase of infection, IL-2 and IL-6 are produced: IL-2 accelerates the growth of Treg cells, but IL-6 inhibits the suppressive activity of Treg cells. Therefore in the early phase of infection, the number of Treg cells increases, but the suppressive function is not enhanced. Once the pathogen is cleared by the immune system, TLR ligands are no longer present, and Treg cells will regain their suppressive capacity, so contributing to the balance between tolerance and immunity. Indeed, we also observed that the addition of anti-IL-6 antibody to the assay medium resulted in recovery of the suppressive activity of nTreg cells (Fig. 5). Therefore, an experiment was carried out to investigate to what extent the NF-κB-mediated signal is involved in inhibition of the suppressive activity of nTreg cells by these streptococcal cells. We found that nTreg cells pretreated with the NF-κB inhibitor BAY11-7082 completely lost suppressive activity regardless of the presence of Sg-wt (Fig. 6A,A'), suggesting that the NF-κB signal in nTreg cells is essential for expression of the suppressive activity. Pretreatment of APCs with the

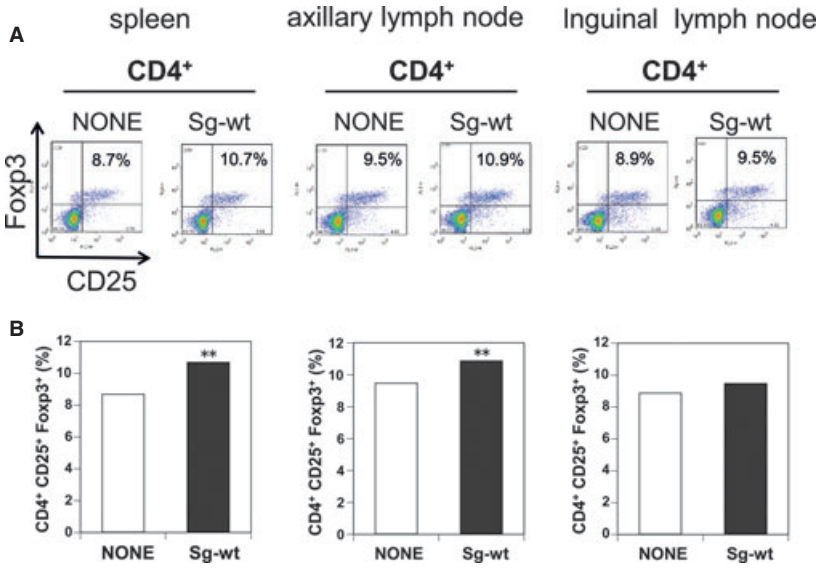

Figure 8 The frequency of regulatory T (Treg) cells in spleens of the chronic infection model mice. We used a modified subcutaneous chamber model described previously. The chamber, which was constructed from coils of stainless-steel wire, was implanted subcutaneously into the back of each of three C57BL/6 female mice (8–10 weeks old). After the healing period, the chamber was used as a biological compartment for inducing inflammation. Wild-type *Streptococcus gordonii* (Sg-wt) cells (1.5×10^8 colony-forming units/mouse) were injected into the chamber four times at 1-week intervals. Single cell suspensions were prepared from spleens, axillary lymph nodes and the inguinal lymph nodes taken out 7 days after the final injection and examined for the frequency of $CD4^+$ $Foxp3^+$ Treg cells in $CD4^+$ cells. The cells falling within the $CD4^+$ gate were analysed with the expression of Foxp3 and/or CD25. Statistically significant difference from control was assessed by 2×2 chi-squared test; $^{**}P < 0.01$.

inhibitor reduced the proliferation of Th cells but enhanced the suppressive activity of Treg cells (Fig. 6B,B'), suggesting that APCs produce something, possibly IL-6, that is regulated by NF-κB and involved in expression of the suppressive activity of nTreg cells toward proliferation of Th cells triggered by anti-CD3ε. The Th cells pretreated with the inhibitor completely lost suppressive activity regardless of the presence of Sg-wt (Fig. 6C,C'), suggesting that the proliferation signal triggered by anti-CD3ε is mediated by NF-κB. Indeed, it has been reported that T-cell receptor ligation leads to the activation of NF-κB (Kingeter *et al.*, 2010).

To confirm whether the frequency of nTreg increases in the early phase of infection, we also designed the acute and chronic infection models and examined the frequency of nTreg cells in spleen. Unexpectedly, in contrast to the report by Sutmuller *et al.* (2006), our data showed that the frequency decreased in the acute phase of infection (Fig. 7) and increased in the chronic infection model (Fig. 8). It remains unknown whether this discrepancy occurs. However, our data seem to be reasonable, because decrease in the frequency of nTreg cells means the acceleration of effector functions to protect the host from bacterial invasion.

Hence, this study suggests that oral streptococci may be able to modulate growth and functions of Treg cells through the TLR2-mediated signal, which plays important roles in oral tolerance (Weiner *et al.*, 2011), if they encounter Treg cells somewhere, for example in the gut.

Further studies are in progress in our laboratory to determine whether Treg cells specific for oral streptococcal antigens exist in gut-associated lymphoid tissue.

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REFERENCES

Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. *Nat Rev Immunol* **4**: 499–511.

Arimoto, T. and Igarashi, T. (2008) Role of prolipoprotein diacylglyceryl transferase (Lgt) and lipoprotein-specific signal peptidase II (LspA) in localization and physiological function of lipoprotein MsmE in *Streptococcus mutans*. *Oral Microbiol Immunol* **23**: 515–519.

Atarashi, K., Tanoue, T., Shima, T. *et al.* (2011) Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* **331**: 337–341.

Belkaid, Y. (2007) Regulatory T cells and infection: a dangerous necessity. *Nat Rev Immunol* **7**: 875–888.

Cheng, G., Yu, A. and Malek, T.R. (2011) T-cell tolerance and the multi-functional role of IL-2R signaling in T-regulatory cells. *Immunol Rev* **241**: 63–76.

Douglas, C.W., Heath, J., Hampton, K.K. *et al.* (1993) Identity of viridans streptococci isolated from cases of infective endocarditis. *J Med Microbiol* **39**: 179–182.

Faria, A.M. and Weiner, H.L. (2005) Oral tolerance. *Immunol Rev* **206**: 232–259.

Fujita, M., Into, T., Yasuda, M. *et al.* (2003) Involvement of leucine residues at positions 107, 112, and 115 in a leucine-rich repeat motif of human Toll-like receptor 2 in the recognition of diacylated lipoproteins and lipopeptides and *Staphylococcus aureus* peptidoglycans. *J Immunol* **171**: 3675–3683.

Gauduchon, V., Benito, Y., Celard, M. *et al.* (2001) Molecular diagnosis of recurrent *Streptococcus mutans* endocarditis by PCR amplification and sequencing. *Clin Microbiol Infect* **7**: 36–37.

Genco, C.A. and Arko, R.J. (1994) Animal chamber models for study of host–parasite interactions. *Methods Enzymol* **235**: 120–140.

Hauet-Broere, F., Unger, W.W., Garssen, J. *et al.* (2003) Functional $CD25^-$ and $CD25^+$ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. *Eur J Immunol* **33**: 2801–2810.

Ishimaru, N., Nitta, T., Arakaki, R. *et al.* (2010) In situ patrolling of regulatory T cells is essential for protecting autoimmune exocrinopathy. *PLoS ONE* **5**: e8588.

Jenkinson, H.F. and Lamont, R.J. (2005) Oral microbial communities in sickness and in health. *Trends Microbiol* **13**: 589–595.

Kingeter, L.M., Paul, S., Maynard, S.K. *et al.* (2010) Cutting edge: TCR ligation triggers digital activation of NF-kappaB. *J Immunol* **185**: 4520–4524.

Kiura, K., Hasebe, A., Saeki, A. *et al.* (2011) In vivo anti- and pro-tumour activities of the TLR2 ligand FSL-1. *Immunobiology* **216**: 891–900.

Liu, H., Komai-Koma, M., Xu, D. *et al.* (2006) Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci USA* **103**: 7048–7053.

Loesche, W.J. (1986) Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* **50**: 353–380.

Mills, K.H. (2004) Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* **4**: 841–855.

Oggioni, M.R., Medaglini, D., Maggi, T. *et al.* (1999) Engineering the gram-positive cell surface for construction of bacterial vaccine vectors. *Methods* **19**: 163–173.

Park, M.J., Min, S.Y., Park, K.S. *et al.* (2008) Indoleamine 2,3-dioxygenase-expressing dendritic cells are involved in the generation of CD4+ CD25+ regulatory T cells in Peyer's patches in an orally tolerized, collagen-induced arthritis mouse model. *Arthritis Res Ther* **10**: R11.

Pasare, C. and Medzhitov, R. (2003) Toll pathway-dependent blockade of CD4+ CD25+ T cell-mediated suppression by dendritic cells. *Science* **299**: 1033–1036.

Round, J.L. and Mazmanian, S.K. (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci USA* **107**: 12204–12209.

Shibata, K., Hasebe, A., Into, T. *et al.* (2000) The N-terminal lipopeptide of a 44-kDa membrane-bound lipoprotein of *Mycoplasma salivarium* is responsible for the expression of intercellular adhesion molecule-1 on the cell surface of normal human gingival fibroblasts. *J Immunol* **165**: 6538–6544.

Sutmuller, R.P., den Brok, M.H., Kramer, M. *et al.* (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest* **116**: 485–494.

Takeda, K., Kaisho, T. and Akira, S. (2003) Toll-like receptors. *Annu Rev Immunol* **21**: 335–376.

Takeuchi, O. and Akira, S. (2010) Pattern recognition receptors and inflammation. *Cell* **140**: 805–820.

Tang, Q. and Bluestone, J.A. (2008) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* **9**: 239–244.

Tsuji, N.M. and Kosaka, A. (2008) Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends Immunol* **29**: 532–540.

Tsuji, N.M., Mizumachi, K. and Kurisaki, J. (2001) Interleukin-10-secreting Peyer's patch cells are responsible for active suppression in low-dose oral tolerance. *Immunology* **103**: 458–464.

Vallabhapurapu, S. and Karin, M. (2009) Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* **27**: 693–733.

Weiner, H.L., da Cunha, A.P., Quintana, F. *et al.* (2011) Oral tolerance. *Immunol Rev* **241**: 241–259.