METHODS

Mice and rats. C57BL/6, BALB/c, IQI/Jic and CB.17 SCID mice and F344 rats kept under SPF or germ-free conditions were purchased from Sankyo laboratories, Japan SLC, or CLEA Japan. IQI germ-free mice were used unless otherwise indicated. Germ-free and gnotobiotic mice were bred and maintained in vinyl isolators within the gnotobiotic facility of Sankyo laboratories. Germ-free *Il10*^{Venus} mice were generated as previously described[4]. OT-I and OT-II T-cell receptor transgenic mice were purchased from Taconic Farms. All animal experiments were approved by the Animal Research Committee of RIKEN Yokohama Institute and the University of Tokyo.

Chloroform treatment of human stool and generation of gnotobiotic mice. Human stool from a healthy volunteer (Japanese, male, age 31 years) was obtained with informed consent. Human stool and mouse caecal contents were directly frozen at −80 °C, or suspended in 4 times volume (w/v) of phosphate-buffered saline (PBS) + 20% glycerol solution, snap-frozen in liquid nitrogen and stored at −80 °C until use. The frozen stocks were thawed, suspended in 10 times volume (w/v) of PBS, and passed through a 70 μm cell strainer to eliminate clumps and debris. Then suspensions were mixed with chloroform (final concentration 3%), and incubated in a shaking water bath for 60 min. After evaporation of chloroform by bubbling with N_2 gas for 30 min, the aliquots containing the chloroform-resistant fraction of intestinal bacteria were inoculated into germ-free mice by intra-gastric administration (250 μl; per mouse). To generate a series of gnotobiotic mice inoculated with diluted samples, caecal contents from exGF mice were treated with chloroform, diluted with PBS, and inoculated into germ-free IQI mice. The caecal suspensions diluted 2×10^4-fold correspond to 2.5×10^4 bacterial cells per mouse. Each group of exGF mice was individually caged in the gnotobiotic isolator for 3–4 weeks at Sankyo Lab service.

Isolation of intestinal lamina propria lymphocytes and flow cytometry. The colons were collected and opened longitudinally, washed with PBS to remove all luminal contents and shaken in Hanks' balanced salt solution (HBSS) containing 5 mM EDTA for 20 min at 37 °C. After removing epithelial cells, muscle layers and fat tissue using forceps, the lamina propria layers were cut into small pieces and incubated with RPMI1640 containing 4% fetal bovine serum, 0.5 mg ml^{-1} collagenase D, 0.5 mg ml^{-1} dispase and 40 μg ml^{-1} DNase I (all Roche Diagnostics) for 1 h at 37 °C in a shaking water bath. The digested tissues were washed with HBSS containing 5 mM EDTA, resuspended in 5 ml of 40% Percoll (GE Healthcare) and overlaid on 2.5 ml of 80% Percoll in a 15-ml Falcon tube. Percoll gradient separation was performed by centrifugation at 800*g* for 20 min at 25 °C. The lamina propria lymphocytes were collected from the interface of the Percoll gradient and suspended in ice-cold PBS. For analysis of T_{reg} cells, isolated lymphocytes were labelled with the LIVE/DEAD fixable dead cell stain kit (Invitrogen) to exclude dead cells from the analysis. The cells were washed with staining buffer containing PBS, 2% FBS, 2 mM EDTA and 0.09% NaN_3 and surface staining was performed with PECy7- or Pacific blue-labelled anti-CD4 antibody (RM4-5, BD Biosciences), PE-labelled anti-ICOS antibody (C938.4A, BioLegend), Alexa488-labelled anti-CD103 antibody (2E7, BioLegend), and PerCP/Cy5.5-labelled anti-integrin-β7 antibody (FIB27, BioLegend). Intracellular staining of FOXP3, CTLA4, Helios and Ki67 was performed using the Alexa647-labelled anti-FOXP3 antibody (FJK-16 s, eBioscience), PE-labelled anti-CTLA4 antibody (UC10-4F10-11, BD Biosciences), PE-labelled anti-Helios antibody (22F6, BioLegend), PECy7-labelled anti-Ki67 antibody (B56, BD Biosciences) and FOXP3 staining buffer set (eBioscience). For analysis of T_H1 and T_H17 cells, isolated lymphocytes were stimulated for 4 h with 50 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 μg ml^{-1} ionomycin (Sigma) in the presence of GolgiStop (BD Biosciences). After incubation for 4 h, cells were washed in PBS, labelled with the LIVE/DEAD fixable dead cell stain kit and surface CD4 was stained with PECy7-labelled anti-CD4 antibody. Cells were washed, fixed in Cytofix/Cytoperm, permeabilized with Perm/Wash buffer (BD Biosciences), and stained with the APC-labelled anti-IL-17 antibody (eBio17B7, eBioscience) and FITC-labelled anti-IFN-γ antibody (XMG1.2, BD Biosciences). The antibody-stained cells were analysed with LSR Fortessa or FACSAriaIII (BD Biosciences), and data were analysed using FlowJo software (Treestar).

Meta 16S rRNA gene sequencing. The caecal contents from exGF mice were suspended in 10 ml of Tris-EDTA containing 10 mM Tris-HCl and 1 mM EDTA (pH 8), and incubated with lysozyme (Sigma, 15 mg ml^{-1}) at 37 °C for 1 h with gentle mixing. A purified achromopeptidase (Wako) was added (final concentration 2,000 unit ml^{-1}) and further incubated at 37 °C for another 30 min. Then, sodium dodecyl sulphate (final concentration 1%) was added to the cell suspension and mixed well. Subsequently, proteinase K (Merck) was added (final concentration 1 mg ml^{-1}) to the suspension and the mixture was incubated at 55 °C for 1 h. High-molecular-mass DNA was isolated and purified by phenol/chloroform extraction, ethanol and finally polyethyleneglycol precipitation[25]. PCR was performed using Ex Taq (TAKARA) and (1) the 454 primer A (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG (454 adaptor sequence) + barcode (10 bases) + AGRGTTTGATYMTGGCTCAG-3′ (27Fmod)) and (2) the 454 primer B (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG (454 adaptor sequence) + TGCTGCCTCCCGTAGGAGT-3′ (338R)) to the V1–V2 region of the 16S rRNA gene. Amplicons generated from each sample (~330 bp) were subsequently purified using AMPur XP (Beckman Coulter). The amount of DNA was quantified using Quant-iT Picogreen dsDNA assay kit (Invitrogen) and TBS-380mini fluorometer (Turner Biosystems). Then, the amplified DNA was used as template for 454 GS Junior (Roche) pyrosequencing using GS Junior Titanium emPCR Kit-Lib-L, GS Junior Titanium Sequencing Kit and GS Junior Titanium PicoTiterPlate Kit (all Roche) according to the manufacturer's instructions. Quality-filter-passed reads were obtained by removing reads that did not have both primer sequences, had the average quality value (QV) <25, and were possibly chimaeric[26]. Of the filter-passed reads, 3,000 reads trimming off both primer sequences for each sample were used and subjected to OTU analysis with the cutoff similarity of 96% identity. Representative sequences from each OTU were blasted to Ribosomal Database Project (RDP) of bacterial isolates, our genome database constructed from publically available genome sequences in NCBI and HMP databases, and 16S sequences of the 23 strains obtained in this study.

Isolation of bacterial strains. The frozen stocks of caecal contents from exGF mice were serially diluted with PBS and seeded onto non-selective agar plates (blood liver (BL) agar (Eiken Chemical) or Eggerth-Gagnon (EG) agar plates). EG agar plates contain the following components (quantities expressed per litre): Lab-Lemco Powder (2.8 g, Oxoid); proteose peptone no. 3 (10.0 g, Difco); yeast extract (5.0 g, Difco); Na_2HPO_4 (4.0 g); D(+)-glucose (1.5 g); soluble starch (0.5 g); L-cystine (0.2 g); L-cysteine-HCl-H_2O (0.5 g); Tween 80 (0.5 g); Bacto agar (16.0 g, Difco); and defibrinated horse blood (50 ml). After culture under aerobic conditions or strictly anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37 °C for 2 or 4 days, individual colonies were picked up and cultured for an additional 2 or 4 days at 37 °C in ABCM broth (Eiken Chemical) or EG agar plate. The isolated strains were collected into EG stock medium (10% glycerol) and stored at −80 °C. To identify the isolated strains, 16S rRNA gene sequences were determined. The 16S rRNA gene was amplified by colony-PCR using KOD FX (TOYOBO) and GeneAmp PCR System9700 (Applied Biosystems) using 16S rRNA gene-specific primer pairs: 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-ATTACCGCGGCKGCTG-3′) or 1513R (5′-ACGGCTACCTTGTTACGACTT-3′). The amplification program consisted of one cycle at 98 °C for 2 min, followed by 40 cycles at 98 °C for 10 s, 57 °C for 30 s and 68 °C for 1 min 30 s. Each amplified DNA was purified from the reaction mixture using Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). Sequence analysis was performed using BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems) and Applied Biosystems 3730xl DNA analyser (Applied Biosystems). The resulting sequences were compared with sequences in RDP database and genome database using BLAST to determine close species/strains.

Bacterial culture of isolated strains. The isolated strains of Clostridia and Erysipelotrichi were cultured in EG broth without horse blood under a strictly anaerobic condition (80% N_2, 10% H_2, 10% CO_2) at 37 °C in an anaerobic chamber (Coy Laboratory Products). To prepare the bacterial mixture, bacterial strains were individually grown in EG broth to confluence and mixed at equal amounts of media volume.

Scanning electron microscopy. Scanning electron microscopy was performed by Filgen, Inc., Japan. The proximal colon was removed from +23-mix mice, cut open longitudinally, prefixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C, and then postfixed with 2% osmium tetroxide for 1 h at 4 °C. Fixed samples were dehydrated for 5 min each in sequential baths of 50%, 70%, 90% and 100% ethanol, inserted into a critical point dryer until dry and coated with osmium in an OPC-80N osmium plasma coater (Filgen). Scanning electron micrographs were taken by a JEOL JSM-6320F instrument.

Measurement of organic acids. Organic acid concentrations in caecal contents were determined by gas chromatography-mass spectrometry (GC-MS). Caecal contents (10 mg) were disrupted using 3-mm zirconia/silica beads (BioSpec Products) and homogenized in extraction solution containing 100 μl of internal standard (100 μM crotonic acid), 50 μl of HCl and 200 μl of ether. After vigorous shaking using a Shakemaster neo (Bio Medical Science) at 1,500 r.p.m. for 10 min, homogenates were centrifuged at 1,000*g* for 10 min and then the top ether layer was collected and transferred into new glass vials. Aliquots (80 μl) of the ether extracts were mixed with 16 μl of *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). The vials were sealed tightly by screwing and heated at 80 °C for 20 min in a water bath, and left at room temperature for 48 h for derivatization. The derivatized samples were run through a 6890N Network GC System (Agilent Technologies) equipped with HP-5MS column (0.25 mm × 30 m × 0.25 μm) and 5973 Network Mass Selective Detector (Agilent Technologies). Pure helium (99.9999%) was used as a carrier gas and delivered at a flow rate of 1.2 ml min^{-1}.

The head pressure was set at 10 p.s.i. with split 10:1. The inlet and transfer line temperatures were 250 °C and 260 °C, respectively. The following temperature program was used: 60 °C (3 min), 60–120 °C (5 °C min^{-1}), 120–300 °C (20 °C min^{-1}). One microlitre quantity of each sample was injected with a run time of 30 min. Organic acid concentrations were quantified by comparing their peak areas with the standards.

Genome sequencing and gene prediction. The genome sequences of 17 T_{reg}-cell-inducing strains were determined by the whole-genome shotgun strategy using a 454GS FLX Ti or Ion PGM sequencer. Each 1–5 μg of the genomic DNA was sheared to obtain DNA fragments. Template DNA was prepared according to the supplier's protocol. The generated sequence data were assembled using Newbler v2.8 software to obtain the draft genome sequences. All genome sequence data were deposited in DDBJ BioProject ID: PRJDB521-543. Protein-encoding genes were predicted using MetaGeneAnnotator software[27]. Putative toxins and virulence factors were searched using the BLASTP program and virulence factor databases, VFDB (http://www.mgc.ac.cn/VFs/main.htm) and MvirDB (http://mvirdb.llnl.gov), with the *e*-value cutoff of 1.0×10^{-10}, the identity >30% and the length coverage >60%.

Phylogenetic tree. Sequences concatenated with genes encoding 26 ribosomal proteins (large subunit L10, L11, L14, L16, L17, L19, L20, L23, L24, L29, L31, L32, L35, L7/L12, and small subunit S10, S12, S13, S15, S16, S17, S20, S20, S21, S3, S4, S7, S8) predicted from the genomes of each strain were used to construct a phylogenetic tree. The sequences of other bacterial species used for the tree construction were obtained from the ribosomal multi-locus sequencing typing (MLST) database[28]. The calculation was performed using the MEGA v5.0 package and the neighbour-joining method with a bootstrap of 1,000 replicates.

Cognate antigen-driven T_{reg} cell suppression assay. Preparation of antigens in caecal contents was performed as previously reported[15]. Caecal contents from germ-free mice or +17-mix mice were collected and suspended in PBS (500 mg ml^{-1}); they were then filtered through a 70-μm mesh, and autoclaved at 121 °C for 15 min. To prepare antigens of bacterial components, the 17 strains of Clostridia were cultured *in vitro*, mixed, washed and suspended with 1 ml PBS, and autoclaved at 121 °C for 20 min. $CD11c^+$ cells were isolated by FACSAriaIII from spleens of SPF C57BL/6 mice and pulsed for 1 h with 0.5 μM SIINFEKL OT-I peptide alone or in combination with either of 5 μM ISQAVHAAHAEINEAGR OT-II peptide, autoclaved caecal contents from +17-mix mice or germ-free mice (diluted 1:200), or autoclaved 17 strains of bacteria cultured *in vitro* (diluted 1:200). The antigen-pulsed $CD11c^+$ cells were plated at 5×10^4 per well in 96-well round-bottomed plates. CD8 T cells (T_{eff} cells) were sorted from spleens of SPF OT-I mice by FACSAriaIII and added to the $CD11c^+$ cell-seeded plates at 5×10^4 per well. Then, $CD4^+CD25^+$ T cells (T_{reg} cells) sorted from colonic lamina propria of +17-mix mice or from spleens of SPF OT-II mice were added to the culture at the indicated ratio of T_{reg} to T_{eff} cells. After 3 days, all cells were harvested, stained with anti-CD4 and anti-CD8 antibodies, and analysed by FACSAriaIII to enumerate the number of CD8 OT-I T cells.

Intestinal epithelial cell stimulation with caecal extracts and SCFAs. To prepare caecal extracts, frozen caecal contents from germ-free, +17-mix or SPF mice were thawed and well suspended in 4 volumes of sterile water. After centrifugation (5,000 r.p.m. for 15 min), transparent supernatants were collected, filtered through 0.22 μm filter and used as caecal extracts. In some experiments, caecal extracts were treated with proteinase K (2 mg ml^{-1}, 55 °C for 1 h; Roche) or nuclease that degrades all forms of DNA and RNA (125 unit ml^{-1}, 37 °C for 4 h; Thermo), and subsequently heated at 95 °C for 5 min to inactivate the enzymes. Human intestinal epithelial cell lines (HCT8, HT29, Caco2, T84 and Colo205) and a mouse epithelial cell line (CMT93) were obtained from ATCC and maintained at 37 °C (5% CO2) in RPMI containing 10% heat-inactivated horse serum (Invitrogen). Cells were cultured at 1.5×10^5 cells in 150 μl medium in 48-well plates and stimulated with 4.5 μl caecal extract for 24 h. Human primary intestinal epithelial cells were obtained from Lonza and maintained at 33 °C (5% CO2) in SmGM-2 medium containing 10% FBS (Lonza) for 1–2 weeks (6×10^4 cells in 48-well plates). The medium was changed to 150 μl SmGM-2 containing 1% FBS before stimulation. Caecal extracts (4.5 μl) were added to the culture and incubated for 24 h. Culture supernatants were collected and the level of the active form of TGF-β1 (Promega), TNF-α (R&D) and IL-6 (R&D) was measured by ELISA. To stimulate epithelial cell lines with SCFAs, sodium salts of acetate, butyrate, propionate and isobutyrate were dissolved in PBS. SCFAs were added to the culture individually (final 0.5 mM) or in combination (final 0.5 mM each), and incubated for 24 h.

TNBS colitis. C57BL/6 SPF adult mice were orally inoculated with 17-mix or control PBS every 2 or 3 days for 3 weeks. 2,4,6-Trinitrobenzene sulphonic acid (TNBS)-induced colitis was induced by the intracolonic administration of 2.5 mg of TNBS (Sigma) in 50% ethanol into anaesthetized mice via a thin round-tip needle. The tip of the needle was inserted 4 cm proximal to the anal verge, and mice were held in a vertical position for 30 s after the injection. All the mice were observed daily and were killed on day 4 after TNBS administration. Colons were fixed with 4% paraformaldehyde, sectioned, and stained with haematoxylin and eosin. The degree of inflammation in the distal part of colon was graded from 0 to 4 as follows: 0, normal; 1, ulcer with cell infiltration limited to the mucosa; 2, ulcer with limited cell infiltration in the submucosa; 3, focal ulcer involving all layers of the colon; 4, multiple lesions involving all layers of the colon, or necrotizing ulcer larger than 1 mm in length.

Allergic diarrhoea. BALB/c SPF adult mice were primed by subcutaneous injection with 1 mg of OVA (Fraction V; Sigma) in 100 μl of Complete Freund Adjuvant (CFA, DIFCO). One week after priming, mice were given 50 mg of OVA dissolved in 200 μl of PBS by intra-gastric administration three times per week. 17-mix or control PBS was orally administered to mice every 2 or 3 days for the entire period of the experiments. Diarrhoea was monitored visually 1 h after each oral OVA challenge. Diarrhoea was scored as follows: 0, normal faeces (solid); 1, moist faeces (semi-solid); 2, mild diarrhoea (loose); and 3, severe diarrhoea (watery). Serum was collected from the cheek vein 1 h after the last OVA challenge and OVA-specific IgE levels were measured by ELISA (Chondrex).

Adoptive $CD4^+CD45RB^{hi}$ T-cell transfer model of colitis. Germ-free CB.17 SCID mice were orally inoculated with SPF faeces together with or without 17-mix of Clostridia. One week later, exGF SCID mice received 4×10^5 $CD4^+CD45RB^{hi}$ T cells by intraperitoneal injection. Naive $CD4^+CD45RB^{hi}$ T cells were isolated from spleens of SPF BALB/c mice by FACS sorting. All the mice were observed daily and were killed on day 14 after T-cell transfer.

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Overcoming regulatory T-cell suppression by a lyophilized preparation of *Streptococcus pyogenes*

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Cancer vaccines have yet to yield clinical benefit, despite the measurable induction of humoral and cellular immune responses. As immunosuppression by $CD4^+CD25^+$ regulatory T (Treg) cells has been linked to the failure of cancer immunotherapy, blocking suppression is therefore critical for successful clinical strategies. Here, we addressed whether a lyophilized preparation of *Streptococcus pyogenes* (OK-432), which stimulates Toll-like receptors, could overcome Treg-cell suppression of $CD4^+$ T-cell responses in vitro and in vivo. OK-432 significantly enhanced in vitro proliferation of $CD4^+$ effector T cells by blocking Treg-cell suppression and this blocking effect depended on IL-12 derived from antigen-presenting cells. Direct administration of OK-432 into tumor-associated exudate fluids resulted in a reduction of the frequency and suppressive function of $CD4^+CD25^+Foxp3^+$ Treg cells. Furthermore, when OK-432 was used as an adjuvant of vaccination with HER2 and NY-ESO-1 for esophageal cancer patients, NY-ESO-1–specific $CD4^+$ T-cell precursors were activated, and NY-ESO-1–specific $CD4^+$ T cells were detected within the effector/memory T-cell population. $CD4^+$ T-cell clones from these patients had high-affinity TCRs and recognized naturally processed NY-ESO-1 protein presented by dendritic cells. OK-432 therefore inhibits Treg-cell function and contributes to the activation of high-avidity tumor antigen-specific naive T-cell precursors.

Keywords: Cancer · Treg cells · Tumor immunology · Vaccination

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Introduction

Many tumor-associated antigens recognized by the immune system are normal self-constituents, and tumor immunity is considered to be in part an autoimmune response [1–3]. Therefore, mechanisms for maintaining immunological self-tolerance hamper effective anticancer immunity. $CD4^+CD25^+$ Treg cells are one of the major components in maintaining immunological self-tolerance in hosts by suppressing a wide range of immune responses [4–7]. Indeed, depletion of Treg-cell populations enhances spontaneous and vaccine-induced antitumor immune responses [6, 8, 9], and the stimulation of $CD4^+CD25^+$ Treg cells by immunization with self-antigens induces enhanced chemically induced primary tumor development and increased numbers of pulmonary metastasis following injection of transplantable tumor cells [10–12]. In human cancers, the presence of high numbers of $CD4^+CD25^+$ Treg cells or low ratio of $CD8^+$ T cells to $CD4^+CD25^+$ Treg cells in tumors is correlated with unfavorable prognosis [13, 14]. In addition, the depletion of $CD4^+CD25^+$ Treg cells in patients receiving a DC vaccine enhances the stimulation of tumor-specific T-cell responses, indicating a crucial role for Treg cells in the regulation of antitumor immune responses in humans [15].

NY-ESO-1, a germ cell protein, was found by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient [16, 17]. We have previously shown that NY-ESO-1–specific $CD4^+$ T cells are detectable in cancer patients with spontaneous NY-ESO-1 serum Ab responses [17, 18]. In addition, NY-ESO-1–specific $CD4^+$ T-cell precursors can expand and become detectable in healthy individuals after in vitro antigenic stimulation of peripheral $CD4^+$ T cells, but only following depletion of $CD4^+CD25^+$ T cells [19, 20]. These results suggested that NY-ESO-1–specific $CD4^+$ T-cell precursors are actually present at relatively high frequencies in healthy individuals, and that the activation/expansion of NY-ESO-1–specific naive $CD4^+$ T cells is suppressed by $CD4^+CD25^+$ Treg cells. In healthy donors and in cancer patients with NY-ESO-1–expressing tumors but without spontaneous anti-NY-ESO-1 Ab (seronegative), naturally arising NY-ESO-1–specific T-cell responses are susceptible to Treg-cell suppression and are exclusively detected from naive populations ($CD4^+CD25^-CD45RA^+$). In contrast, most NY-ESO-1–specific $CD4^+$ T cells in cancer patients with spontaneous anti-NY-ESO-1 Ab (seropositive) are derived from memory populations ($CD4^+CD25^-CD45RO^+$) and are detectable even in the presence of $CD4^+CD25^+$ Treg cells [20, 21]. After vaccination with HLA-DPB1*0401/0402-restricted NY-ESO-$1_{157–170}$ peptide in incomplete Freund's adjuvant, ovarian cancer patients develop NY-ESO-1–specific $CD4^+$ T cells with only low avidity to antigen and low sensitivity to Treg cells, even though they have an effector/memory phenotype ($CD4^+CD25^-CD45RO^+$) [21]. Still, high-avidity naive NY-ESO-1–specific T-cell precursors are present in the peripheral blood of vaccinated patients, but they are subjected to continuous $CD4^+CD25^+$ Treg-cell suppression throughout vaccination [21]. Thus, a strategy to overcome Treg-cell suppression on preexisting high-avidity naive T-cell precursors is an essential component for effective cancer vaccines.

Accumulating data shed light on recognition of pathogen-associated molecular patterns through TLRs to break the suppressive environment in tumors [22]. It has been reported that TLR stimulants, such as lipopolysaccharide or CpG, block the suppressive activity of $CD4^+CD25^+$ Treg cells partially by an IL-6–dependent mechanism [23]. TLR2 signaling was reported to stimulate the proliferation of $CD4^+CD25^+$ Treg cells and to induce temporal loss of suppressive activity of $CD4^+CD25^+$ Treg cells [24]. TLR2 signaling has also been shown to increase IL-2 secretion by effector T cells, thereby rendering them resistant to $CD4^+CD25^+$ Treg-cell–mediated suppression [25]. We and others have recently reported that vaccination of tumor antigens by TLR stimulating viral or bacterial vectors was able to not only inhibit the suppressive function of $CD4^+CD25^+$ Treg cells but also break tolerance or hyporesponsiveness of effector T cells to tumor antigens even in the presence of Treg cells [26–28].

OK-432 is a lyophilized preparation of *Streptococcus pyogenes* that binds TLR-2, TLR-4, and/or TLR-9 and activates APCs, making it attractive for potential use as an adjuvant of cancer vaccine [29–33]. OK-432–matured DCs effectively prime antigen-specific T cells in vitro [29, 34]. Importantly, OK-432 has already been used for many years as a direct anticancer agent, particularly in Japan, and has a well-established clinical safety profile. However, while it is considered that OK-432 may inhibit Treg-cell suppressive activity by stimulating several TLR signaling pathways, its influence on Treg cells has not yet been shown. In this study, we addressed whether OK-432 inhibits Treg-cell suppressive function and could be a promising adjuvant of cancer vaccines.

Results

OK-432 inhibits the suppressive activity of $CD4^+CD25^+$ Treg cells

To address whether OK-432 inhibited $CD4^+CD25^+$ Treg-cell suppression, we employed the standard in vitro suppression system. $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells (highest 3% of $CD4^+CD25^+$ cells) were isolated from PBMCs of healthy individuals. $CD4^+CD25^-$ T cells were cultured with irradiated autologous APCs (CD4-depleted PBMCs) and anti-CD3 Ab in the presence or absence of $CD4^+CD25^{high}$ Treg cells. $CD4^+CD25^-$ T-cell proliferation was analyzed as described in the *Materials and methods*. In accordance with previous reports [7], $CD4^+CD25^{high}$ Treg cells markedly suppressed the proliferation of $CD4^+CD25^-$ T cells (Fig. 1A and B). In sharp contrast, when OK-432 was added in the culture, suppressive activity of $CD4^+CD25^{high}$ T cells was significantly inhibited (Fig. 1A and B). In addition, OK-432 did not induce death of $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD^+ cells was not significantly increased in the presence of OK-432 (data not shown). Instead, $CD4^+CD25^{high}$ Treg cells exhibited marginal proliferation in the presence of

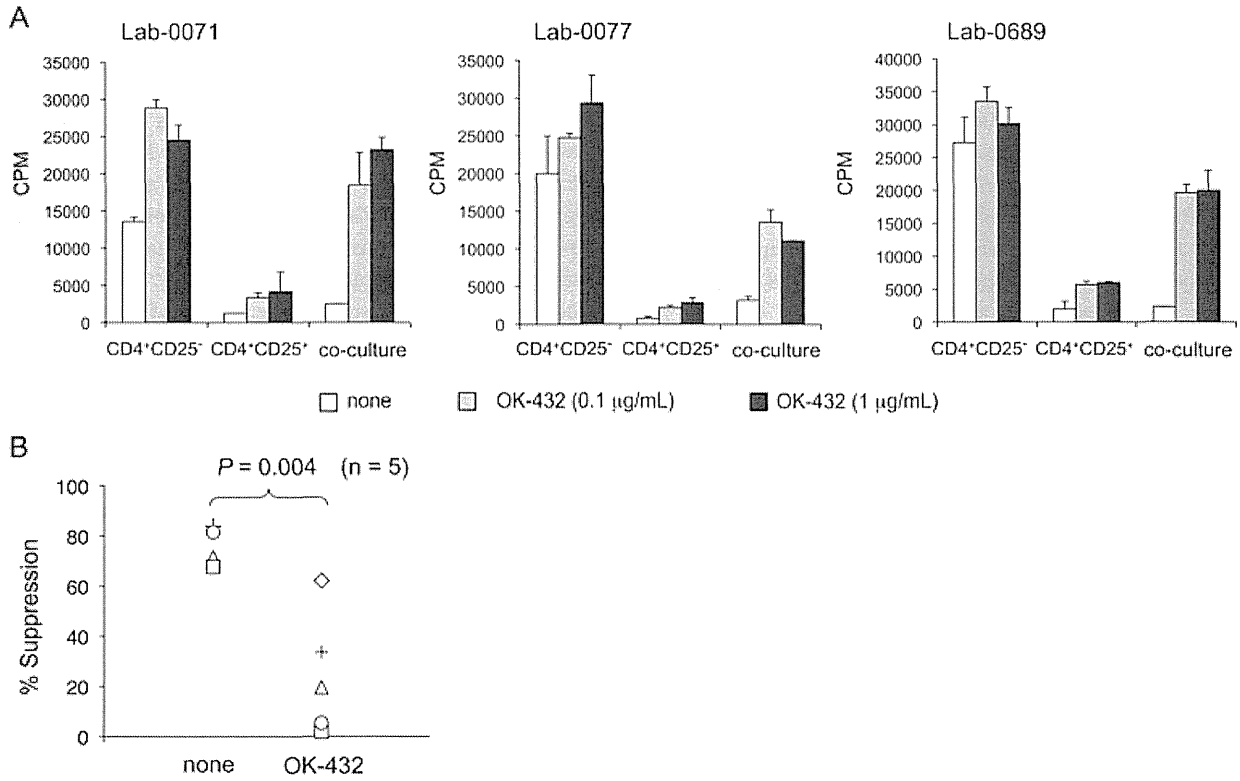

Figure 1. OK-432 overcomes Treg-cell suppression in vitro. (A, B) $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells were collected from PBMCs of healthy individuals as described in the *Materials and Methods*. 1×10^4 $CD4^+CD25^-$ T cells were cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 5×10^3 $CD4^+CD25^+$ Treg cells with/without OK-432. Proliferation was analyzed by ^{3}H-thymidine incorporation. Data of three donors are shown as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. (B) Summary of percent suppression in five healthy individuals. Percent suppression was calculated as: (1 – (cpm in coculture)/(cpm in $CD4^+CD25^-$ T cells)) × 100. Data shown are from one experiment representative of at least two performed.

OK-432 (Fig. 1A). These data indicate that addition of OK-432 impairs the suppressive activity of $CD4^+CD25^{high}$ Treg cells and partially reverses anergy status of Treg cells.

Inhibition of the suppressive activity of $CD4^+CD25^+$ Treg cells by OK-432 is dependent on IL-12

Since OK-432 reportedly induces TLR-2, TLR-4, and/or TLR-9 activation and subsequent production of proinflammatory cytokines [29–33], we examined the involvement of cytokines in this inhibition of Treg-cell suppression. To this end, Abs against several candidate cytokines were added to cultures. Among cytokines tested, only blocking Ab against IL-12 significantly abrogated the inhibition of Treg-cell suppression by OK-432 (Fig. 2A).

To confirm the importance of IL-12, we next analyzed whether the addition of IL-12 could inhibit Treg-cell suppression as observed by OK-432. $CD4^+CD25^-$ T cells were cultured with $CD4^+CD25^{high}$ Treg cells, irradiated autologous APCs and anti-CD3 Ab in the presence of IL-12. Treg-cell suppressive activity was significantly inhibited by the addition of IL-12, but not IL-6 or IFN-γ (Fig. 2B). Again, IL-12 did not kill $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD^+ cells was not significantly increased in the presence of IL-12 (data not shown). Instead, $CD4^+CD25^{high}$ Treg cells slightly proliferated in the presence of OK-432 (Fig. 2B). These data suggest a critical role for IL-12 in the inhibition of Treg-cell suppression by OK-432.

OK-432 induces higher amounts of IL-12 but not IL-10 from APCs compared with other stimuli

To gain insight into the cellular target(s) of OK-432, we explored the origin of IL-12 after OK-432 treatment based on the essential role of IL-12 in the inhibition of Treg-cell suppression by OK-432. We then analyzed whether OK-432 stimulation indeed induced IL-12 production from APCs, such as CD3-depleted PBMCs used in the standard Treg-cell suppression assays. CD3-depleted PBMCs from healthy donors were stimulated with OK-432, LPS, or TNF-α, and cytokine production was examined. OK-432 induced significantly higher amounts of IL-12 from CD3-depleted PBMCs than LPS or TNF-α (Fig. 3A). In addition, CD3-depleted PBMCs stimulated with OK-432 induced much less IL-10 production than LPS (Fig. 3A). Similar results, i.e. IL-12 rather than IL-10 was dominantly produced by CD3-depleted PBMCs stimulated with OK-432, were obtained from four esophageal cancer patients (Fig. 3B).

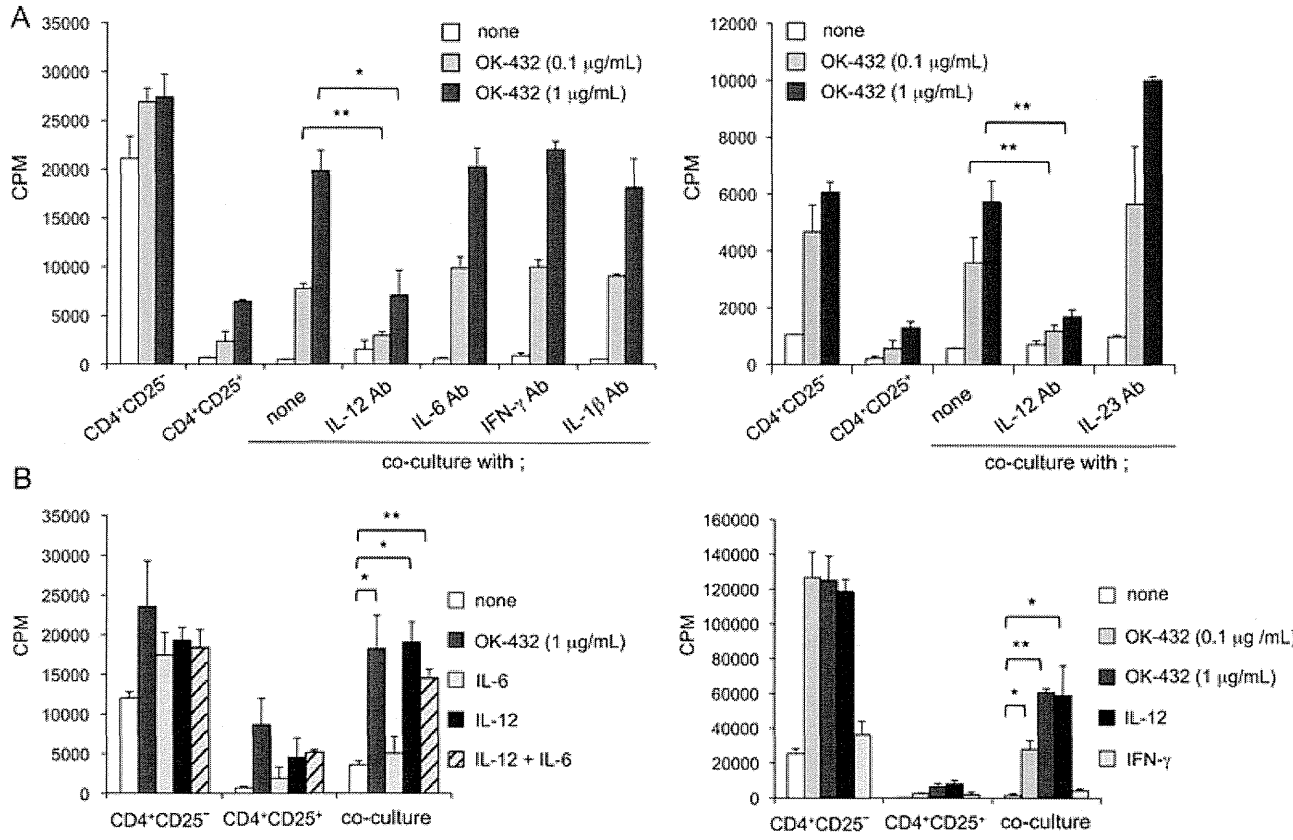

Figure 2. IL-12 is a critical cytokine to overcome Treg-cell suppression by OK-432. $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells were collected from PBMCs of healthy individuals. 1×10^4 $CD4^+CD25^-$ T cells were cultured with 1×10^5 irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 2.5×10^3 $CD4^+CD25^{high}$ Treg cells with/without OK-432. (A) Blocking Abs (10 μg/mL) against several cytokines as indicated were added in the culture and proliferation was measured. (B) Recombinant cytokines (IL-12; 5 ng/mL, IL-6; 5 ng/mL, IFN-γ; 100 U/mL) were added into the cultures and proliferation was analyzed. Data for one representative donor among three donors are expressed as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with control, Student's t-test.

We next examined which cell types in PBMCs produced IL-12 after OK-432 stimulation. The major sources of IL-12 in PBMCs after OK-432 stimulation were $CD11c^+$ and $CD14^+$ cells, and neither NK cells nor T cells produced IL-12 (Fig. 3C). Taken together, APCs, such as monocytes, macrophages, and DCs are considered to be the cellular targets of OK-432 to induce IL-12 which is a crucial component for the inhibition of Treg-cell suppression by OK-432.

OK-432 administration to tumor-associated exudates reduces local Treg-cell accumulation and function

As OK-432 is available as an anticancer agent in Japan and has been used for controlling tumor-associated exudate fluids by direct injection to the cavity, we next investigated its influence on Treg cells following in vivo treatment of OK-432. We analyzed the local Treg-cell accumulation and function of tumor-associated sites before and 2–3 days after local OK-432 administration. Cells were isolated from tumor-associated exudate fluids, such as pleural effusions and ascites. The frequency of Treg cells before and after treatment with OK-432 was examined by staining with Abs for CD4, CD25, and Foxp3. The $Foxp3^+$ T-cell population in $CD4^+$ T cells was markedly reduced (Fig. 4A). Furthermore, the proportion of $Foxp3^+$ T cells in $CD4^+CD25^+$ T cells was also significantly reduced after OK-432 administration (Fig. 4A and B), indicating that the balance of helper T cells to Treg cells had changed.

We next addressed the suppressive activity of $CD4^+CD25^{high}$ T cells in tumor-associated exudate fluids. $CD4^+CD25^{high}$ T cells (highest 3% gate of $CD4^+CD25^+$ cells defined with peripheral blood was applied) were isolated from tumor-associated exudate fluids and cultured with $CD4^+CD25^-$ T cells from PBMCs with irradiated autologous APCs and anti-CD3 Ab. After OK-432 administration, as the volume of tumor-associated exudate fluids decreased, sufficient amounts of $CD4^+CD25^{high}$ T cells for proliferation assays were available only from two patients. $CD4^+CD25^-$ T-cell proliferation was analyzed as described in the *Materials and Methods*. There was a trend, albeit not significant, toward a decrease in Treg-cell function after OK-432 administration (Fig. 4C). In contrast, we did not observe any differences in frequency and function of Treg cells in PBMCs

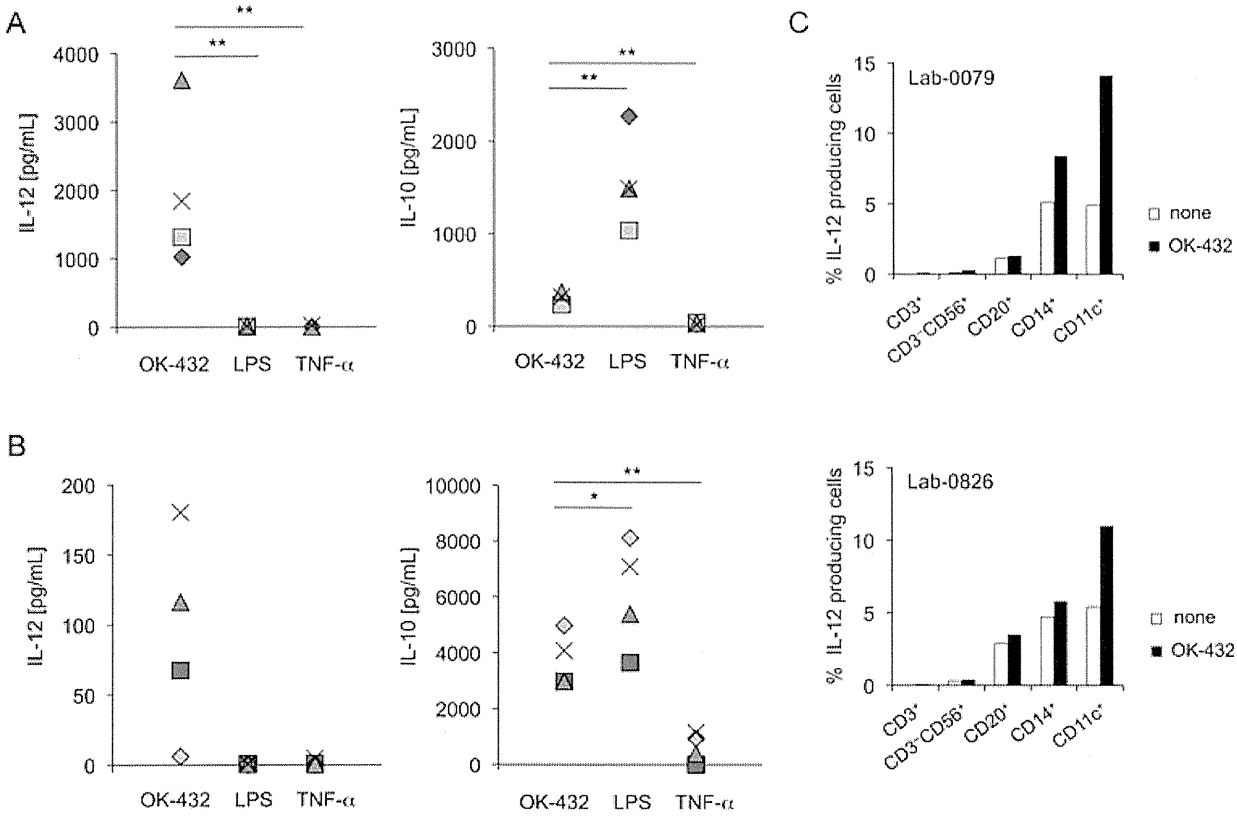

Figure 3. APCs stimulated with OK-432 produce significantly higher amounts of IL-12. (A) 1×10^5 CD3-depleted PBMCs from four healthy individuals were cultured with TNF-α (100 ng/mL), LPS (1 mg/mL), or OK-432 (1 μg/mL) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (B) 1×10^5 CD3-depleted PBMCs from four esophageal cancer patients were cultured as in (A) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (A, B) Each symbol represents an individual donor; data shown are from one experiment representative of at least two performed. (C) PBMCs from two healthy individuals were cultured with/without OK-432. Cells were subjected to staining with the indicated surface markers and then intracellular IL-12, and were analyzed by flow cytometry. Data of two donors from one experiment representative of at least two performed. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with control, Student's t-test.

before and after OK-432 administration (data not shown). These data propose that in vivo injection of OK-432 decreases the local Treg-cell accumulation and function.

Origin of the repertoire of CD4$^+$ T-cell effectors elicited by vaccination with NY-ESO-1 and OK-432

To further explore the effect of OK-432 on the inhibition of in vivo Treg-cell activity, we also examined the potential of OK-432 as an adjuvant in a cancer vaccine. We have reported that high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors are present in naive CD45RA$^+$ populations and that their activation is rigorously suppressed by CD4$^+$CD25$^+$ Treg cells [20, 21]. We also found that synthetic peptide vaccination with incomplete Freund's adjuvant induces only peptide-specific CD4$^+$ T cells with low-avidity TCRs (recognition of >1 μM peptide but not naturally processed NY-ESO-1 protein), but not high-avidity CD4$^+$ T cells (recognition of naturally processed NY-ESO-1 protein or <0.1 μM peptide) that are susceptible to Treg-cell suppression [21]. Together, these data highlight the importance of blocking Treg-cell activity to allow activation/expansion of high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors. For this reason, we investigated whether high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors were activated by NY-ESO-1 protein vaccination with OK-432 as an adjuvant and were present in memory CD45RO$^+$ populations.

Samples from two patients who received vaccination with cholesteryl hydrophobized pullulan (CHP)-HER2 and NY-ESO-1 with OK-432 (Supporting Information Fig. 1) were available for this analysis. Whole CD4$^+$ T cells or CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T cells before and after vaccination were presensitized with NY-ESO-1–overlapping peptides covering the entire sequence of NY-ESO-1 and specific CD4$^+$ T-cell induction was analyzed with ELISPOT assays. As the sample size was not sufficient to analyze specific CD4$^+$ T-cell induction within CD4$^+$CD25$^-$CD45RA$^+$ (naive) T cells, we analyzed whether NY-ESO-1–specific high-avidity CD4$^+$ T cells were induced from the CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T-cell population after vaccination in Pt #1 (HLA-DR 4, 12 and HLA -DQ 4, 8) and #2 (HLA-DR 9, 15 and HLA-DQ 6, 9). Pt #1 exhibited spontaneously induced CD4$^+$ T-cell responses against NY-ESO-1_{91-110} before vaccination and the responses were maintained after extensive vaccination (Fig. 5A). These spontaneously

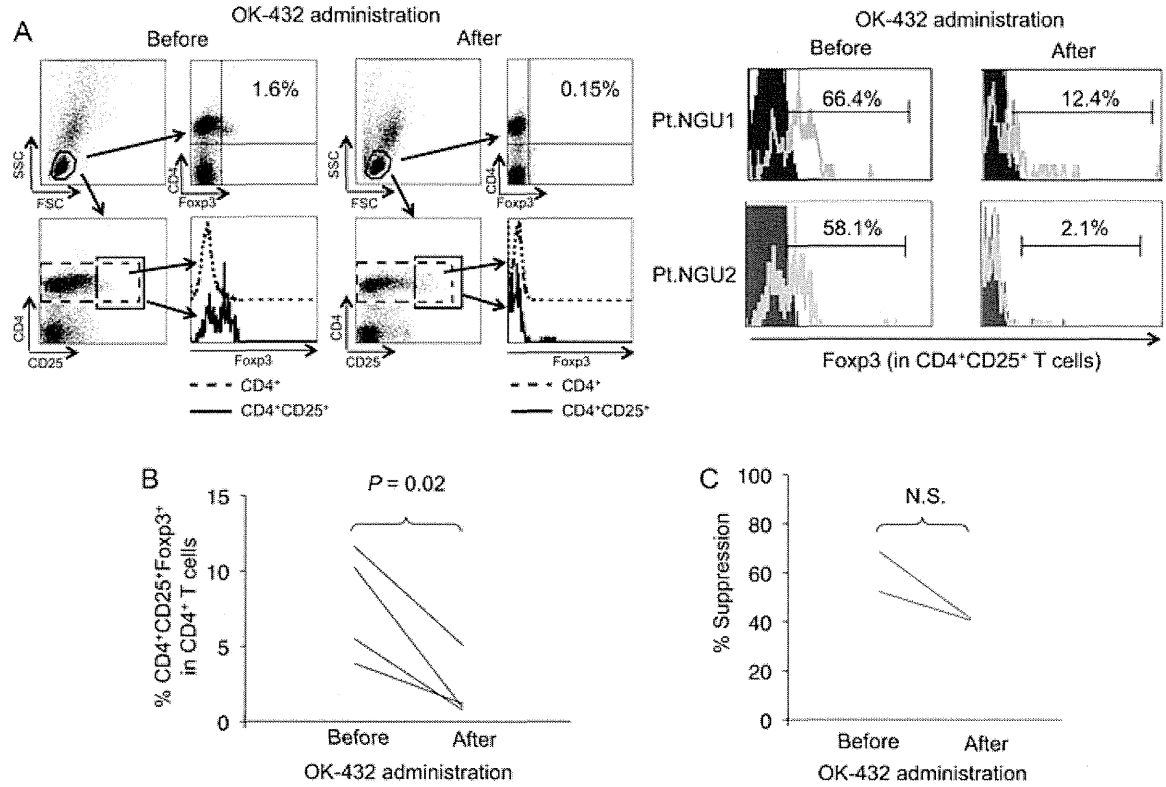

Figure 4. OK-432 administration into tumor-associated exudate fluids elicits reduction of local Treg-cell accumulation and function. Cells were isolated from tumor-associated exudate fluids (two pleural effusions or two ascites) and peripheral blood before and 2 days after OK-432 administration. (A) Cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 Abs and were analyzed with flow cytometry. The staining pattern and gating method of a representative patient is shown (left); solid line histogram, Foxp3 expression in $CD4^+$ $CD25^+$ T cells; dotted line histogram, Foxp3 expression in $CD4^+$ T cells. Foxp3 expression in $CD4^+CD25^+$ T cells of two representative patients is shown (right); filled histogram, control staining; gray line histogram, Foxp3. (B) The percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ T cells is shown for $n = 4$ patients' samples. Statistical significance determined by Student's *t*-test. (C) 1×10^5 $CD4^+CD25^-$ T cells were isolated from PBMCs before OK-432 administration and cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 1×10^5 $CD4^+CD25^{high}$ T cells isolated from tumor-associated exudate fluids before and after OK-432 administration ($n = 2$). Data shown are from one experiment representative of two independent experiments.

induced NY-ESO-1_{91-110}–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population before and after vaccination. Following vaccination with NY-ESO-1 protein in the presence of OK-432, $CD4^+$ T-cell immune responses against NY-ESO-$1_{111-130}$ were newly elicited (Fig. 5A). These vaccine-induced NY-ESO-$1_{111-130}$–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population only after vaccination (Fig. 5A). In Pt #2, while specific $CD4^+$ T cells were not observed before vaccination, NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were elicited after vaccination. The vaccine-induced NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were also detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population, as observed in Pt #1 (Fig. 5B).

NY-ESO-1 vaccination with OK-432 activates high-avidity preexisting NY-ESO-1–specific $CD4^+$ T-cells

We then asked whether vaccine-induced T cells had a high-affinity TCR that recognized naturally processed antigens [21, 28]. We established NY-ESO-1–specific $CD4^+$ T-cell clones. Four clones and a single clone that recognized different epitopes were generated from Pt #1 and Pt #2, respectively. Four minimal epitopes (NY-ESO-$1_{83-96,\ 94-109,\ 119-130,121-134}$) were defined from $CD4^+$ T-cell clones derived from Pt #1 (Fig. 6A and data not shown). Both spontaneously induced (#2–11) and vaccine-induced (#3–1) $CD4^+$ T-cell clones recognized naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6A). One minimal epitope defined from Pt #2 was NY-ESO-$1_{122-133}$ and the vaccine-induced $CD4^+$ T-cell clone (#1–1) again recognized both the naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6B), indicating that these T-cell clones had high-affinity TCRs against NY-ESO-1. Together, OK-432 as an adjuvant could overcome Treg-cell suppression and activate high-affinity preexisting NY-ESO-1–specific $CD4^+$ T-cell precursors.

Discussion

While a subset of patients treated with immunotherapy has been shown to experience objective and durable clinical responses, it is becoming increasingly clear that several mechanisms

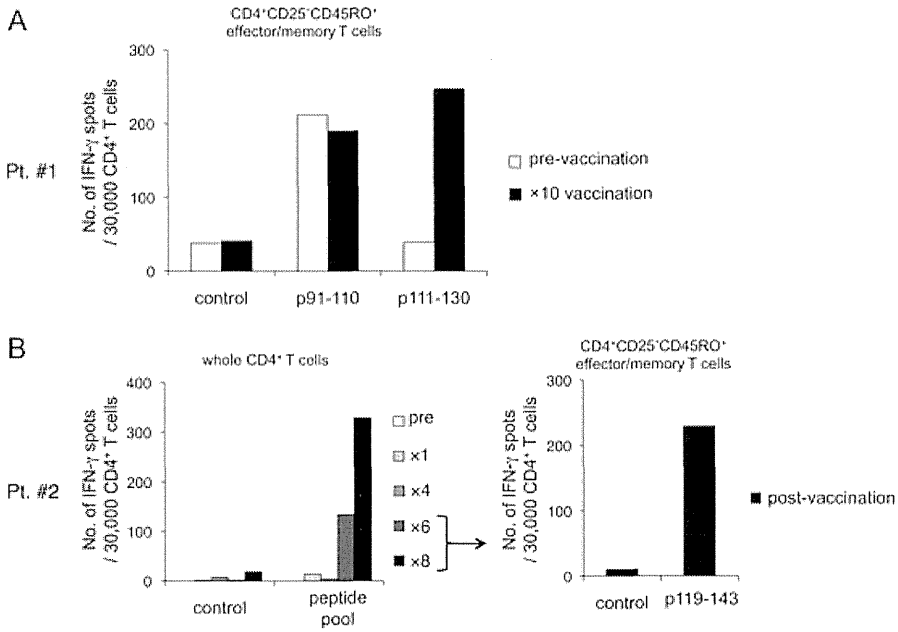

Figure 5. Vaccination with OK-432 elicits NY-ESO-1–specific effector/memory $CD4^+$ T cells $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were prepared from PBMCs of (A) Pt #1 (HLA-DR 4, 12 -DQ 4, 8) and (B) Pt #2 (HLA-DR 9, 15 -DQ 6, 9) before and after vaccination as described in the *Materials and Methods*; 3–5 × 10^5 $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were cultured with 3–5 × 10^5 CD4-depleted PBMCs pulsed with 10 μM pooled peptides covering the entire sequence of NY-ESO-1 for 3 weeks. Induction of NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data shown are from one experiment representative of at least two experiments performed.

downregulate antitumor immunity during the course of the immune response and play a major role in limiting the effectiveness of cancer immunity [6, 35, 36]. A plethora of cell types, cell surface molecules, and soluble factors mediate this suppressive activity [3, 6, 35, 36]. Among them, $CD4^+CD25^+Foxp3^+$ Treg cells play a crucial role by suppressing a wide variety of immune responses, and finding ways to control Treg-cell suppression is a major priority in this field [6, 7]. In this study, we showed the potential of OK-432 (a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*) which stimulates TLR signals [30, 33, 34] to control Treg-cell suppression, supporting the idea that OK-432 may be a promising adjuvant for cancer vaccines by inhibiting Treg-cell suppression and by augmenting induction of tumor-specific T cells against coadministered protein antigens.

Appropriate adjuvant combinations, such as those that are MyD88-dependent or MyD88-independent, or those that are TRIF-coupled and include endosomal signals, are known to synergistically activate DCs with regard to the production of inflammatory cytokines [37, 38]. As OK-432 is derived from bacterial components, its capacity to bind a combination of various TLRs makes it attractive. It has been shown that OK-432 exhibits antitumor effects through TLR-2, TLR-4, and TLR-9 using knockout mice for each TLR [30, 33, 34]. Alternatively, OK-432 reportedly stimulates DCs through the β_2-integrin system rather than via TLR signals [29]. In the presence of OK-432, Treg cells slightly proliferated with TCR stimulation. TLR2 triggering results in a temporary loss of the anergic status of Treg cells and is associated with loss of Treg-cell suppressive function [24, 25]. The perturbation of Treg-cell anergy by OK-432 through TLR2 stimulation may play a role, at least in part, in the inhibition of Treg-cell suppressive function.

In accordance with previous reports [29, 34], we showed that APCs, including $CD11c^+$ and $CD14^+$ cells (monocytes, macrophage, and DCs), stimulated with OK-432 exhibited significantly higher production of IL-12 as compared with that of LPS- or TNF-α–matured APCs, and that OK-432–induced IL-12 from these APCs was a critical component for abrogating Treg-cell activity. Additionally, we found that monocyte-derived DCs stimulated with OK-432 produced significantly higher amounts of IL-12 compared with DCs stimulated with LPS or TNF-α (Supporting Information Fig. 2). It has been reported that IL-12 receptor expressed on effector T cells, but not on Treg cells has a critical role for abrogating Treg-cell suppression by IL-12 in mice [39, 40]. In accordance with this, downregulation of IL-12 receptors by siRNA on effector cells partially abrogated the OK-432–induced inhibition of Treg-cell suppressive activity (Supporting Information Fig. 3). IL-12 receptor was induced in both effector T cells and Treg cells after activation (Supporting Information Fig. 3). We attempted to downregulate the IL-12 receptor on Treg cells with siRNA to explore the exact target(s) of IL-12, however, the limitation in the availability of human materials hampered these analyses. Thus, IL-12 produced by APCs on the OK-432 stimulation could have two (or more) mutually compatible activities, (i) rendering effector cells resistant to Treg-cell suppression and (ii) inhibiting Treg-cell suppressive function directly, though the in vivo data argue against direct inhibition of Treg-cell suppression [39, 40].

Local administration of OK-432 reduced the number of $CD4^+CD25^+Foxp3^+$ Treg cells in tumor-associated exudate fluids. After administration of OK-432, local chemokine gradient may be changed and infiltration of Treg cells may be blocked [6, 13]. Alternatively, the inflammatory environment after OK-432 administration may be suitable for effector T-cell activation and IL-2, that is critical for Treg-cell survival and function [41], may not be adequately provided, as observed during severe *Toxoplasma gondii* infection [42]. In addition, suppressive function of $CD4^+CD25^{high}$ T cells in tumor-associated

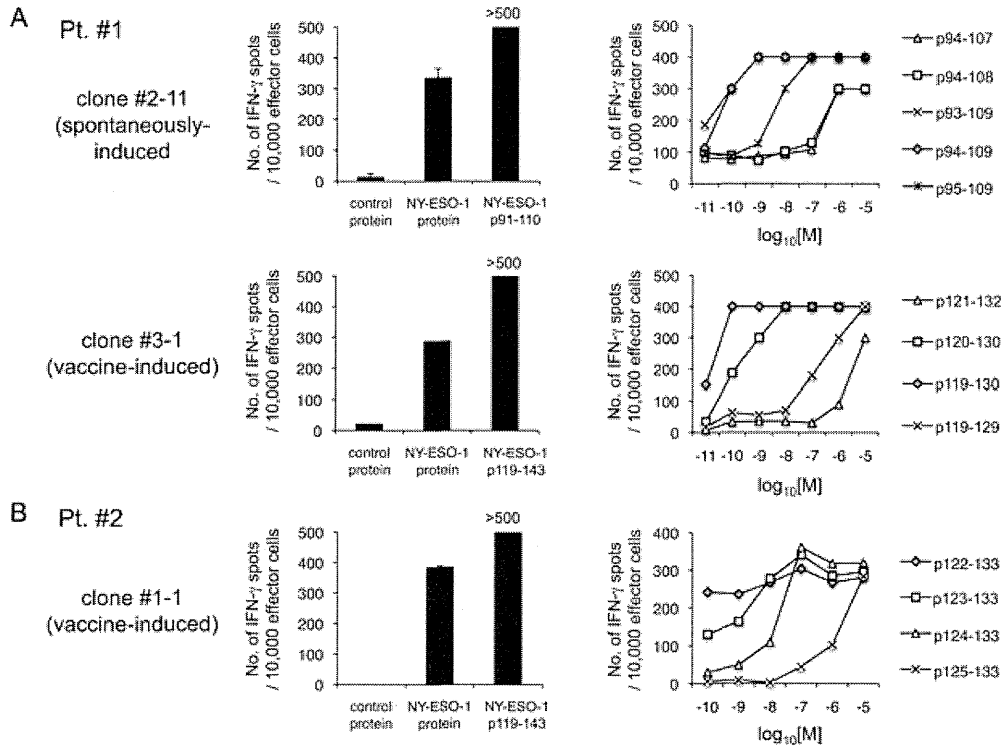

Figure 6. Vaccination with OK-432 activates high-avidity NY-ESO-1–specific CD4$^+$ T cells. NY-ESO-1–specific CD4$^+$ T-cell clones were generated from PBMCs of patients harboring NY-ESO-1–specific CD4$^+$ T cells. CD4$^+$ T-cell clones from (A) Pt. #1 and (B) Pt. #2 were stimulated and expanded by 30 ng/mL anti-CD3 Ab in the presence of 20 U/mL IL-2 and irradiated PBMCs and EBV-transformed human B lymphocytes as feeder cells. A couple of weeks later, these NY-ESO-1–specific CD4$^+$ T cell clones (1 × 10^4/well) were cultured with 1 × 10^4 NY-ESO-1 protein-pulsed DCs or 2 × 10^4 EBV-B cells pulsed with graded amounts of peptides and NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data are shown as mean ± SD of two replicates and are from one experiments representative of at least two experiments performed.

exudate fluids was reduced after OK-432 treatment in accordance with decreased expression of Foxp3 [43]. Considering the fact that IL-12, a main effector molecule induced by OK-432, renders effector cells resistant to Treg-cell suppression, direct administration of OK-432 may change the immunological balance in the local microenvironment from suppression by Treg cells to activation of helper T cells by augmenting helper T-cell activity. However, the sample size of patients analyzed in this study was relatively small and warrants cautious interpretation.

We have previously shown that while naive NY-ESO-1–specific CD4$^+$ T-cell precursors are present in wide range of healthy individuals and cancer patients, their activation is kept under stringent CD4$^+$CD25$^+$ Treg-cell control [20, 21, 28]. Using OK-432 as an adjuvant, we detected high-affinity NY-ESO-1–specific CD4$^+$ T cells in effector/memory population after vaccination in two esophageal cancer patients. In Pt #1, we found two responses; spontaneous and vaccine-induced NY-ESO-1–specific CD4$^+$ T cells. Both of them exhibited a similar efficiency to recognize titrated peptide, indicating that these NY-ESO-1–specific CD4$^+$ T cells had TCRs with similar affinity and were likely activated from naive high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors. Vaccination with minimal peptide in incomplete Freund's adjuvant fails to activate high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, rather it dominantly expands low-avidity effector/memory CD4$^+$ T cells that cannot recognize naturally processed antigens [21]. In addition, following DNA vaccination covering the entire sequence of NY-ESO-1, high-avidity NY-ESO-1–specific CD4$^+$ T cells were not detected persistently because of rapid suppression by Treg cells [44]. While these data suggest a critical role for the inhibition of Treg-cell suppression by OK-432 in the activation of high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, it is still difficult to obtain conclusive evidence without direct in vivo Treg-cell inhibition/depletion. To formally address this issue, clinical trials using Treg-cell depletion reagents and another clinical trial having two arms of patients receiving NY-ESO-1 with/without OK-432 would be required.

Certain types of immunization methods or DC stimulations elicit/augment CD4$^+$CD25$^+$ Treg cells in vivo [10–12, 45]. As many tumor-associated antigens recognized by autologous tumor-reactive lymphocytes are antigenically normal self-constituents [1–3], they also could be recognized with CD4$^+$CD25$^+$ Treg cells. Given that a proportion of cancer/testis antigens are targets of Treg cells [46], it is necessary to avoid unwanted activation of cancer/testis antigen-specific CD4$^+$CD25$^+$ Treg cells. Though the sample size of patients analyzed in this study was small and warrants cautious interpretation, including OK-432 in vaccine components as an adjuvant would be a promising strategy to establish favorable circumstances for stimulating effector T cells by inhibiting Treg-cell activation. Furthermore, since this agent has a long history and is widely applied as an anticancer drug, particularly

in Japan, its clinical safety profile has been already established. Our data provide a critical cue for effective cancer vaccines and immunotherapy during antigen priming through modulation of $CD4^+CD25^+$ Treg-cell function.

Materials and methods

Blood samples

All healthy donors were subjects with no history of autoimmune disease. PBMCs, pleural effusions, or ascites from cancer patients were collected before and after local administration of OK-432 based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Nagasaki University Graduate School of Medicine. PBMCs from esophageal cancer patients enrolled in a clinical trial of CHP-NY-ESO-1 and CHP-HER2 vaccination with OK-432 [47] (Supporting Information Fig. 1) were collected based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Kitano Hospital. The clinical trial was conducted in full conformity with the current version of the Declaration of Helsinki and was registered as NCT00291473 of Clinical Trial.gov, and 000001081 of UMIN Clinical Trial Registry. All samples were collected after written informed consent.

Abs and reagents

Synthetic peptides of NY-ESO-1_{1-20} (MQAEGRGTGGSTGDADGPGG), NY-ESO-1_{11-30} (STGDADGPGGPGIPDGPGGN), NY-ESO-1_{21-40} (PGIPDGPGGNAGGPGEAGAT), NY-ESO-1_{31-50} (AGGPGEAGATGGRGPRGAGA), NY-ESO-1_{41-60} (GGRGPRGAGAARASGPGGGA), NY-ESO-1_{51-70} (ARASGPGGGAPRGPHGGAAS), NY-ESO-1_{61-80} (PRGPHGGAASGLNGCCRCGA), NY-ESO-1_{71-90} (GLNGCCRCGARGPESRLLEF), NY-ESO-1_{81-100} (RGPESRLLEFYLAMPFATPM), NY-ESO-1_{91-110} (YLAMPFATPMEAELARRSLA), NY-ESO-$1_{101-120}$ (EAELARRSLAQDAPPLPVPG), NY-ESO-$1_{111-130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-$1_{119-143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-$1_{131-150}$ (NILTIRLTAADHRQLQLSIS), NY-ESO-$1_{139-160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-$1_{151-170}$ (SCLQQLSLLMWITQCFLPVF), NY-ESO-$1_{161-180}$ (WITQCFLPVFLAQPPSGQRR), and HIV $P17_{37-51}$ (ASRELERFAVNPGLL) [48] were obtained from Invitrogen (Carlsbad, CA, USA). Recombinant NY-ESO-1 protein was prepared using similar procedures as described previously [49]. OK-432 was purchased from Chugai Pharmaceutical (Tokyo, Japan). LPS (*Escherichia coli* 055:B5) was obtained from Sigma (St. Louis, MO, USA). Purified and FITC-conjugated anti-IL-12 (C8.6; mouse IgG1), purified anti-IL-6 (MQ2–13A5; rat IgG1), purified anti-IFN-γ (NIB42; mouse IgG1), purified anti-IL-23 (HNU2319; mouse IgG1), PE-conjugated anti-CD20 (2H7; mouse IgG2b) and PE-conjugated anti-CD56 (MEM188; mouse IgG2a) Abs were purchased from eBioscience (San Diego, CA, USA). Purified anti-IL-1β Ab (8516; mouse IgG1) was purchased from R&D Systems (Minneapolis, MN, USA). PE-conjugated anti-CD14 (MφP9; mouse IgG2b), PE-conjugated anti-CD45RA (HI100; mouse IgG2b), PerCP-conjugated anti-CD4 (RPA-T4; mouse IgG1), and FITC-conjugated anti-CD4 (RPA-T4; mouse IgG1), Foxp3 (259D; mouse IgG1), and CD45RO (UCHL1; mouse IgG2a) Abs were purchased from BD Biosciences (Franklin Lakes, NJ, USA). PerCP-Cy5.5-conjugated anti-CD11c Ab (3.9; mouse IgG1) was obtained from Biolegend (San Diego CA, USA). PE-conjugated anti-CD25 Ab (4E3; mouse IgG2b) was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant IL-6, IL-12, and TNF-α were purchased from PeproTech (Rocky Hill, NJ, USA).

Intracellular cytokine staining

PBMCs were cultured with/without OK-432 and GolgiStop reagent (BD Biosciences) for 20 h. Cells were stained for cell surface markers and then for intracellular cytokine (IL-12) after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences).

Generation of NY-ESO-1–specific $CD4^+$ T cells

NY-ESO-1–specific $CD4^+$ T cells were elicited as described previously [20]. Briefly, $CD4^+$ T cells and $CD4^+CD25^-$ T cells were isolated from PBMCs using a $CD4^+CD25^+$ Treg Isolation Kit (Miltenyi Biotec). $CD4^+CD25^-$ T cells were further separated into $CD45RO^+$ T cells or $CD45RA^+$ T cells by FACSAria (BD Bioscience) after staining with anti-CD45RO and CD45RA Abs. $CD4^-$ PBMCs pulsed with 10 μM of peptide overnight were used as APCs. After irradiation, 5×10^5 APCs were added to round-bottom 96-well plates (Nunc, Roskilde, Denmark) containing 1–5 $\times 10^5$ unfractionated $CD4^+$ or $CD4^+CD25^-CD45RO^+$ T cells and were fed with 10 U/mL IL-2 (Kindly provided by Takeda Pharmaceutical, Osaka, Japan) and 20 ng/mL IL-7 (R&D Systems). Subsequently, one-half of medium was replaced by fresh medium containing IL-2 (20 U/ml) and IL-7 (40 ng/mL) twice per week.

Generation of NY-ESO-1–specific $CD4^+$ T-cell clones

Cloning was performed by limited dilution as described previously [50]. Briefly, NY-ESO-1–specific $CD4^+$ T cell lines (0.3 cells/well) were stimulated and expanded in the presence of irradiated 5×10^4 cells/well PBMCs and 1×10^4 cells/well irradiated EBV-transformed human B lymphocytes with 10% AB serum, 20 U/ml IL-2, and 30 ng/mL anti-CD3 Ab (OKT3; eBioscience) in 96-well round-bottom plates.

Proliferation assay

$CD4^+CD25^-$ T cells were cultured with 1×10^5 irradiated CD4-depleted PBMCs and stimulated with 0.5 μg/mL anti-CD3 Ab

(OKT3, eBioscience) in round-bottom 96-well plates. $CD4^+CD25^{high}$ Treg cells (highest 3% of $CD4^+CD25^+$ cells) were purified with FACSAria (BD Biosciences), and graded numbers of them added in the culture as indicated in figure legends. Proliferation was evaluated by ^{3}H-thymidine with 1 μCi/well for the last 18 h of 6-day culture. ^{3}H-thymidine incorporation was measured by a scintillation counter.

ELISPOT (enzyme-linked immunospot) assay

The number of IFN-γ secreting antigen-specific $CD4^+$ T cells was assessed by ELISPOT assays as described [20, 21]. Briefly, flat-bottomed, 96-well nitrocellulose-coated microtiter plates (Millipore, Bedford, MA, USA) were coated with anti-IFN-γ Ab (1-D1K; MABTECH, Stockholm, Sweden). The presensitized T cells and phytohaemagglutinin (PHA HA15; Murex Diagnostics, Dartford, UK) activated $CD4^+$ T cells, EBV-transformed human B lymphocytes or DCs pulsed with 10 μM of peptides or 25 μg/mL protein overnight were added to each well and incubated for 24 h. Spots were developed using biotinylated anti-IFN-γ Ab (7-B6–1-biotin; MABTECH), alkaline phosphatase conjugated streptavidin (Roche, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) and counted with C.T.L. Immunospot analyzer and software (Cellular Technologies, Cleveland, OH, USA).

Preparation of monocyte-derived DCs

Monocyte-derived DCs were generated from PBMCs as previously described with some modifications [51]. Briefly, $CD14^+$ monocytes were enriched by positive selection using CD14 Microbeads (Miltenyi Biotec). Monocytes were cultured in the presence of 20 ng/mL GM-CSF (Immunex, Seattle, WA, USA) and 20 ng/mL IL-4 (R&D systems) in RPMI1640 supplemented with 2.5% fetal calf serum. Medium was replaced by fresh medium containing cytokines 3 days later. On day 6, cells were harvested and used for subsequent experiments.

ELISA

The concentration of IL-12p70 and IL-10 was measured by ELISA Kit (eBioscicence) according to the instruction provided by the manufacturer.

Statistical analysis

Statistical significance was evaluated by Student's *t*-test; *p* values less than 0.05 are considered significant.

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Abbreviation: **CHP**: cholesteryl hydrophobized pullulan

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Natural and induced T regulatory cells in cancer

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CD4+Foxp3+ T regulatory (Treg) cells control many facets of immune responses ranging from autoimmune diseases, to inflammatory conditions, and cancer in an attempt to maintain immune homeostasis. Natural Treg (nTreg) cells develop in the thymus and constitute a critical arm of active mechanisms of peripheral tolerance particularly to self antigens. A growing body of knowledge now supports the existence of induced Treg (iTreg) cells which may derive from a population of conventional CD4+ T cells. The fork-head transcription factor (Foxp3) typically is expressed by natural CD4+ Treg cells, and thus serves as a marker to definitively identify these cells. On the contrary, there is less consensus on what constitutes iTreg cells as their precise definition has been somewhat elusive. This is in part due to their distinct phenotypes which are shaped by exposure to certain inflammatory or "assault" signals stemming from the underlying immune disorder. The "policing" activity of Treg cells tends to be uni-directional in several pathological conditions. On one end of the spectrum, Treg cell suppressive activity is beneficial by curtailing T cell response against self-antigens and allergens thus preventing autoimmune diseases and allergies. On the other end however, their inhibitory roles in limiting immune response against pseudo-self antigens as in tumors often culminates into negative outcomes. In this review, we focus on this latter aspect of Treg cell immunobiology by highlighting the involvement of nTreg cells in various animal models and human tumors. We further discuss iTreg cells, relationship with their natural counterpart, and potential co-operation between the two in modulating immune response against tumors. Lastly, we discuss studies focusing on these cells as targets for improving anti-tumor immunity.

Keywords: Tregs, Foxp3, natural, induced, cancer, tumor, Interleukin-10, transforming growth factor β

INTRODUCTION

Early studies of T regulatory (Treg) cells, defined as a subset of CD4+ cells that co-express high levels of CD25, the high affinity IL-2 receptor α-chain, demonstrated unequivocally that these cells are crucial for maintenance of peripheral self tolerance as their elimination led to development of multiple organ-specific autoimmune diseases (1). Subsequent studies identified foxp3, a member of the fork-head/winged-helix family of transcriptional factor as uniquely expressed by Treg cells and allowed for more precise phenotypic identification of these cells as CD25 alone was insufficient due to its upregulation on activated T cells (1–3). Endowed with highly suppressive machinery, it is now well established that CD4+Foxp3+ Treg cells regulate a diverse array of immune responses ranging from autoimmune disease, allergies, and transplant rejection, to infections and cancers (4). While generally beneficial in the former conditions, the inhibitory activity of Treg cells often antagonizes protective immunity in the latter settings. Depending on the microenvironment in which they are found, and potential stimuli eliciting their recruitment or presence at such sites, CD4+Foxp3+ Treg cells are now broadly described as natural or adaptive (5, 6). Natural CD4+Foxp3+ Treg cells are the better understood of the two with the central dogma being that the adaptive or "induced" cells are generally derived from existing pool of naïve conventional CD4+ T cells. Regardless of their origin, they share one key feature: their ability to potently suppress effector T cells (5). Although expression of Foxp3 generally identifies natural, thymus-derived CD4+ Treg cells, adaptive Treg cells may or may not express this transcription factor (5, 7, 8).

Recent years have seen a surge in studies of cancer models and in humans highlighting the elevated levels of Treg cells in the tumor and/or in circulation (9, 10). This often correlates with poor anti-tumor effector response, hence compromised tumor immunity (11, 12). Whether the Foxp3+ cells widely described in many cancer settings are of natural or adaptive/induced type remains largely a bone of contention. This review focuses on the current knowledge about both subsets of Treg cells, their generation, phenotypic characteristics, and ill-defined roles as described in various tumor models and human cancers. Current therapeutic modalities geared toward Treg depletion and how they may impinge on recruited natural versus tumor-induced Treg (iTreg) cells are discussed.

INDUCED/ADAPTIVE TREGS, MORE THAN JUST Foxp3+ CELLS

Adaptive Tregs encompass a number of CD4+ cells with regulatory/suppressive capabilities (7, 8, 13). Although "iTregs" is commonly used interchangeably with "adaptive Tregs," the former is perhaps a better nomenclature for all extrathymically derived CD4+ Treg cells. In this context, iTreg cells range from Tr1 cells, which are induced by IL-10, and secrete both IL-10 and TGF-β (7), to TGF-β-producing Th3 cells (induced by oral antigen tolerizing

conditions) (8), to peripheral naïve CD4+CD25−Foxp3− cells that become converted to Foxp3-expressing cells (13). Tr1 cells regulate immune responses against ubiquitous commensal organisms and promote tolerance in the gut, and accumulating evidence reveal they play key roles in other facets of adaptive immune response (7). Th3 cells on the other hand, appear critical in tolerance induced by oral antigen delivery (8). Both adaptive Treg cell types are induced in peripheral sites and have been described to generally lack expression of Foxp3 which distinctively identifies natural Treg (nTreg) cells of thymic origin (2, 3, 14). In most tumor studies however, these cells have not been extensively described. Most of the attention on iTregs in tumor settings has largely focused on converted Foxp3-expressing cells mentioned above. Since both peripherally induced Foxp3+ as well as Foxp3 non-expressing CD4+ regulatory T cells (e.g., Tr1− cells) are often discussed under the umbrella of "induced" Treg cells, for simplicity sake, the term iTreg in this review will be restricted to CD4+CD25−Foxp3− cells that have acquired Foxp3 expression. In order to do justice to their contributions in tumor settings, other Foxp3 non-expressing, peripherally induced CD4+ regulatory cells, specifically Tr1 cells, will be discussed separately as such in one section and the rest of our discussion will focus on Foxp3+ peripherally converted iTregs.

NATURAL VERSUS INDUCED TREGS IN CANCER: ORIGIN AND ACCUMULATION

Several lines of evidence reveal an accumulation of Treg cells both at peripheral sites (spleen, peripheral blood), and within the local tumor microenvironment [reviewed in Ref. (10, 12, 15)]. This often correlates with persistent tumor burden and poor anti-tumor effector response (11, 12). Importantly, a low CD8+ effector T cell number is also noted relative to the high proportion of Foxp3+ Treg cells in the peripheral blood and tumor tissue in many cancer patients (12) suggesting active recruitment of Foxp3+ Treg cells is a key feature of many tumors. Thus, a "guilty-by-association" analogy means that these tumor-infiltrating Treg cells must at least, in part, be responsible for dampening anti-tumor immunity, namely preventing effective tumor immunosurveillance. One outstanding issue however is the source of these cells, and this issue is currently a subject of debate within the tumor immunology community.

From current knowledge, the composition of Foxp3+ Treg cells within tumors and/or in circulation in human cancer patients remains poorly understood. There are a few possibilities: (1) They are nTregs recruited to the tumor site and actively expanding (16–18); (2) They are a pool of induced, Foxp3-acquired Treg cells (iTregs) derived from converted CD25− cells (19, 20); (3) They are Tr1 cells (discussed in the following section). In support of the first possibility, studies performed by Zou and colleagues demonstrated specific recruitment of pre-existing human Treg cells into tumors in a manner that was dependent on tumor-mediated CCL22 production and gradient (16). Another study demonstrated that Treg cells underwent substantial proliferation at tumor site and draining lymph node in response to TGF-β secreted by immature DCs which themselves were a result of tumor cell modification (18). In either study however, the possibility that iTregs were also recruited or expanded at tumor site could not be excluded. The notion that tumor-infiltrating Tregs are likely expanded nTreg cells was further purported in a study that examined the TCR repertoire analysis of tumor-infiltrating Treg and T conventional cells (17). In this report, authors concluded that since the TCR repertoires of either population were largely non-overlapping, the tumor-infiltrating Tregs are likely of natural origin as a significant overlap would have been observed if a fair amount of CD25− cell conversion to Foxp3+ cells occurred.

Data supporting the second possibility comes from a number of studies (19–21). One of these demonstrated that in thymectomized, and anti-CD25-treated tumor-bearing mice, a population of Treg cells converted from CD25− cells developed (20). Anti-CD25 Treg depletion strategy has been described not to efficiently eliminate Treg cells (22). So the possibility remains that nTreg cells not touched by the treatment regimen expanded in this system. In any case, the thymectomy would have at least reduced any potential contribution by newly generated nTreg cells after anti-CD25 treatment cessation. Many tumors secrete TGF-β that may directly or indirectly induce naïve T cell conversion to Foxp3+ iTregs (19, 20, 23) Consistent with this, another group demonstrated that in a mouse prostate tumor model, tumor-derived TGF-β potentiated the conversion of CD4+CD25− T conventional cells into Foxp3-expressing, CD25+ iTreg cells (19). However, sole presence of iTreg or nTreg cells within the tumor need not be mutually exclusive as demonstrated by Zhou et al. Using an influenza hemagglutinin (HA)-expressing tumors along with HA TCR-transgenic T cells in an adoptive transfer system, they were able to demonstrate that both *de novo* generated adaptive and nTreg cells contributed to the pool of tumor-Treg cells (24). Thus, a more realistic view of their composition is that both adaptive and nTreg cells contribute to the total Treg pool affiliated with tumor microenvironment.

Tr1 CELLS IN CANCER

Not all regulatory CD4+ cells are endowed with Foxp3 suppressive machinery. As mentioned previously, IL-10-producing Tr1 cells fall under this umbrella of Foxp3-non-expressing cells. Tr1 cells by their original description in the early literature are CD4+CD25−, IL-10, and TGF-β-producing cells (7). The general consensus is that they are derived from a pool of naïve CD4+ T cells that are distinct from thymus-derived Foxp3+ cells. Suffice to say, they are seemingly low in frequency in an unperturbed immune environment but are readily detected in an environment rich in cytokines such as IL-10, justifying their label as adaptive or induced regulatory T cells.

Unlike CD4+Foxp3+ Treg cells, the involvement of Tr1 cells in tumors has not received as much attention. There are a number of studies showcasing the importance of these cells in tempering anti-tumor response, some dating back to pre-Foxp3 years (25–30). In a cohort of Hodgkins lymphoma patients, an argument was made by Marshall and colleagues for a contributory role of CD4+ IL-10+ Tr1 cells toward ineffective clearance of Hodgkins lymphoma. This was in part based on their finding that these cells were present at elevated proportions in associated lymph nodes, and could suppress T cell response in corresponding PBMCs (26). The co-existence of the Tr1 cells with CD4+CD25+ (presumably natural Foxp3+) both of which were enriched in the lymph

nodes in this particular study makes it difficult to ascertain to what extent, if any, the Tr1 cells played an inhibitory role. Whiteside and colleagues have reported extensively the presence of Tr1 cells in head and neck squamous-cell carcinoma (HNSCC) patients (10). Although relatively low in frequency in circulation, they were present in a sizable proportion in tumor-infiltrating lymphocytes (28). *In vitro* analysis of peripheral CD4+ cells in glioblastoma patient also revealed a prominent Tr1 response against tumor cells suggestive of an enriched population of Tr1 cells in this setting (27). In a protocol involving adoptive transfer of *in vitro*-cultured Th1-like cells to ovarian cancer patients, Tr1 cells were also shown to contribute to the total circulating Treg pool (30). In general, many of the analyses performed in these studies were dependent on stimulation of patient's PBMC with or without tumor antigens plus Tr1 cell-enhancing cytokines to showcase their existence, and demonstrate that cancer patients harbor more Tr1 cells than healthy individuals. Perhaps, most of the Tr1 cells in the periphery exist in precursor form and are only expanded at tumor site where antigen is ubiquitous and key cytokines such as IL-10 are abundant, similar to the *in vitro* simulations. The study performed by Bergmann et al., certainly is in agreement with this notion (28).

The mechanisms by which Tr1 cells might be induced within the tumor remains unclear. Some lines of evidence suggest that certain factors uniquely produced by tumor cells could facilitate an IL-10-rich environment that ultimately fosters Tr1 cell induction (10, 27). In one report, cyclooxygenase-2 (COX-2) overexpressing glioma via Prostaglandin E2 (PGE2) synthesis induced mature DCs to express high levels of IL-10, which in turn induced CD4+ T cells that secreted copious amounts of IL-10 and TGF-β (27). Furthermore, CD4+ T cells isolated from peripheral blood of glioblastoma patient showed marked IL-10 production against tumor cells indicating an enrichment of Tr1 cells within the peripheral CD4+ T cell pool in this patient. This sentiment was echoed by another study which demonstrated that *in vitro*, highly suppressive Tr1 cells were generated from CD4+CD25− T cells in the presence of autologous DCs and irradiated COX-2+ HNSCC cells or exogenous PGE2, with a cytokine cocktail that included IL-10 (29). Like the afore-mentioned study, the overall conclusion here is that COX-2 overexpression, and PGE2 production by HNSCC plays a key role in the induction of Tr1 cells in this malignancy. The Tr1 cells in this study however, were shown to have some Foxp3 expression.

One important point is that a unifying phenotype that definitively identifies these CD4+ Tr1 cells is yet to be agreed upon. Besides being CD25 negative, IL-10, and TGF-β-producing, their Foxp3 status remains a divisive subject. Some studies showed they express variable Foxp3 levels (28, 29, 31), others described them as Foxp3 negative, or foxp3 status was not addressed (26, 27, 30, 32, 33). The differences between these studies may likely stem from experimental designs although it can be argued that the stimulatory conditions used in some of the *in vitro* assays to amplify Tr1 cells are also conducive to Foxp3 induction in lieu of the fact that conventional human T cells can upregulate FOXP3 upon activation (34). Regardless of how they are described, Tr1 cells, like their natural counterparts, are capable of exhibiting potent suppressive functions as demonstrated in some of the above-mentioned studies.

With respect to their perceived function within the tumor microenvironment, it remains a possibility that they co-operate with nTregs, a notion that has been suggested by others (35). The dichotomy that Tr1 cells are increased in frequency in advanced cancer stage and also in patients who had no evidence of active disease following oncologic treatments when compared with early stage raises the possibility that they may play differing roles under varying tumor burdens (28). On the far end of the spectrum of possibilities is that Tr1 cells actually may play beneficial roles that are masked by the over-representation of their "natural cousins" within the tumor microenvironment. Perhaps the ratio between nTregs and Tr1 iTregs may be key to understanding their contribution to shaping the course of tumor progression. In support of this idea, *ex vivo* stimulated PBMCs of ovarian cancer patients who had better survival outcomes upon previous infusion with Th1-like CD4+ cells, contained higher fractions of both CD4+CD25+CD45RO+FoxP3+ and CD4+CD25−FoxP3− IL-10-producing cells compared to cells derived from short-term survivors (30). Importantly, the ratio of the Foxp3+ nTregs versus IL-10+ Tr1 cells was touted to be key to better outcome as the one patient that remained cancer-free showed a dwindling pattern in the frequency of CD4+Foxp3+ cells while the Tr1 cell numbers steadily increased with each cycle of T-cell infusion and *ex vivo* PBMC stimulation. Could induced regulatory cells that present in the form of IL-10-producing Tr1 cells be beneficial in the context of tumor immunity? Perhaps some studies in the foreseeable future may specifically tackle this question. IL-10 being a cytokine that appears to play both inhibitory and immunostimulatory roles (25, 26, 32, 36), an anti-tumor immunity-boosting role for IL-10+ Tr1 cells is thus, not unimaginable and the above study certainly leaves room for such deduction. Consistent with this notion, IL-10-producing CD4+ cells have been demonstrated to effect tumor rejection in a murine glioma model by augmenting CTL and NK cell response (32). Perhaps, "curative" outcome seen from a combination of standard cancer treatments and immune modulatory protocols favor an increase in a discrete, unobstructive, Tr1 cell population with a concomitant decrease in a tampering nTreg subset. At any rate, more studies are warranted to better understand how Tr1 cells shape the course of anti tumor immunity, and by extension, tumor progression. In addition, identification of reliable markers to pin-point categorically their existence in tumor mass and in circulation of cancer patients without a need to amplify them *in vitro* is necessary.

DIFFERENTIATING NATURAL TREGS FROM INDUCED TREGS

HELIOS

Expression of Helios, a member of the Ikaros transcription factor family has been described to be a part of Treg genetic signature based on a number of gene array analysis (37, 38). In a recent report, essentially all thymic Treg cells were Helios+ but only about 70% of the peripheral pool retained their expression (39). Furthermore, *in vitro* and *in vivo*-generated iTregs failed to express Helios. An argument was thus made that Helios expression may mark the bona fide nTregs of thymic origin (39). Building on this observation, studies in tumor-bearing mice and human cancers have also explored the composition of tumor-infiltrating Treg cells with respect to Helios expression (40–42). Treg cells from peripheral

blood of renal cell carcinoma (RCC) patients were found to consist of a population that expressed Helios (40). In human ovarian carcinomas, CXCR3+ Treg cells were reported to be abundantly represented in the majority of tumor-Treg cells and they co-express Helios (41). In another study that used a xenogeneic mouse model of malignant human brain tumor, it was demonstrated that majority of tumor-associated Treg cells expressed Helios, and their frequency decreased when tumor-bearing mice were thymectomized prior to tumor cell implantation (42). In all of these studies, the consensus was that the Treg cells within the tumors are most likely natural due to their expression of this transcription factor. On the contrary, it was reported that the vast majority of tumor-infiltrating Treg cells in a murine colon adenocarcinoma expressed low levels of Helios and the authors concluded that based on this phenotype, coupled with additional markers, these are likely to be iTregs (43). In the absence of any immune pathology in the colon however, it should be pointed out that colonic Treg cells may be predominantly thymus-derived nTreg cells as recently demonstrated (44). When weighed together, these observations only reinforce the possibility that the expression of Helios on tumor-infiltrating Treg cells may not necessarily be an indication that they are derivatives of nTreg cells. Further putting into question the reliability of Helios in resolving the dichotomy of "i" versus "n" Treg cells are some existing reports (45–47). Using polyclonal or antigen-specific stimulation methods to activate T cells derived from TCR-transgenic $Rag^{-/-}$ mice (hence, no endogenous Tregs), Wraith and colleagues demonstrated that a substantial fraction of *in vitro*-generated iTregs expressed Helios under the latter stimulation condition (47). Another group also described transient expression of Helios on activated human and murine T conventional and Treg cells (45). Whether Helios positive versus negative Foxp3+ cells simply represent different versions of the same Treg group (i.e., n Tregs) is of particular interest given that the profile of iTreg cells generated in adoptively transferred lymphopenic mice based on gene expression analysis was found to be relatively similar to nTreg cells from normal mice (48). As Treg cells encounter tumor-associated antigens (TAA), it remains a possibility that they become activated and upregulate Helios expression. In this context, expression of Helios simply is not sufficient to distinguish the origin of tumor-Tregs.

NEUROPILIN-1

Neuropilin-1 (Nrp-1), a type-1 transmembrane protein is yet another molecule that is being implicated in the iTreg versus nTreg identification issue (43, 49, 50). Using microarray analysis, Haribhai and team demonstrated that iTreg cells induced *in vitro* under TGF-β and IL-2 expressed very low levels of *Nrp1* compared to nTregs cells (49). In an MBP-specific TCR-transgenic mouse model under Rag deficiency background, another report demonstrated the existence of Foxp3+ iTreg cells in peripheral compartments, which persisted even in athymic mice suggesting that they were extrathymically derived (50). These cells expressed low levels of Nrp-1. In a model of iTreg cell generation via mucosal routes, Lafaille and colleagues demonstrated that mucosal iTreg cells or iTreg cells generated *in vivo* under non-inflammatory conditions also express low levels of Nrp-1 unlike nTreg cells in which high expression levels were noted. Under inflammatory conditions however, iTreg cells upregulated its expression (43). In tumor settings, there is only scant data describing Nrp-1 expression in association with sub-phenotypes of Treg cells. In one report, there was a positive trend toward increased presence of a sizable fraction of Foxp3+ cells which exhibited low expression levels of Nrp-1 in the tumor tissue of tumor-bearing mice. In contrast, Nrp-1hi cells predominated in the spleen suggesting that the Nrp-lo phenotype may represent a population of iTreg cells induced locally within the tumor (43). Taken together, these studies allude to the possibility that Nrp-1 expression may be a good indicator for distinguishing between peripherally induced adaptive Treg cells and may be particularly suitable in deciphering the composition of tumor-infiltrating Foxp3+ Treg cells.

OTHER MARKERS

Worth mentioning are a myriad of cell surface molecules and receptors that have also been associated with tumor-infiltrating Treg cells (41, 51–56). Garpin (GARP; glycoprotein A repetitions predominant) was found in one study to be significantly higher on Foxp3+ Treg cells in hepatocellular carcinoma patients (55). Lymphocyte activation gene-3 (LAG-3), a CD4 homolog that binds MHC class II is yet another molecule that has been described to distinguish a unique sub-population of CD4+Foxp3+ Treg cells that expand at tumor sites (51). This study analyzed the frequency and phenotype of Foxp3+ cells in melanoma and colorectal cancer patients at different stages of disease and discovered that increased percentages of LAG-3-expressing Foxp3+ Treg cells preferentially expanded in the peripheral blood and tumor sites raising the notion that these cells represent a subset of tumor-iTreg cells (51). Other studies identified TNFR2, TIM-3, and ICOS as upregulated on Treg cells at tumor sites suggesting they may represent a distinct Treg cell subset that are generated specifically in response to TAA (52–54, 56). In a human melanoma study, for example, CD4+Foxp3+ Treg cells infiltrating tumor tissue not only displayed upregulated expression of ICOS but also exhibited a more potent suppressive activity compared to those derived from circulating blood cells (54). While these assessments were not particularly geared toward separating tumor-infiltrating Treg cells into natural or induced subset, it could be insightful if their expression patterns are considered in tandem with analysis focused at determining the composition of tumor-Treg cells with respect to their origin. (See **Table 1** for a number of cancer studies in which some of these markers or TCR repertoire pattern were implicated in the suggested origin of tumor-infiltrating Treg cells.)

nTREG VERSUS iTREG IN TUMORS; A FUNCTION OF ACTIVATION/DIFFERENTIATION STATUS?

Perhaps, a healthy dose of objectivity is ideal in our trying to piece together the different phenotypes exhibited by Foxp3+ Treg cells in different tumors and finding a unifying phenotype that specifically identifies subsets. The increased expression of some of the afore-mentioned molecules upon T cell activation (57, 58) raises the possibility that the various unique phenotypes as observed in many tumor models and human cancers may simply represent an activation state and not an indication of a different cohort of iTreg cells generated from peripheral non-Treg cells. For instance, a recent study reported that the expression of GARP identifies

Table 1 | Natural and induced Treg cells in cancer.

Species	Cancer type/tumor model	Treg phenotype	Suggested origin	Origin indicator	Reference
Human	Ovarian carcinoma	CD4+FOXP3+; CXCR3+, T-bet+	Natural	Helios expression	(41)
Human	Colon adenocarcinoma	CD4+FOXP3+; CCR4+CTLA-4hi	Unknown		(39)
Human	Ovarian cancer	CD4+FOXP3+; Helios+, CCR4^{lo}	Unknown		(61)
Mice/Rats	Colon carcinoma/melanoma	CD4+CD25+/Foxp3+	Natural	Expansion via mDC-TGF-β	(18)
Mice	Fibrosarcoma	CD4+Foxp3+	Natural	Distinct TCR repertoire versus CD4+CD25−	(17)
Mice	Colon carcinoma	CD4+Foxp3+	Induced	Foxp3 induction in CD4+CD25−	(20)
Mice	Renal cell carcinoma	CD4+CD25+/Foxp3+	Induced	Foxp3 induction via TGF-β	(19)
Human	Renal cell carcinoma	CD4+FOXP3+	Natural	Helios expression	(40)
Mice, human	Glioblastoma	CD4+Foxp3+	Natural	Helios expression	(42)
Mice	Colon adenocarcinoma	CD4+Foxp3+; Nrp-1^{lo}, Helios^{lo}	Induced	Helios and Nrp-1 expression	(43)
Mice	Tumor cell line/melanoma	CD4+Foxp3+	Natural	Distinct TCR sequence versus CD4+CD25−	(70)
Human	Hodgkin lymphoma	CD4+IL-10+ Tr1 and CD4+CD25+	Unknown		(26)
Human	Ovarian cancer	CD4+CD25−FOXP3− IL-10+ Tr1 and CD4+CD25+Foxp3+	Induced and natural	IL-10 production or Foxp3 status	(30)

activated human CD4+Foxp3+ Treg cells especially upon *in vitro* stimulation (58). Although very few studies have demonstrated the antigen specificity of tumor-infiltrating Treg cells (59, 60), one might speculate that the bulk of the Treg cells infiltrating the tumor have encountered and been activated by some TAA, hence are antigen-experienced. Therefore, it remains plausible that the different phenotypes as observed in different tumor models and human cancers is a reflection of their activation status and a factor of antigen repertoire to which the Treg cells are exposed in the tumor and/or draining lymph nodes. In sync with this notion, a recent study in late stage ovarian cancer patients noted a dominant population of Helios+ activated Treg cells in disseminated tumors (61). Another issue is whether the expression of these molecules signals a terminal differentiation stage. We previously reported that in humans, CD45RA-Foxp3hi cells are activated and terminally differentiated (62). In a murine study, KLRG1-expressing Treg cells were identified and also deemed to be terminally differentiated (63). Thus, tumor-infiltrating Treg cells may well be derived from pre-existing pool of peripheral nTreg cells but exhibit unique phenotypic properties reflective of their activation status and/or differentiation stage as opposed to being generated from non-Treg precursors, hence induced.

Expanding on this issue, it has been said that tumor-infiltrating Treg cells appear to display an effector phenotype that likely emanates from chronic exposure to TAA (10, 64, 65). Could expression of an effector phenotype distinguish between nTregs from iTregs? This is unlikely given that both potentially co-inhabit the tumor and are subjected to similar antigenic cues. Cretney et al., opined that activated/effector Treg cells display unique phenotypic features that distinguishes them from naïve cells (66, 67). In one of their studies, they described a distinct population of Blimp-1-expressing Treg cells with an effector phenotype (67). Given that IL-2 and inflammatory signals was shown to facilitate their production, one might speculate that the prevalence of such inflammatory cytokines/signals in the tumor surroundings may favor the recruitment or generation of these functionally mature effector Treg cells. In this context, Blimp-1 could be useful to identify effector Tregs which are derived from the natural pool versus those induced from CD25− cells *in situ*. Perhaps, an evaluation of a plethora of activation-associated markers such as described by Cretney and colleagues may yield some clues as to which subset of tumor-infiltrating Treg cells are natural or induced regardless of their antigen experience.

At the genetic level, molecular analysis has revealed that while nTreg cells show a stable hypomethylation pattern at the Foxp3 locus, iTregs generated *in vitro* and *in vivo* are fickle, presenting with unstable Foxp3 expression with partial hypomethylation pattern (68, 69). Although both iTreg and nTreg in the tumor may be indistinguishable in terms of having an effector phenotype, assessing Foxp3 epigenetic modification patterns could be useful to differentiate nTregs from iTregs.

TCR REPERTOIRE DIVERSITY AND ANTIGEN SPECIFICITY OF TUMOR-INFILTRATING TREG CELLS

Currently, there is paucity of data addressing the issue of antigen specificity and TCR repertoire within tumor-associated Treg cells and how this information may define induced versus nTreg cells. The notion that Treg cells accumulating within tumors might be nTreg cells was presented by Gallimore's lab. In one of their studies as mentioned previously, they analyzed the TCR repertoires of Treg cells and T conventional cells within the tumor tissue and found that they were largely distinct concluding that based on this finding, tumor-Tregs are likely derivatives of nTregs (17). In another study using non-TCR-transgenic mice, immunoscope-based analysis of the TCR repertoire of tumor-infiltrating Treg cells and T effector cells revealed that each population exhibited a skewed and distinct repertoire indicative of clonal expansion, hinting that the tumor-infiltrating Tregs are likely a few clones

that proliferate extensively in the tumor (70). Further analysis of CDR3 sequences revealed some public sequences that were unique to Treg cells obtained from multiple tumor tissues but had little overlap with T effector cells arguing against the possibility that the Treg cells were converted from T effector cells, although based on the limited scope of the work, such possibility still cannot be excluded.

Treg cells are selected with TCRs specific for self peptide: MHC constituents (71, 72) and many TAA are self antigens (73). Furthermore, Treg cells can recognize an array of tumor-associated immunogenic self antigens (74, 75). So, it is possible that tumor-infiltrating Treg cells exhibit unique TCR repertoire highly reactive against some of the TAA. Supporting this notion, a human-melanoma-infiltrating Treg clone specific for LAGE-1, a cancer/testis antigen that is expressed in many types of tumors was identified in a study (76). It should be reiterated here that the expression of cancer/testis antigens is normally restricted to male germ cells but not in adult somatic tissues. On that account, they are cancer tissue-specific self antigens. In another study, the same group reported the establishment of CD4+ Treg clones generated from tumor-infiltrating lymphocytes of cancer patients which were reactive against another tumor-derived ARTC1 peptide (77). In another unrelated study, NY-ESO-1 (New York esophageal squamous-cell carcinoma-1)-specific CD4+ T cells were generated from naïve T cell preparation upon Treg cell depletion suggesting that Treg cells, presumably an antigen-specific subset suppressed NY-ESO-1-specific T cell induction in cancer patients (78). Thus, circulating tumor-antigen-specific Treg cells exist at least in patients with certain cancers (79). While these studies suggest to a certain extent, the self specificity of tumor-infiltrating Treg cells, the issue of their origin was not addressed. How might iTreg cells and nTreg cells in the tumor differ with respect to their antigen specificity and repertoire? Answering this question requires a clear understanding of which of these two subsets predominates in specific cancers. Then, our efforts could expand to deciphering their peptide specificity, immunodominant epitopes of such peptides, and TCR diversity of Treg cells that may recognize them through combination of techniques including but not limited to cloning, proteomics, and spectratyping analysis.

TUMOR-TREG CELL RECRUITMENT AND TRAFFICKING

The recruitment of Treg cells (natural or induced) into tumors likely involves complex, multi-step processes that ultimately culminate in the high frequencies observed in many cancers. Perhaps, the expression of certain receptors may be key to unraveling some of these processes and sorting the suppressor cells. One potential candidate protein is Neuropilin-1 (Nrp-1), the expression of which was found to be low on *in vivo*-generated iTreg cells under non-inflammatory conditions unlike nTreg cells which preferentially expressed this protein at high levels (43). In tumor-bearing mice, Nrp-1 expression on Treg cells was demonstrated to promote their recruitment to tumor site via tumor-derived VEGF gradient (80). Anecdotally, Nrp-1, the expression of which is very low in naïve T conventional cells is under Foxp3 control as ectopic expression of Foxp3 in these cells led to induction of Nrp-1 (37, 81). Given that TGF-β can bind Nrp-1 in addition to inducing Foxp3 expression (35, 82), it remains plausible that TGF-β-induced Foxp3+ iTreg cells, armed with Foxp3-induced Nrp-1 expression, respond to further TGF-β binding in a positive feedback loop, and ultimately become recruited across similar gradient as the nTreg cells.

Chemokine receptor pattern while largely unexplored, could be another critical aspect of tumor-affiliated Treg cells that could be useful in determining Tumor-Treg sub-groups. For example, in human ovarian carcinomas, selective accumulation of Treg cells expressing high levels of chemokine receptor CXCR3 was noted (41). Similarly, Treg cells that infiltrated colorectal tumor mass preferentially expressed CCR6 which appeared to promote their recruitment via tumor-associated macrophage production of CCL20 (83). In skin tumor-bearing mice, CCR5 was preferentially expressed on tumor-infiltrating Treg cells, which seemed to be recruited to the tumor via its ligands, CCL3, 4, and 5 that was produced by myeloid-derived suppressor cells (MDSCs) (84). Similarly, CCR5 signaling appeared to facilitate the recruitment of Treg cells to pancreatic adenocarcinoma (85). Other chemokine receptors implicated in Treg trafficking to tumor sites include CXCR4, which drives Treg cells toward tumor site via interactions with CXCL12 that is produced within the tumor microenvironment, as well as CCR8 and CCR10 (86–88). In the case of CCR10, hypoxia within ovarian tumor environment promotes the secretion of CCL28 by cancer cells which in turn enhances the recruitment of Foxp3+ Treg cells via CCR10 (87). Furthermore, in studies of oral squamous-cell carcinoma and colon adenocarcinoma, increased frequencies of tumor-associated CCR4hi cells were reported (89, 90). Consistent with this and other reports (16, 91, 92), we have recently identified CCR4 to be highly expressed on the majority of tumor-infiltrating Treg cells in a human melanoma study (manuscript in preparation). Notably, their phenotype was unique and distinct from their counterparts in non-tumor-associated peripheral blood. Whether these Treg cells are peripherally recruited by tumor-derived factors such as CCL22, which is a chemokine that is widely produced by a number of tumors, and a ligand for CCR4 (12, 65) remains to be determined and is a subject of our ongoing investigations.

In contrast to our observations and that of others mentioned above, one report found that tumor-infiltrating Treg cells exhibited markedly reduced levels of CCR4 in HNSCC relative to circulating Tregs (61). One obvious explanation for variabilities between these studies is that differences in tumor type, infiltrating immune cells, and stage of disease likely impacts the phenotype of Treg cells prevalent within tumors at time of investigation. Despite the lack of any extrapolation from all these studies as to the natural or induced status of tumor-Treg cells, they bring to light, the notion that the tumor milieu likely shapes the composition of Treg cells present within it as different Treg cell subsets express different homing receptors based on the environmental cues to which they are subjected (93). Thus, different tumors may exhibit distinct Treg cell composition that reflects such properties. In this regard, evaluation of homing receptor expression pattern in various human cancers may thus shed more light to whether they are locally induced, or are expanded from a recruited natural population.

INDUCED/ADAPTIVE TREG GENERATION IN TUMORS

The mechanisms involved in *de novo* generation of adaptive Treg cells are still unclear. Several lines of evidence point to the