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Characterization and functional studies of forkhead box protein 3$^-$ lymphocyte activation gene 3$^+$ CD4$^+$ regulatory T cells induced by mucosal B cells

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Summary

The induction of mucosal tolerance has been demonstrated to be an effective therapeutic approach for the treatment of allergic diseases. Our previous study demonstrated that Peyer's patch B cells could convert naive T cells into regulatory T cells (so-called T_{reg}-of-B(P) cells); however, it is important to characterize this particular subset of T_{reg}-of-B cells for future applications. This study aimed to investigate the role of lymphocyte activating gene 3 (LAG3) in mediating the regulatory function of T_{reg}-of-B(P) cells induced by mucosal follicular B (FOB) cells. Microarray analysis and real-time polymerase chain reaction (PCR) were used to assess the gene expression pattern of T_{reg}-of-B(P) cells. To evaluate the role of LAG3, the *in-vitro* suppressive function and the alleviation of airway inflammation in a murine model of asthma was assessed. Our data indicated that FOB cells isolated from Peyer's patches had the ability to generate more suppressive T_{reg}-of-B cells with LAG3 expression, compared with CD23^{lo}CD21^{lo} B cells. LAG3 is not only a marker for T_{reg}-of-B(P) cells, but also participate in the suppressive ability. Moreover, CCR4 and CCR6 could be detected on the LAG3$^+$, not LAG3$^-$, T_{reg}-of-B(P) cells and would help cells homing to allergic lung. In the murine model of asthma, the adoptive transfer of LAG3$^+$ T_{reg}-of-B(P) cells was able to sufficiently suppress T helper type 2 (Th2) cytokine production, eosinophil infiltration and alleviate asthmatic symptoms. LAG3 was expressed in T_{reg}-of-B(P) cells and was also involved in the function of T_{reg}-of-B(P) cells. In the future, this particular subset of T_{reg}-of-B cells might be used to alleviate allergic symptoms.

Keywords: airway hyperresponsiveness, mucosal tolerance

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Introduction

Mucosal tolerance, which induces immunological tolerance to non-pathogenic antigens in the mucosa of the respiratory, gastrointestinal and urogenital tracts, has been used in humans for the treatment of allergic diseases for a century [1,2]. In addition, both oral and nasal tolerance are used to treat several inflammatory diseases, including experimental autoimmune encephalomyelitis, arthritis and food allergies [3–5]. It has been proposed that clonal deletion due to high-dose antigen exposure and regulatory T cell (T_{reg}) production or anergy due to low-dose antigen exposure results in the induction of mucosal tolerance [6–9].

Organized lymphoid tissues are associated with each organ system and are thought to be the site of naive T cell priming and immune response initiation. Cervical lymph nodes (CLNs) and Peyer's patches are the major sites for tolerance induction [10]. Previous studies have indicated that mucosal tolerance cannot be elicited in mice without CLNs or Peyer's patches [11,12]. In addition to the microenvironment in lymph nodes, antigen-presenting cells play an important role in tolerance induction. Interleukin (IL)-10- and transforming growth factor (TGF)-β-producing dendritic cells (DCs) from the mesenteric lymph nodes (MLNs) of antigen-fed mice stimulate antigen-specific CD4$^+$ T cells to produce IL-10 or TGF-β [13,14]. Mucosal macrophages have been found to exert anti-inflammatory effects that inhibit T helper type 17 (Th17) cell differentiation [15]. Recently, the function of B cells in tolerance has been noted. It has been reported that mucosal tolerance cannot be induced in B cell-deficient mice [3,16]. The mucosal administration of antigen to B cell-deficient μMT mice resulted in a reduced number of forkhead box protein 3 (FoxP3)$^+$ T_{reg} cells and deficient T_{reg} cell function

[17]. In addition, naive B cells can generate T_{reg} cells without increasing FoxP3 expression [18]. Our previous study demonstrated that mucosal B cells have a better ability to convert naive T cells into T_{reg} cells, so-called T_{reg}-of-B(P) cells [19]. These T_{reg}-of-B(P) cells, which produce more IL-10 and express cytotoxic T lymphocyte antigen 4 (CLTA-4), inducible co-stimulator (ICOS), OX40 (CD134), programmed death-1 (PD-1) and tumour necrosis factor (TNF)-RII, alleviate allergic airway inflammation.

Recently, lymphocyte activation gene 3 (LAG3) has been identified as a marker of T_{reg} cells. LAG3 mRNA is expressed selectively by naturally occurring T_{reg} (nT_{reg}) cells and is not found in CD4$^+$ CD25$^-$ T cells [20]. In addition to modulating T_{reg} cell function *in vitro* and *in vivo*, the ectopic expression of LAG3 confers a regulatory function to CD4$^+$ T cells. A clinical study demonstrated that one particular T_{reg} cell population, CD4$^+$CD25^{hi}FoxP3$^+$LAG3$^+$ cells, expanded preferentially in peripheral blood mononuclear cells (PBMCs) and tumour-infiltrating lymphocytes (TILs) in cancer patients and might contribute to tolerance at tumour sites [21]. In Peyer's patches, CD4$^+$LAG3$^+$ T cells are enriched to approximately 8%, compared with 2% in the spleen, implying that LAG3 might participate in mucosal tolerance [22]. In the present study, our data showed that LAG3 was expressed in T_{reg}-of-B(P) cells and modulated the suppressive function of these cells. The number of LAG3$^+$CD4$^+$ T cells in the Peyer's patches increased after the oral administration of ovalbumin (OVA). We also demonstrated that follicular B (FOB) cells in Peyer's patches had a better ability to generate T_{reg}-of-B(P) cells compared with CD23^{lo}CD21^{lo} B cells. Finally, the results showed that sorted LAG3$^+$FoxP3$^-$ T_{reg}-of-B(P) cells could alleviate allergic airway inflammation and hypersensitivity.

Materials and methods

Animals

Male BALB/c mice, OVA-T cell receptor (TCR) transgenic (DO11·10) mice and FoxP3–green fluorescent protein (GFP) transgenic mice aged 6–8 weeks were obtained and maintained in the National Laboratory Animal Center. The DO11·10 mice had transgenic TCRs that recognize the 323–339 peptide fragments of OVA. DO11·10 mice were crossed with FoxP3–GFP mice to generate heterozygous OVA–TCR transgenic mice (F_1 mice), in which GFP was detected along with FoxP3 expression. The Animal Research Committee of the college approved the animal study protocol.

Preparation of T_{reg}-of-B cells

The protocol for T_{reg}-of-B cell generation and the characteristics of these cells are as described previously [19]. Naive CD4 T cells from spleens of DO11·10 × FoxP3–GFP F_1 mice were enriched by negative isolation via immunomagnetic depletion (IMag; BD Pharmingen, San Diego, CA, USA) to purities of more than 90%. Separation of B cells from BALB/c mice resulted in 90–95% purity by B220 expression via immunomagnetic-positive selection (IMag; BD Pharmingen). Peyer's patches DCs are enriched to >90% purity by positive magnetic affinity cell sorter (MACS) selection using CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Different subsets of B cells are sorted by the expression of CD21 and CD23. First, B cells are isolated from Peyer's patches and spleen then stained with fluorescein isothiocyanate (FITC)-anti-CD21 and phycoerythrin (PE)-anti-CD23 (BD Pharmingen). These labelled cells were sorted on a fluorescence activated cell sorter (FACS)Aria (BD Biosciences, San Jose, CA, USA) through the service provided by the Cell Sorting Core Facility (the First Core Laboratory, National Taiwan University College of Medicine). Bone marrow-derived dendritic cells (BMDCs) were prepared as described previously [23]. Briefly, bone marrow cells were cultured in RPMI-1640/5% fetal bovine serum (FBS) in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (500 U/ml) and IL-4 (1000 U/ml) (PeproTech Inc., Rocky Hill, NJ, USA).

The protocol of T_{reg}-of-B cells is as described previously [19]. To assess the ability of Peyer's patch DCs and B cells to generate regulatory T cells, B cells and DCs were isolated from Peyer's patches and pulsed with $OVA_{323–339}$ 10 μg/ml and 1 μg/ml, respectively, in culture medium (RPMI-1640 supplemented with 5% FBS, 2·5 mM HEPES, 4 mM L-Gln and 100 U/ml penicillin, 100 μg/ml streptomycin and 0·25 μg/ml amphotericin) overnight, then co-cultured with CD4$^+$CD25$^-$ T cells (B : T = 1:1, DCs : T = 1:10) for 3 days. BMDCs are harvested on day 8 and added with $OVA_{323–339}$ 1 μg/ml 4 h prior to culturing with naive T cells. As part of the LAG3 induction, T cells were cultured with anti-CD3 plus anti-CD28 1 μg/ml for 3 days then applied for LAG3 detection. B-cell-primed T cells (labelled as T_{reg}-of-B) were applied to the following experiments.

Microarray analysis

RNA was extracted from nT_{reg}, naive CD4 T and T_{reg}-of-B(P) cells by the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA samples (0·2 μg for each) were amplified by low RNA Input Quick Amp Labeling kit (Agilent Technologies, Foster City, CA, USA) and labelled with cyanin 3–cytidine 5′-triphosphate (Cy3-CTP). All Cy3-labelled cRNAs were hybridized to Agilent Mouse G3 Whole Genome Oligo 8 × 60 K microarray. After washing and drying by nitrogen blowing, microarrays were scanned with an Agilent microarray scanner at 535 nm for Cy3. The scanned images were analysed by Feature Extraction version 10·5.1·1 software (Agilent Technologies) to quantify the signal and background intensity for each feature, and normalization by the 75 percentile method. Only the features

with a signal-to-noise ratio of >2·6 were retrieved for further analysis.

Determination of the frequency of $CD4^+FoxP3^-LAG3^+$ T cells in Peyer's patches and spleen

Mice were administered OVA 0·5 mg per day orally for 5 consecutive days and killed on days 0, 6 and 8. Cells were isolated from Peyer's patches and spleen then applied for FACS analysis.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from T_{reg}-of-B cell using Trizol reagent (Invitrogen, Life Technologies, Paisley, UK) and then reverse-transcribed into cDNA using random hexamers [SMART reverse transcription–polymerase chain reaction (RT–PCR) kit; BD Biosciences Clontech, Palo Alto, CA, USA]. Gene expression of LAG3 was determined in triplicate by quantitative real-time PCR using SYBR Gene Expression Assays according to the manufacturer's protocol on an ABI 7500Fast (Applied Biosystems, Life Technologies, CA, USA). Amplification of the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed in order to standardize the amount of sample cDNA added.

Determination of cytokine levels

Quantitative enzyme-linked immunosorbent assay (ELISA) was performed to assay IL-2, IL-4, IL-5, IL-10, eotaxin (R&D Systems, Abingdon, UK) and IFN-γ (BD OptEIA) using paired monoclonal antibodies (mAbs) specific for the corresponding cytokines.

Cultured supernatants of splenocytes, T_{reg}-of-B cells and nT_{reg} cells

In supernatants of splenocytes, cells isolated from spleen were cultured at 1×10^6 cells per well with OVA 5 μg/ml for 48 h. To determine the cytokine secreted by T_{reg}-of-B cells, nT_{reg} cells and naive T cells, 5×10^5 cells per well were stimulated with $OVA_{323–339}$ 1 μg/ml presented by irradiated splenocytes for 48 h. The supernatant was collected and stored at −20°C until further experiment.

Suppression function

To address the suppressive function of T_{reg}-of-B cells, after 3 days of stimulation T_{reg}-of-B cells were harvested and cultured with $CD25^-CD4^+$ T cells isolated from DO11·10 mice (as responder T cells) and irradiated splenocytes in the presence of $OVA_{323–339}$ 5 μg/ml for 96 h. Ten μg/ml neutralizing antibodies, including anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) (9H10; Biolegend, San Diego, CA, USA), anti-ICOS (7E, 17G9; Biolegend), anti-IL-10R (1B1·3a; BD Pharmingen) and anti-LAG3 (C9B7W; BD Pharmingen) antibodies, were added 1 h prior to adding responder T cells. Proliferative response was measured by addition of 1 μCi $[^3H]$-thymidine incorporation into the culture for the last 16 h. Thymidine uptake was determined using a β-counter (Packard Instrument Co., Meriden, CT, USA) and expressed as counts per minute (cpm).

FACS analysis and cell sorting

For infiltrated eosinophil determination, isolated bronchoalveolar lavage fluid (BALF) cells were stained with fluorescence labelled mAbs. mAb against major histocompatibility complex (MHC)-II, CD3, B220 and CD11c were purchased from BD Pharmingen; mAb against CCR3 was purchased from R&D Systems). To prevent non-specific binding to Fc receptors, 2·4G2 blocking reagent (BD Pharmingen) was added. For cell surface marker staining, mAbs against CD4, CD194 (CCR4), CD196 (CCR6) and CD49b were purchased from Biolegend; mAbs against LAG3, latency associated peptide (LAP), CD25, CD44 were purchased from BD Pharmingen, mAbs against CD103 was purchased from eBioscience. For the detection of different B cell subpopulations, Peyer's patch B cells were stained with mAbs against B220, CD23 and CD21 (BD Pharmingen) [24]. These labelled cells were sorted on a FACSAria (BD Biosciences) through the service provided by the Cell Sorting Core Facility (the First Core Laboratory, National Taiwan University College of Medicine).

Adoptive transfer of T_{reg}-of-B cells for the alleviation of OVA-induced allergic airway inflammation

OVA-induced airway inflammation was established as described previously [19]. Six to 8-week-old BALB/c mice were sensitized by intraperitoneal injections of 50 μg OVA emulsified in 4 mg of alum on day 0, and 25 μg OVA mixed with 4 mg of alum on days 14, 21 and 28. On days 42–44, mice were challenged with OVA 100 μg/mouse (in total volume 40 μl) by intranasal administration. On day 45, airway hyperresponsiveness was measured and mice were killed on day 46. $FoxP3^-LAG3^+$ T_{reg}-of-B(P) cells (2×10^6 cells per mouse) were sorted (Supporting information, Fig. S1) and injected intravascularly into mice on day −1. Asthmatic control mice were injected in a similar manner with PBS. The naive group received challenge but without sensitization.

Measurement of OVA-specific antibodies

OVA-specific immunoglobulin (Ig)E, IgG1, IgG3, IgG2b and IgG2a titres in serum were determined by ELISA without pretreatment. Levels of antibodies were compared

with standard serum and the concentration of all immunoglobulin subsets in standard serum was arbitrarily assigned one ELISA unit (EU): EU = (a sample – a blank)/(a positive – a blank).

Measurement of airway hyperresponsiveness

The airway responsiveness to aerosolized methacholine (MCh) (Sigma, St. Louis, MO, USA) was measured as described previously [25]. The mice were placed into the main chamber (Buxco Electronics. Inc., Sharon, CT, USA) and challenged with aerosolized 0·9% normal saline, accompanied by increasing doses of MCh (6·25–50 mg/ml). The Penh [enhanced pause = pause × (peak expiratory box flow/peak inspiratory box flow)] values were determined. The Penh value was expressed as a relative increase ratio in response to PBS challenge.

BALF study

BALF was collected from each mouse, as described previously [19]. Supernatant derived from the first lavage was stored at −20°C until measurement. Levels of cytokines were determined by ELISA and the cell pellet was resuspended to two subsequent lavages for cell counting and cell subset determination. Infiltrated cells were counted and classified as macrophages, lymphocytes, neutrophils or eosinophils with the expression of MHC-II, CD11c, B220, CD3 and CCR3 [26] to analyse the inflammatory cell population in the BALF.

Cells were analysed on a FACSCalibur (BD Biosystems, Franklin Lakes, NJ, USA) using CellQuest (BD Immunocytometry Systems, San Jose, CA, USA). Lymphocytes were identified as forward-scatter (FSC)^{lo}/side-scatter (SSC)^{lo} and expressing CD3 or B220; B cells were distinguished from T cells by MHC-II expression in the (B220/CD3)$^+$ gate. Granulocytes were recognized as non-autofluorescent highly granular (SSC^{hi}) cells, and within this gate eosinophils were defined as cells expressing the CCR3 [27], intermediate levels of CD11c and very low to undetectable expression of MHC-II, B220 and CD3. Neutrophils had a similar scatter profile to eosinophils, but lacked CCR3 expression. DCs were identified as (CD3/B220)$^-$, and expressing high levels of MHC-II and CD11c. Alveolar macrophages cells were identified as large autofluorescent cells.

Histopathological study

After lavage, the lungs were removed immediately, fixed in 10% buffered formalin, and embedded in paraffin. Sections (5 μm thick) were stained with haematoxylin and eosin (H&E) and examined by light microscopy for histological changes.

Statistical analysis

All statistical analyses were performed with Prism version 6·0 (GraphPad Software, San Diego, CA, USA) software. Dual comparisons were made with Student's *t*-test. Groups of three or more were analysed by analysis of variance (ANOVA), with Dunnett's post-tests for experiments comparing treatments to controls. *P*-values < 0·05 were considered statistically significant.

Results

T_{reg}-of-B(P) cells express LAG3

Similar to the previous study [19], CD4$^+$FoxP3$^-$ T_{reg}-of-B(P) (T_{reg}-of-B(P) cells, which are generated by Peyer's patch B cells cultured with naive CD4$^+$CD25$^-$ T cells in the presence of $OVA_{323–339}$, suppressed the proliferation of responder T cells (Fig. 1a). In contrast to CD4$^+$FoxP3$^+$ nT_{reg} cells, T_{reg}-of-B(P) cells secreted higher levels of IL-10 (Fig. 1e). Thus, we hypothesized that certain molecules are involved in the regulatory function of T_{reg}-of-B(P) cells. Microarray analysis was performed with single-colour mouse chips, and pairs of T_{reg}-of-B(P) and naive T cells or T_{reg}-of-B(P) and nT_{reg} cells were compared. Previous studies have shown that the expression of both IKAROS family zinc finger (Ikzf)4 and Ikzf2, which are also known as Eos and Helios, respectively, and are highly expressed in nT_{reg} cells [28,29], are decreased more than twofold in T_{reg}-of-B(P) cells, compared with the naive T or nT_{reg} groups (Fig. 1b). Compared with nT_{reg} cells, T_{reg}-of-B(P) cells expressed a higher level of LAG3, and this result was confirmed by immunofluorescence staining and real-time PCR (Fig. 1c and Supporting information, Fig. S2a). A similar result was also observed in B lymphocyte-induced maturation protein 1, Blimp1 (prdm1) expression (Supporting information, Fig. S2b). It has been reported that LAG3$^+$CD4$^+$ T cells are enriched in Peyer's patches to a greater degree than in the spleen [22], implying that LAG3$^+$ T cells might be associated with oral tolerance. In the present study, our data showed that a greater number of FoxP3$^-$LAG3$^+$ T cells were present in Peyer's patches. In contrast to the number of FoxP3$^-$LAG3$^+$ CD4$^+$ T cells in the spleen, the number of FoxP3$^-$LAG3$^+$CD4$^+$ T cells in Peyer's patches increased after the administration of OVA 0·5 mg for 5 days, implying that naive T cells exposed to OVA presented by Peyer's patch antigen-presenting cells can become LAG3$^+$ T cells (Fig. 1d).

Because T_{reg}-of-B(P) cells could secrete higher amounts of IL-10 and express LAG3, we further investigated the expression of CD25, CD44, CD49b and CD103, which were expressed on the Tr1-type cells and inducible regulatory T cells [30–32]. FACS data showed that T_{reg}-of-B(P) cells expressed higher levels of CD25 and CD44. In contrast, CD49b, CD103 and LAP were not detected (Fig. 1f). Chemokine receptors, which mediated cell migration, were

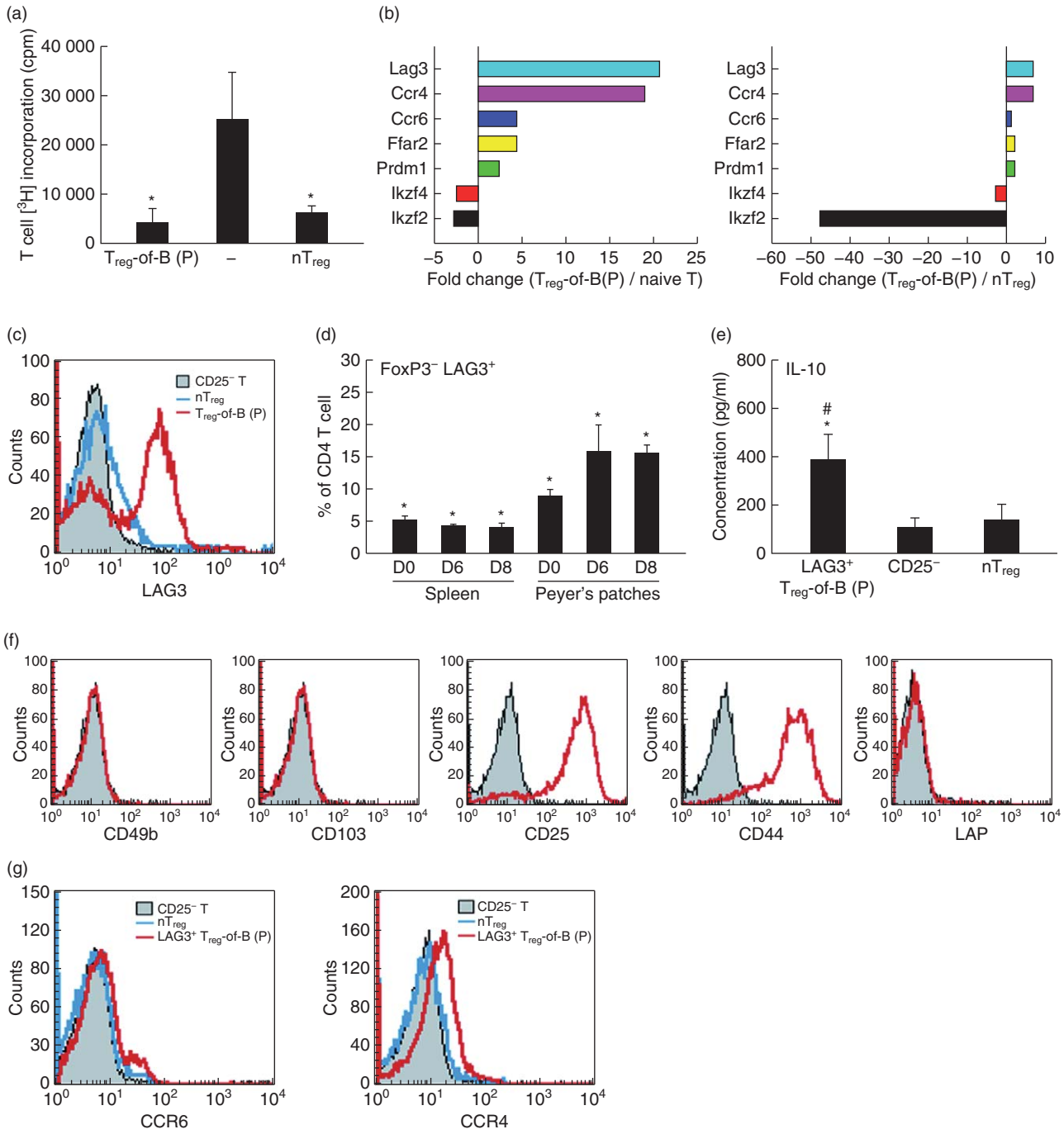

Fig. 1. (a) The suppressive function of Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells]. Freshly isolated $CD4^+CD25^+$ naturally occurring regulatory T cells (nT_{reg}) served as a positive control (nT_{reg}), and $CD4^+CD25^-$ T cells served as responder T cells (–). (b) The gene expression profile was evaluated by microarray analysis, and the results are shown as the fold change between T_{reg}-of-B(P) cells and naive T cells (left) and between T_{reg}-of-B(P) and nT_{reg} cells (right). (c) Lymphocyte activation gene 3 (LAG3) expression was detected in T_{reg}-of-B(P) cells by fluorescence staining. (d) The frequency of $CD4^+$ forkhead box protein 3 $(FoxP3)^-$ $LAG3^+$ T cells in Peyer's patches and spleen by mice fed with ovalbumin (OVA) was determined on days 0, 6 and 8 (right, labelled D0, D6 and D8, respectively). (e) Interleukin (IL)-10 production by $LAG3^+$ T_{reg}-of-B(P), $CD4^+CD25^-$ naive T cells ($CD25^-$) and nT_{reg} cells. (f) Representative expression of CD49b, CD103, CD25, CD44 and latency-associated peptide (LAP) (red line) with isotype controls (grey-shaded) on T_{reg}-of-B(P) cells. (g) Representative expression of CCR6 and CCR4 on $LAG3^+$ T_{reg}-of-B(P) cells, $n = 5$ per experiment. The same results were obtained in three other experiments. The results are expressed as the mean ± standard error of the mean (s.e.m.). $^*P < 0{\cdot}05$ compared with the responder T only group, Peyer's patches D0 group or $CD25^-$ group. $^\#P < 0{\cdot}05$ compared with the nT_{reg} group.

also investigated. Microarray data showed that CCR4 and CCR6 are more highly expressed in T_{reg}-of-B(P) cells. CCR6 regulates cell migration to lung and gut [33]. CCR4 is critical for cell migrating to allergic lungs [34]. Our data showed that T_{reg}-of-B(P) cells, which gated on the LAG3$^+$ population, display increased CCR4 and CCR6, compared with naive T cell and nT_{reg} cell. In contrast, the LAG3$^-$ population did not express these receptors (Fig. 1g and Supporting information, Fig. S2c). This finding indicated that LAG3$^+$ T_{reg}-of-B(P) cells would be chemoattracted to inflammatory lung though these two receptors. Moreover, free fatty acid receptor 2 (FFAR2) is highly expressed in T_{reg}-of-B(P) when compared to CD25$^-$ cells, and in LAG3$^-$ compared to LAG3$^+$ cells (Supporting information, Fig. S2d).

T_{reg}-of-B(P) cells generated by follicular B cells in Peyer's patches have better suppressive function than by CD21^{lo} CD23^{lo} B cells

B cells play an important role in mucosal tolerance induction and maintain the microenvironment in the intestine [3]. Different subsets of B cells express CD23 and CD21, including CD21^{lo}CD23^{hi} follicular B (FOB) cells and CD21^{hi}CD23^{lo} marginal zone B (MZB) cells. In several models of infectious diseases, splenic MZB cells are the major source of IL-10 and play a protective role in mice with severe susceptibility to bacteria [24,35]. In this study, we found that B cells in Peyer's patches, in contrast to B cells in the spleen, comprise FOB cells and CD23^{lo}CD21^{lo} B cells, and FOB cells accounted for the largest cell population (Fig. 2a). To determine which B cell subset was able to generate T_{reg}-of-B(P) cells, FOB and CD23^{lo}CD21^{lo} B cells were sorted and cultured with naive T cells. The other two types of antigen-presenting cells, Peyer's patch dendritic cells (DCs) and bone marrow-derived dendritic cells (BMDCs), were used as controls [36]. Figure 2b shows that compared with CD23^{lo}CD21^{lo} B cells, FOB cells were better in inducing T_{reg} cells. As expected, naive T cells activated by mature BMDCs did not suppress the proliferation of responder T cells. Peyer's patch DCs, which have been suggested to be able to capture oral antigens and induce the homing of T_{reg} cells to the gut [37], also generated T_{reg} cells, although FOB cells seemed to be more efficient. The higher level of IL-10 production could be detected in T_{reg} cells generated by FOB and CD21^{lo}CD23^{lo} Payer's patch B cells; however, increased IL-4 was shown in T_{reg} cells induced by CD23^{lo}CD21^{lo} B cells (Fig. 2c). The higher level of IL-4 might lead to less effective suppression [38,39]. LAG3 expression is another candidate to give T_{reg}-of-B(P) cells suppressive ability. Similar to T cells cultured with Peyer's patch DCs, T_{reg} cells generated by Peyer's patch FOB cells had increased LAG3 on the cell surface, compared with T cells cultured with CD23^{lo}CD21^{lo} B cells or activated by anti-CD3 and anti-CD28 antibodies (Fig. 2d). Based on the better suppressive ability of T_{reg} cells, which are generated by Peyer's patch FOB cells and Peyer's patch DCs and could express LAG3 and IL-10, we speculated that IL-10 and LAG3 might be involved in the regulatory function of T_{reg}-of-B(P) cells.

LAG3 and IL-10 participate in the suppressive function of T_{reg}-of-B(P) cells

Because the level of LAG3 expression was elevated in T_{reg}-of-B(P) cells, we determined whether LAG3 was required for the regulatory function of T_{reg}-of-B(P) cells. First, CD4$^+$FoxP3$^-$ naive T cells were isolated and cultured with Peyer's patch B cells. To exclude contaminating nT_{reg} cells, FoxP3–GFP × DO11·10 F_1 male mice were used. In these mice, nT_{reg} cells express GFP and CD4$^+$ T cells recognize the OVA peptide. After 3 days of co-culturing, CD4$^+$LAG3$^+$ T_{reg}-of-B(P) cells were sorted and used in the suppressive function assay in the presence of anti-LAG3 antibodies. Due to the elevated expression of CTLA-4 and ICOS by T_{reg}-of-B(P) cells, and the higher IL-10 production, we also determined whether CTLA-4, ICOS and IL-10 contribute to the suppressive function of these cells. The results indicated that in contrast to blocking CTLA-4 or ICOS, blocking LAG3 and IL-10R abrogated the suppressive function of T_{reg}-of-B(P) cells (Fig. 3). This result suggests that LAG3 is not only a marker of T_{reg}-of-B(P) cells, but is also involved in their function.

LAG3$^+$ T_{reg}-of-B(P) cells decrease OVA-specific IgE and Th2 cytokine production and alleviate asthmatic symptoms

An OVA-immunized murine model of asthma was used to determine the biological effect of LAG3$^+$ T_{reg}-of-B(P) cells *in vivo*. LAG3$^+$ T_{reg}-of-B(P) cells were sorted and transferred adoptively into mice on day −1. Mice were sensitized on day 0, boosted on days 14, 21 and 28 and challenged on days 42–44. One day later, airway hyperresponsiveness was evaluated, and the mice were killed on day 46 (Fig. 4a). Mice transferred with PBS and sensitized with OVA comprised the asthma group. The naive group was composed of mice transferred and sensitized with PBS. In the presence of LAG3$^+$ T_{reg}-of-B(P) cells, the mice had decreased OVA-specific IgE, IgG1 and IgG2b levels and increased OVA-specific IgG2a and IgG3 levels (Fig. 4b). Cytokine production by splenocytes stimulated with OVA was also determined by ELISA. In comparison with the asthmatic group, the LAG3$^+$ T_{reg}-of-B(P) cell-transferred group had decreased levels of IL-4 (Fig. 4c). This result suggests that LAG3$^+$ T_{reg}-of-B(P) cells have a regulatory effect on systemic Th2 responses, including antibody class-switch and cytokine production.

The effect of LAG3$^+$ T_{reg}-of-B(P) cells on asthmatic symptoms was evaluated based on the severity of airway hyperresponsiveness, the extent of inflammatory cell infiltration in the lungs and pulmonary histology. When LAG3$^+$ T_{reg}-of-B(P) cells were present, OVA-sensitized mice had

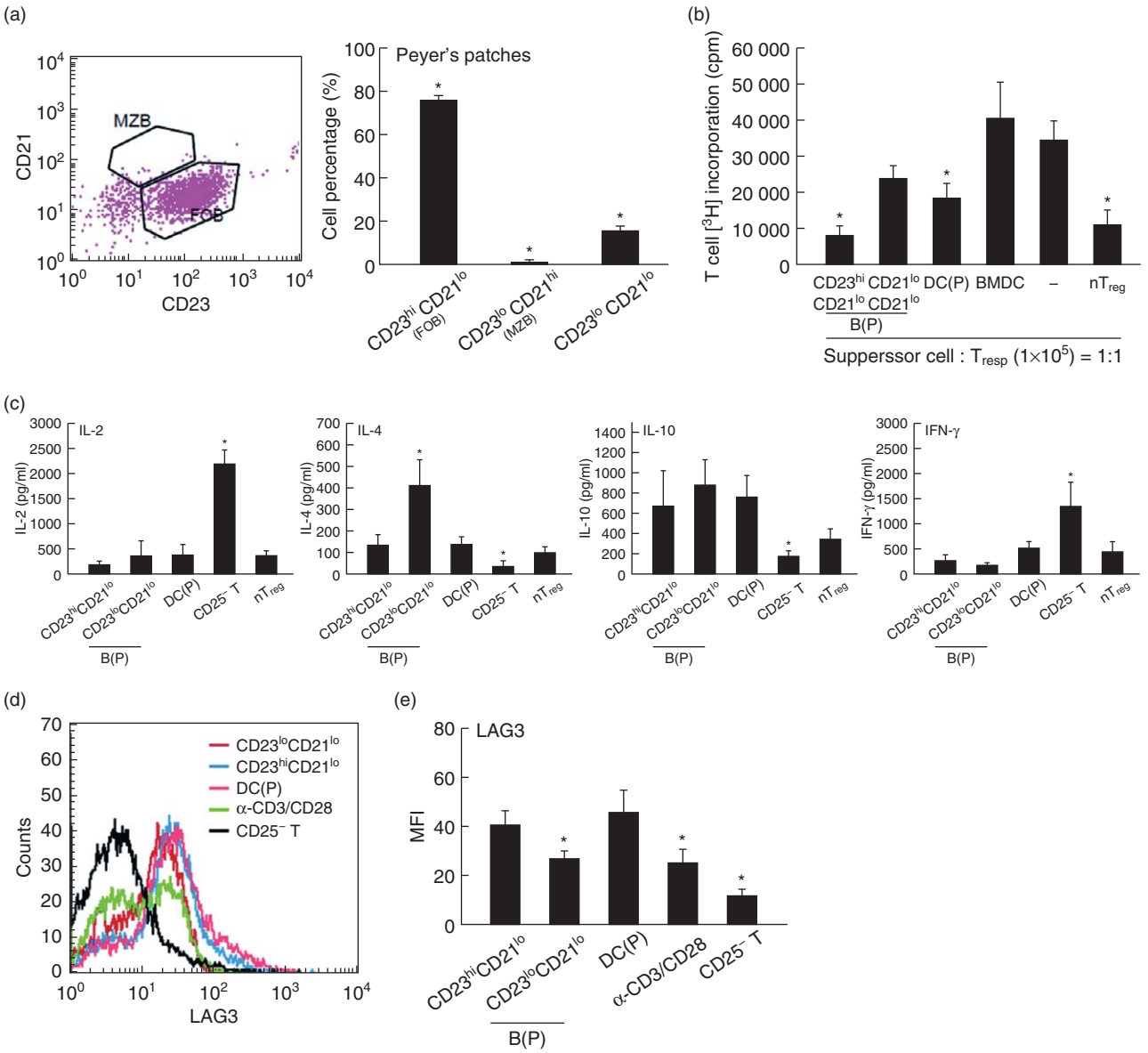

Fig. 2. Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells] generated by follicular B cells in Peyer's patches have better suppressive function. (a) Different B cell subsets that express CD23 and CD21, including follicular B (FOB) cells, marginal zone B (MZB) cells and CD23^{lo} CD21^{lo} cells, are found in Peyer's patches. FOB cells are the most prevalent cell population in Peyer's patch B cells. T_{reg}-of-B cells generated by FOB cells had a better suppressive function (b). (c) After 48 h of restimulation, T_{reg}-of-B(P) cells generated by FOB cells secreted lower interleukin (IL)-2, IL-4 and interferon (IFN)-γ levels and higher IL-10 levels, in contrast to the higher IL-4 levels secreted by CD23^{lo}CD21^{lo} B cells generating T_{reg} cells. (d) Lymphocyte activation gene 3 (LAG3) expression by T cells cultured with Peyer's patch FOB cells, Peyer's patch MZB cells, Peyer's patch DCs and anti-CD3 plus anti-CD28 antibodies, $n = 4$ per experiment. The same results were obtained in five other experiments. The results are expressed as the mean ± standard error of the mean (s.e.m.). *$P < 0{\cdot}05$ compared with CD23^{hi}CD21^{lo} cells or responder T cells only (labelled as –) or naive CD4$^+$CD25$^-$ T cells (labelled as CD25$^-$ T).

lower increases in the Penh value when stimulated with higher concentrations of methacholine, suggesting that these mice exhibited lower airway hyperresponsiveness (Fig. 5a, left). This lower reaction might be resulted from decreased eosinophil infiltration due to the diminished IL-5 and eotaxin production in the lungs (Fig. 5a–c). In addition, these results indicated that LAG3$^+$ T_{reg}-of-B(P) cells generated by Peyer's patch B cells were capable of alleviating allergic airway inflammation (Fig. 6).

Discussion

Recently, the role of LAG3 in T_{reg} cell function has been investigated more extensively. LAG3, which is a homologue

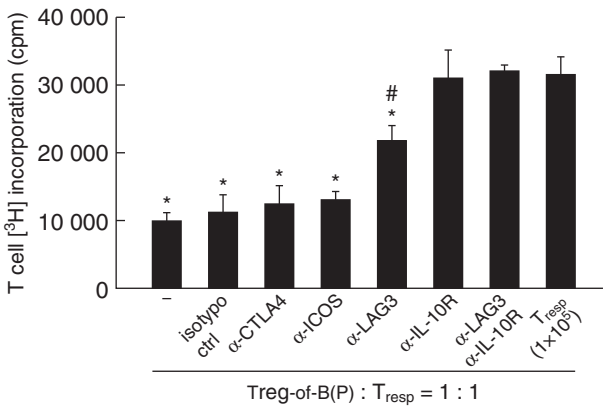

Fig. 3. Lymphocyte activation gene 3 (LAG3) participates in the suppressive function of Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells]. T_{reg}-of-B(P) cells were cultured with responder T cells, ovalbumin (OVA) peptide-pulsed irradiated antigen-presenting cells (APCs) and neutralizing antibodies. In the presence of the anti-LAG3 and anti-interleukin (IL)-10R antibody, the suppressive function of T_{reg}-of-B(P) cells was abrogated, in contrast to the effect of blocking the cytotoxic T lymphocyte antigen 4 (CTLA-4) and inducible co-stimulatory (ICOS) signals, $n = 3$ per experiment. The same results were obtained in five other experiments. The results are expressed as the mean ± standard error of the mean (s.e.m.). *$P < 0{\cdot}05$ compared with responder T cells only (labelled as T_{resp}). #$P < 0{\cdot}05$ compared with T_{reg}-of-B(P) cells (–).

of CD4, could bind to MHC-II molecules with a higher affinity [40,41] and result in the suppression of DC maturation [42]. It has been demonstrated that LAG3 is expressed highly selectively in induced T_{reg} cells and modulates the function of T_{reg} cells both *in vitro* and *in vivo* [20]. In this study, we found that naive CD4$^+$ T cells stimulated by Peyer's patch B cells became T_{reg}-of-B(P) cells and expressed higher LAG3 levels, which participated in the suppressive ability (Figs 1 and 3). It has been reported that, compared with the spleen, Peyer's patches are enriched in CD4$^+$LAG3$^+$ T cells (approximately 8%) [22]. This T cell population is hypoproliferative and is able to inhibit the induction of colitis. Similar to the results of a previous study, higher numbers of LAG3$^+$ T cells were observed in Peyer's patches than in the spleen in the present study. Furthermore, after the oral administration of OVA for 5 days, the proportion of LAG3$^+$CD4$^+$ T cells was increased in Peyer's patches (approximately 15%), although this phenomenon was not found in the spleen (Fig. 1d). These data implied that when antigens enter the intestines, they might be loaded on Peyer's patch B cells and presented to naive T cells. This would help naive T cells to become LAG3$^+$FoxP3$^-$ regulatory T cells.

Several studies indicate that different subsets of inducible T_{reg} cells participate in regulating immune responses. Tr1 cells, which co-express CD49b and LAG3, are shown to maintain immune tolerance in several diseases with higher IL-10 production [30]. CD4$^+$FoxP3$^-$LAP$^+$ T_{reg} cells, which are induced by nasal tolerance, could suppress asthmatic lung inflammation [31]. In the present study, our T_{reg}-of-B(P) cells express LAG3, CD25 and CD44; however, CD49b, LAP and CD103 are not detectable. In addition, the amounts of TGF-β are undetectable in Payer's patch cells and T_{reg}-of-B(P) cells cultured supernatants with OVA stimulation (data not shown). This implies that T_{reg}-of-B(P) cells do not belong to these T_{reg} cell subsets.

A previous study showed that the LAG3 gene is also expressed in nT_{reg} cells; however, the protein expression is lower in nT_{reg} cells [20], as shown in our data, and up-regulation of LAG3 expression requires contact by nT_{reg} cells and antigens presented by APCs (Supporting information, Fig. S3). Our observations showed that, in contrast to naive T cells stimulated with anti-CD3 and anti-CD28, naive T cells cultured with Peyer's patch B cells express higher levels of LAG3 on the cell surface, suggesting that B cells might provide some molecules that are required for LAG3 expression. Another point to consider is that in the human system, T_{reg} cells might suppress activated T cells through the binding of LAG3 to MHC-II molecules expressed by activated T cells and APCs [43]. However, murine T cells do not express MHC-II after activation [44]. Thus, it is unclear whether there are pathways other than the inhibition of DC maturation.

B cells are important in the induction of mucosal tolerance [3,16]. Our previous study indicated that Peyer's patch B cells can generate T_{reg} cells [19]. In the present study, we further investigated the ability of different subsets of Peyer's patch B cells to induce the production of T_{reg} cells. The major Peyer's patch B cell population is comprised of FOB cells (approximately 80%), and MZB cells account for fewer than 1% (Fig. 2a). The main function of FOB cells is to differentiate into antibody-secreting cells in response to thymus-dependent (TD) and thymus-independent (TI) antigens [45,46]. In this study, we found that compared with CD23^{lo}CD21^{lo} B cells, Peyer's patch FOB cells can be useful APCs to generate T_{reg}-of-B(P) cells with higher LAG3 expression. In comparison with Peyer's patch B cells, the expression of LAG3 by different splenic B cells was also determined. B220$^+$ splenic B cells are composed of MZB, FOB and CD23$^-$CD21$^-$ B cells. Our data indicate that LAG3 expressed by T cells cultured with splenic MZB cells is higher, compared with splenic FOB and CD23$^-$ CD21$^-$ B cells (Supporting information, Fig. S4); however, the suppressive ability of these T cell cells remains to be determined. In addition to LAG3 mediating the suppressive function, IL-10 is involved in the suppressive ability of T_{reg}-of-B(P) cells (Fig. 3). Furthermore, the levels of IL-10 are similar in cultures with Peyer's patch FOB cells and DCs, and IL-10 might participate not only in the suppressive function of T_{reg}-of-B(P) cells but also in the suppression function of T_{reg} generated by DCs.

We would like to emphasize the important role of B cells in mucosal tolerance. As found in another study, Peyer's

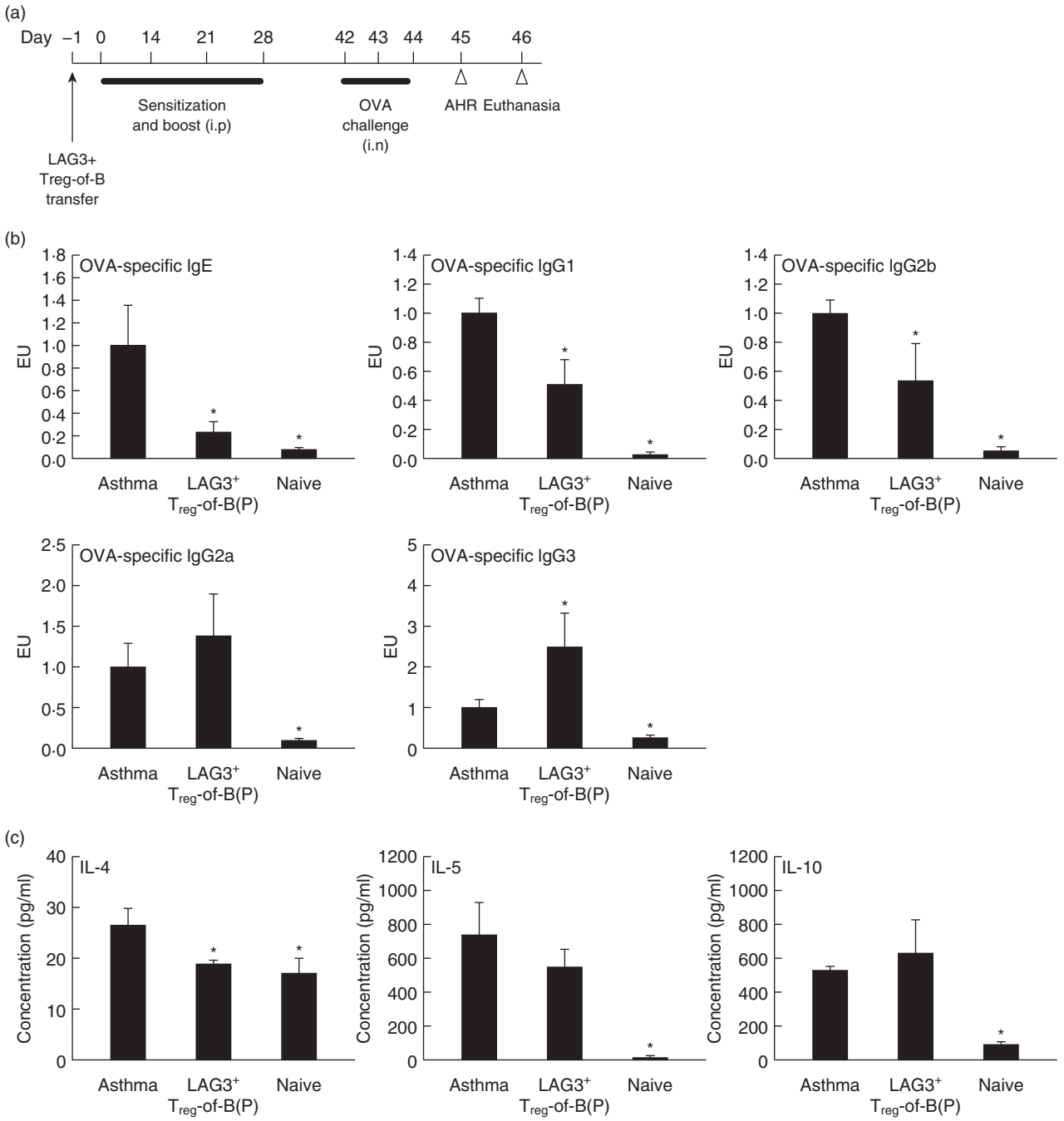

Fig. 4. Lymphocyte activation gene 3 (LAG3$^+$) Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells] suppress ovalbumin (OVA)-specific immunoglobulin (Ig)E and T helper type 2 (Th2) cytokine production. (a) Sensitization protocol. (b) Serum was collected 1 day after aryl hydrocarbon receptor (AHR) detection (day 46) to measure the levels of OVA-specific IgE, IgG1, IgG2b, IgG2a and IgG3. The antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) and compared with the levels in standard serum. The Ig concentrations in the standard serum were set to arbitrarily one ELISA unit (EU), where EU = (a sample – a blank)/(a positive – a blank). (c) Splenocytes were collected 48 h after the last challenge and cultured with OVA for 48 h. The levels of interleukin (IL)-4, IL-5 and IL-10 in the splenocyte culture supernatants were determined by ELISA, $n = 6$ per group. The same results were obtained in two independent experiments. The results are expressed as the mean ± standard error of the mean (s.e.m.). $^*P < 0{\cdot}05$ compared with the asthma group.

patch DCs can generate T_{reg} cells [36] (Fig. 2b); however, B cells are the major cell population in Peyer's patches and are capable of presenting antigens to naive T cells and generating T_{reg} cells [19]. T cells stimulated by Peyer's patch DCs express α4β7 and CCR9, which are required for the homing of T cells to the gut [47]. We found that Peyer's patch B cells induce naive T cells express FFAR2, which is related to gut-homing [48]; however, this receptor is detected in only

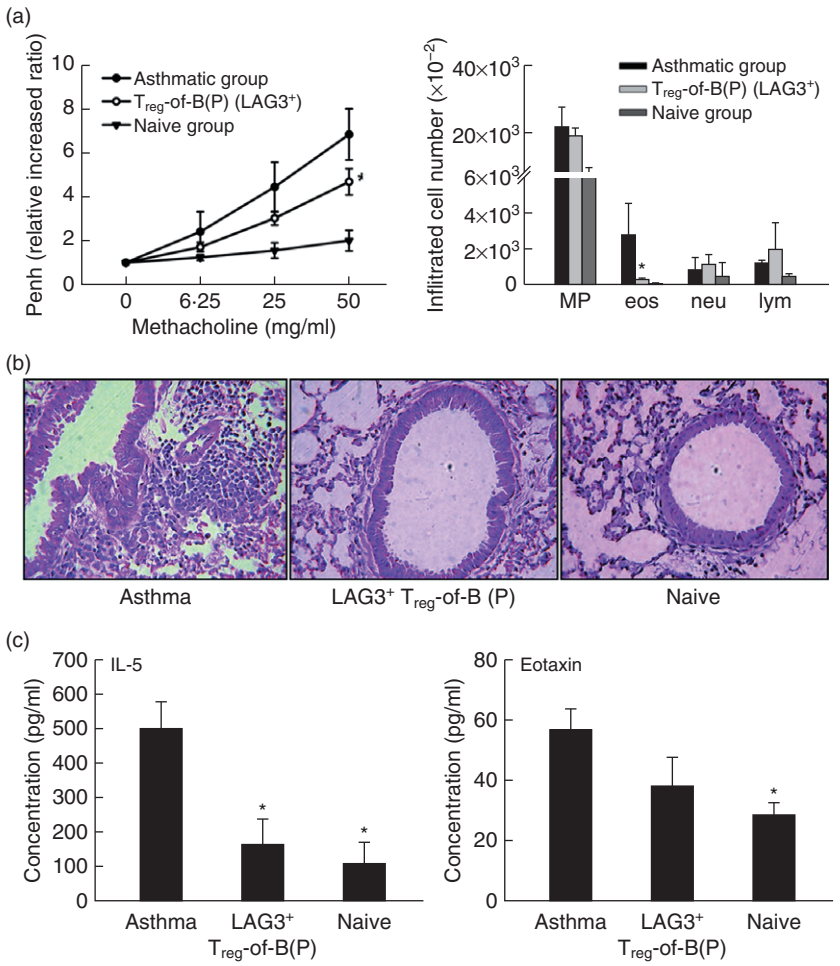

Fig. 5. The effect of lymphocyte activation gene 3 (LAG3$^+$) T_{reg}-of-B cells on pulmonary inflammation. Mice were transferred adoptively with LAG3$^+$ Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells]. Asthmatic mice were transferred with phosphate-buffered saline (PBS) and then immunized with ovalbumin (OVA). Naive mice were transferred adoptively and immunized with PBS. (a) The LAG3$^+$ T_{reg}-of-B(P) group had decreased airway hyperresponsiveness (left) and fewer infiltrated eosinophils in the bronchoalveolar lavage (BALF) (right). (b) Non-sensitized mice had normal pulmonary tissue sections. In sensitized and challenged mice, cells infiltrating the airway were observed; in contrast, the adoptive transfer of LAG3$^+$ T_{reg}-of-B cells decreased the level of cell infiltration. (c) The interleukin (IL)-5 and eotaxin levels were decreased in the adoptive transfer group. Sections stained with haematoxylin and eosin (H&E) (original magnification, c–e: ×200), $n = 6$ per group. The same results were obtained in two independent experiments. The results are expressed as the mean ± standard error of the mean (s.e.m.). $^*P < 0{\cdot}05$ compared with the asthma group.

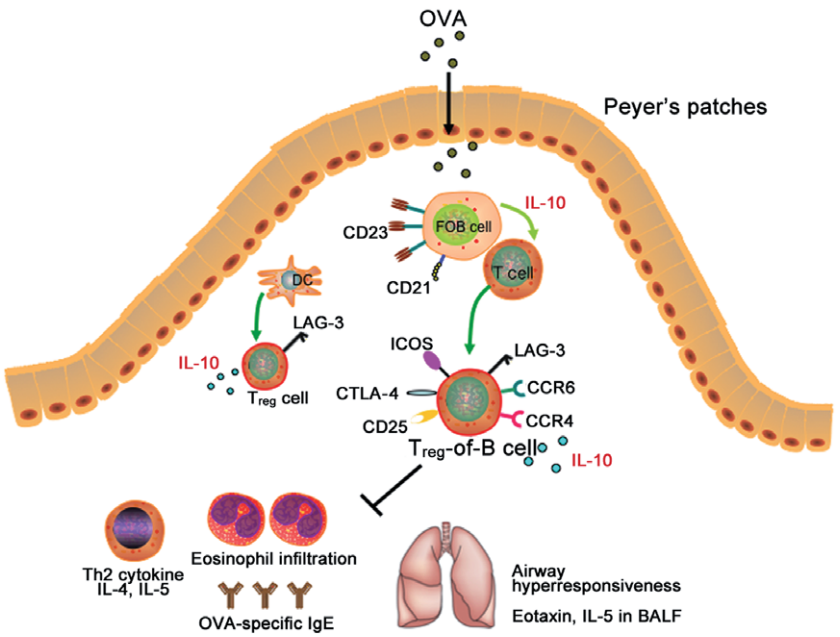

Fig. 6. Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells] express lymphocyte activation gene 3 (LAG3) and have therapeutic effects, alleviating allergic airway hypersensitivity. In our study, we proposed a model of oral tolerance induction: when an antigen [ovalbumin (OVA)] enters the intestines, it is captured by B cells in Peyer's patches and presented to naive T cells. Through cell–cell contact and interleukin (IL)-10 production by B cells [19], naive T cells are converted into regulatory T cells (T_{reg}-of-B(P) cells) that express cytotoxic T lymphocyte antigen 4 (CTLA-4), inducible co-stimulatory (ICOS), LAG3 and CCR6. LAG3 regulates the function of T_{reg}-of-B(P) cells, and CCR4 and CCR6 helps T_{reg}-of-B(P) cells to migrate to inflamed lungs and alleviate airway inflammation.

LAG3$^-$ T_{reg}-of-B(P) cells, not LAG3$^+$ T_{reg}-of-B(P) cells. In contrast, CCR4 and CCR6 are expressed in LAG3$^+$ T_{reg}-of-B(P) cells (Fig. 1g). CCL20 is the only ligand of CCR6 and is expressed by pulmonary epithelial cells and intestinal epithelial cells [33]. The Th2 response, including elevated IL-4 and IL-13 levels, influences CCL20 expression. Given that CCR6-deficient mice suffer from lung and gut inflammation [49,50] and CCR4 is critical for T cell migration to allergic lung [34], we hypothesized that LAG3$^+$ T_{reg}-of-B(P) cells could be recruited to the airway through CCR4 and CCR6 expression and regulate allergic airway inflammation. Thus, it is suggested that Peyer's patch B cells, which generate T_{reg} cells, might exert a better therapeutic effect on treating airway inflammation than Peyer's patch DCs.

To elucidate the effect of LAG3$^+$ T_{reg}-of-B(P) cells on airway allergic inflammatory disease, we transferred sorted LAG3$^+$ T_{reg}-of-B(P) cells into OVA-sensitized mice and determined the severity of the airway hyperresponsiveness and systemic Th2 responses. Our data suggested that LAG3$^+$ T_{reg}-of-B(P) cells have the ability to alleviate allergic airway inflammation (Figs 4 and 5). It is noted that Th2-type immunoglobulins (IgE, IgG1 and IgG2b) are decreased and Th1-type immunoglobulins (IgG2a and IgG3) [51] are increased in the adoptively transferred group. Therefore, we speculate that LAG3$^+$ T_{reg}-of-B(P) cells have the potential to balance the immune system. Our study has shown that most T_{reg}-of-B(P) cells migrate to the mucosal lymph nodes (cervical lymph nodes and Peyer's patches) and the spleen after transfer into mice [19]. The effects of T_{reg}-of-B(P) cells on other inflammatory diseases remain to be investigated.

Taken together, we found that the oral administration of OVA could increase the number of LAG3$^+$CD4$^+$ T cells in Peyer's patches. Peyer's patch FOB cells convert naïve CD4$^+$FoxP3$^-$LAG3$^-$ T cells into FoxP3$^-$LAG3$^+$CCR4$^+$ IL-10-producing T cells, which have a regulatory function. It would be necessary to define the molecules which are required for T_{reg}-of-B(P) cell induction. Knowing the molecules could help us to generate T_{reg}-of-B(P) cells more efficiently. Although additional experiments needed to be tested, our data indicated that T_{reg}-of-B(P) cells, which are generated *in vitro*, have the potential to apply to allergic disease treatment.

Acknowledgements

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Disclosure

The authors declare no conflicts of interest.

Author contributions

K. H. C. designed the study, performed all the experiments, analysed the data and drafted the manuscript. B. L. C. conceived the study and helped draft the manuscript. Both authors have read and approved the final manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. To examine the role of lymphocyte activation gene 3 (LAG3) in Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells], we purified $CD4^+$ forkhead box protein 3 $(FoxP3)^-$ naive T cells and cultured then with Peyer's patch B cells, as described in Materials and methods. After 3 days, $LAG3^+CD4^+$ T_{reg}-of-B(P) cells were sorted on a fluorescence activated cell sorter (FACS)Aria with the gating strategy to exclude the dead cells and doublets. The purity of $CD4^+$ $LAG3^+$ T cells was approximately 99%.

Fig. S2. Peyer's patch B cell-converted naive T cells into regulatory T cells [T_{reg}-of-B(P) cells] could express lymphocyte activation gene 3 (LAG3), B lymphocyte-induced maturation protein 1 (prdm1), FFAR2 and prdm1. The expressions of LAG3 (a), prdm1 (b) and free fatty acid receptor 2 (FFAR2) (d) on T_{reg}-of-B(P) cells are detected by real-time polymerase chain reaction (PCR). The expression of CCR4 and CCR6 are detected by flow cytometry and represented by mean fluorescence intensity (MFI) (c). The same results were obtained in three other experiments. The results are expressed as the mean ± standard error of the mean. $^*P < 0.05$ compared with $CD4^+CD25^-$ (labelled as $CD25^-$) T cells. $^{\#}P < 0.05$ compared with naturally occurring regulatory T cells (nT_{reg}) cells.

Fig. S3. Lymphocyte activation gene 3 (LAG3) expression by activated $CD25^-$ T cells, naturally occurring regulatory T cells (nT_{reg}) cells. $CD4^+$ $CD25^-$ T cells and nT_{reg} cells were activated by antigen-presenting cells pulsed with ovalbumin $(OVA)_{323–339}$ peptide for 24 h then applied for the detection of LAG3.

Fig. S4. Lymphocyte activation gene 3 (LAG3) expression by T cells cultured with splenic follicular B (FOB) cells, marginal zone B (MZB) and $CD23^-CD21^-$ cells. Naive T cells were cultured with different B cell subsets isolated from spleen. After 3 days, cells were stained with fluorescence conjugated anti-LAG3 antibody.

