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Inducible CD4$^+$LAP$^+$Foxp3$^-$ Regulatory T Cells Suppress Allergic Inflammation

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Regulatory T cells (Tregs) play a critical role in the maintenance of airway tolerance. We report that inhaled soluble Ag induces adaptive Foxp3$^+$ Tregs, as well as a regulatory population of CD4$^+$ T cells in the lungs and lung-draining lymph nodes that express latency-associated peptide (LAP) on their cell surface but do not express Foxp3. Blocking the cytokine IL-10 or TGF-β prevented the generation of LAP$^+$ Tregs and Foxp3$^+$ Tregs in vivo, and the LAP$^+$ Tregs could also be generated concomitantly with Foxp3$^+$ Tregs in vitro by culturing naive CD4$^+$ T cells with Ag and exogenous TGF-β. The LAP$^+$ Tregs strongly suppressed naive CD4$^+$ T cell proliferation, and transfer of sorted OVA-specific LAP$^+$ Tregs in vivo inhibited allergic eosinophilia and Th2 cytokine expression in the lung, either when present at the time of Th2 sensitization or when injected after Th2 cells were formed. Furthermore, inflammatory innate stimuli from house dust mite extract, nucleotide-binding oligomerization domain containing 2 ligand, and LPS, which are sufficient for blocking airway tolerance, strongly decreased the induction of LAP$^+$ Tregs. Taken together, we concluded that inducible Ag-specific LAP$^+$ Tregs can suppress asthmatic lung inflammation and constitute a mediator of airway tolerance together with Foxp3$^+$ Tregs. *The Journal of Immunology*, **2011, 187: 6499–6507.**

The breakdown of immune tolerance in the airways leads to an abnormal response to harmless airborne Ags, characterized by Th2-type inflammation and airway hyperresponsiveness (AHR). Among several possibilities for maintaining the delicate balance between airway tolerance and airway inflammation, regulatory T cells (Tregs) have been proposed to be an essential protective mechanism (1). Several major populations of Tregs have been studied in alternate scenarios with regard to the respiratory environment, including natural CD4$^+$Foxp3$^+$ Tregs (2, 3) and peripheral Ag-induced adaptive CD4$^+$ Tregs that either make IL-10 but do not express Foxp3 (4) or express Foxp3 with or without membrane-bound TGF-β (5–7).

TGF-β has been implicated as a key contributor to either the function or the differentiation of Tregs. TGF-β is produced as part of a large complex, formed by three molecules: a latent TGF-β–binding protein, which tethers the complex to the cell surface or the extracellular matrix; a latency-associated peptide (LAP), which provides a disulfide-linked shell hindering interaction of TGF-β with its cellular receptors; and the TGF-β cytokine itself. LAP regulates TGF-β latency, likely through an RGD sequence that sequesters TGF-β (8). Mutation of LAP leads to Camurati-Engelmann disease, an autosomal-dominant disorder of the long bones (9), suggesting the essential role of LAP in controlling the biological activities of TGF-β. LAP was also shown to promote chemotaxis of human monocytes and block inflammation in a murine model of delayed-type hypersensitivity (10). This implies that LAP could function independently to modulate immune responses, as well as being a marker for a TGF-β–producing cell.

LAP has been found on activated CD4$^+$Foxp3$^+$ Tregs in patients with activated paracoccidioidomycosis (11) and oral squamous cell carcinoma (12). These Tregs exerted their suppressive function in a TGF-β–dependent fashion, suggesting that LAP was a marker for the immunosuppressive cells. Hepatitis C virus-infected hepatocytes were also found to induce CD4$^+$Foxp3$^+$ Tregs that expressed LAP; similarly, TGF-β was found to contribute to the suppressive function of these cells (13). In addition, CD8$^+$ Tregs generated after hematopoietic stem cell transplantation expressed high levels of LAP (14). However, not all Foxp3$^+$ Tregs express LAP, and these data prompted some investigators to suggest that LAP, together with IL-1R type I/II (CD121a/CD121b), may be used to enrich for activated Foxp3$^+$ Tregs (15). Indeed, CD4$^+$CD25$^+$LAP$^+$ cells showed a potent TGF-β–dependent suppressive activity when transferred into an experimental autoimmune encephalomyelitis model (16). Most interestingly, a recent study of human T cells found a small population of LAP$^+$CD4$^+$ T cells that did not express Foxp3 but still exerted suppressive activity (17). Moreover, forced expression of the gene encoding TGF-β in naive CD4$^+$ T cells promoted membrane LAP (18), and exogenous TGF-β induced LAP expression in Foxp3$^-$ T cells (19). Therefore, these data suggested that LAP might mark Tregs with suppressive potential, regardless of Foxp3 expression.

We showed in this study that LAP$^+$CD4$^+$ T cells that lack Foxp3 are generated in vivo in a TGF-β– and IL-10–dependent manner when mice are exposed intranasally (i.n.) to soluble Ag, which leads to airway tolerance. Correlating with recent data (19), similar LAP$^+$Foxp3$^-$CD4$^+$ T cells could be generated in vitro in response to TGF-β, and we demonstrated that these cells are strongly suppressive and can block both the induction and effector phases of asthmatic lung inflammation. Moreover, decreased generation of LAP$^+$ Tregs was associated with exposure to aller-

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Abbreviations used in this article: AHR, airway hyperresponsiveness; Alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; HDM, house dust mite; i.n., intranasal(ly); LAP, latency-associated peptide; LN, lymph node; MAMP, microbial-associated molecular pattern; MDP, muramyl dipeptide; Treg, regulatory T cell.

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gens or inflammatory stimuli that are sufficient for blocking airway tolerance and lead to Th2-driven lung inflammation. Therefore, our data describe a novel population of Tregs that may be relevant for treatment or manipulation of allergic inflammatory diseases.

Materials and Methods

Mice

C57BL/6 and B6.PLThy1a (Thy1.1) mice were from The Jackson Laboratory. OT-II TCR transgenic mice, bred in-house on the BL/6 background, were used as a source of Vβ5/Vα2/Thy1.2 $CD4^+$ T cells responsive to the peptide OVA-323–339. Foxp3/GFP reporter mice, kindly provided by Dr. Alexander Rudensky (Sloan-Kettering Institute, New York, NY), were bred in-house with OT-II TCR transgenic mice and also used as a source of Vβ5/Vα2/Thy1.2 $CD4^+$ T cells. All experiments were conducted following the guidelines of the La Jolla Institute for Allergy and Immunology's Institutional Animal Care and Use Committee.

Airway tolerance and lung inflammation

Airway tolerance was induced similarly to previously described protocols (7). Briefly, on day 0, mice were exposed to 100 μg soluble OVA (Worthington Biochemical) in PBS or to PBS alone, given i.n. on three consecutive days. To assess the extent of tolerance, 9 d later, mice were sensitized by i.p. injection of 20 μg OVA protein (chicken egg albumin; Sigma-Aldrich), adsorbed to 4 mg aluminum hydroxide (Alum; Pierce Chemical). On day 24 or later, mice were challenged via the airways with OVA aerosol in a whole-body Plexiglas box (10 mg/ml in 15 ml PBS) for 20 min, once a day for four consecutive days, by ultrasonic nebulization. Tolerance was shown by a lack of lung inflammation, which was assessed as described below.

To prevent airway tolerance, soluble OVA was mixed with house dust mite (HDM; 100 μg), the Nod2 ligand muramyl dipeptide (MDP; 50 μg), or LPS (1 μg), given i.n. For TGF-β– and IL-10R–blockade experiments, one single dose of 200 μg anti–IL-10R (1B1.3a), 200 μg anti–TGF-β (1D11), or control IgG was given i.p. at the time of initial exposure to OVA.

Lung inflammation and AHR

Bronchoalveolar lavage was performed 24 h after the last OVA aerosol challenge. BAL fluid (BALF) was examined for cytokine content by ELISA (BD Biosciences). For cytological examination, BALF cells were spun on a slide using a Cytospin (Thermo Shandon, Pittsburgh, PA), fixed, and stained with Protocol HEMA3 (Fisher Scientific). Differential cell count was performed on ≥500 cells in each cytospin slide. For lung histology analysis, 5-μm sections were cut and stained with H&E for examining cell infiltration.

AHR was assessed 24 h after the final OVA challenge in intubated and ventilated mice (FlexiVent ventilator; Scireq) anesthetized with i.p. ketamine (100 mg/kg) and xylazine (10 mg/kg). The dynamic airway resistance was determined using Scireq software in mice exposed to nebulized PBS or methacholine (3, 24, or 48 mg/ml).

T cell preparation and adoptive transfer

Naive OVA-specific $CD25^-Foxp3^-CD4^+$ cells were isolated from spleen and lymph nodes (LNs) of OT-II/Foxp3/GFP reporter mice with naive $CD4^+CD62L^+$ T Cell Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell suspensions were incubated with the biotinylated Ab mixture and supplemented with biotinylated anti-mouse CD25, followed by magnetic anti-biotin microbeads and negative selection on LS MACS columns, according to the manufacturer's instructions. The $CD4^+$ cell purity was >90%, with >95% of resulting cells expressing the Vα2Vβ5 transgene and <0.1% $CD4^+CD25^+Foxp3^+$ cells. These cells were either used in vitro or were adoptively transferred (5×10^6 cells) i.v. into B6.PL Thy1.1 congenic mice to allow T cell responses to be analyzed under tolerogenic or inflammatory conditions.

To track the induction of $Foxp3^+$ and LAP^+ $CD4^+$ T cells after adoptive transfer and induction of tolerance, LNs and spleen from individual OVA-challenged mice were harvested, homogenized, and treated with RBC-lysing buffer (Sigma, St. Louis, MO) to prepare single-cell suspensions. Lung tissues were minced into fragments <1 mm in size and digested with Collagenase D (3 mg/ml) and DNase (10 μg/ml) before homogenizing to prepare single-cell suspensions. After Fc block with the 2.4G2 mAb, cells were stained with anti-Thy1.2 (53-2.1), anti-CD4 (RM4–5) (BD Biosciences), and anti-LAP (27232) (R&D Systems) Abs. Foxp3 expression was assessed by analyzing GFP expression. All samples were run on a FACS LSRII (BD Biosciences) with FlowJo (Tree Star) software.

In vitro generation and sorting of $Foxp3^+$ and LAP^+ Tregs

Naive $CD4^+$ T cells from spleen and peripheral LNs of OT-II/Foxp3/GFP reporter mice were isolated, as above. APCs from BL/6 spleen cells were made by depleting T cells using complement fixation with Abs to Thy-1.2 (F7D5 and HO.13.4), CD4 (RL172.4), and CD8 (3.155) and were irradiated with 3000 rad before use. Cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) with penicillin, streptomycin, glutamine, HEPES, 2-ME, and 10% FCS (Omega Scientific). For generation of $Foxp3^+$ and LAP^+ Tregs, naive OT-II cells were plated at 5×10^5 cells/ml with 2×10^6 cells/ml APCs, 1 μM OVA-323–339, and 10 ng/ml recombinant human TGF-β1 (PeproTech). At day 4, expanded OT-II cells were separated into $CD4^+LAP^+$ or $CD4^+Foxp3^+$ (GFP^+) populations using a FACSAria (BD Biosciences). Expression of Treg-associated markers, such as CD25, GITR, CD103, intracellular granzyme B, intracellular CTLA-4, CD44, CD69, and TGF-βRII, were determined by flow cytometry using an LSRII (BD Biosciences) with FlowJo software (Tree Star). Abs used were anti-LAP (BAF246) and anti–TGF-βRII (R&D Systems), anti-CD4 (RM4–5), anti-CD25 (PC61), anti-GITR (DTA-1), anti-CD103 (2E7), anti-granzyme B (16G6), anti–CTLA-4 (UC10-4B9) and anti-CD44 (IM7) (BD Biosciences), and anti-CD69 (H1.2F3) (eBioscience).

In vitro suppression assay

Naive $CD4^+$ T cells were purified from $CD45.1^+$ OT-II TCR-Tg B6 mice and labeled with PKH26-GL (Sigma), according to the manufacturer's instructions. To evaluate the suppressive function of $Foxp3^+$ and LAP^+ T cells, 5×10^4 PKH26-labeled $CD45.1^+$ naive OT-II cells were cultured with 4×10^5 irradiated T-depleted APCs and 0.5 μM OVA-323–339 for 3 d in the presence of varying numbers of $CD45.2^+$ Tregs. Cell division of responder T cells was assessed by dilution of PKH26 dye in the gated $CD4^+CD45.1^+$ populations. In some experiments, naive OT-II $CD4^+$ T cell proliferation was assessed by incorporation of $[^3H]$thymidine (1 μCi/well), which was added for the last 8 h of culture.

In vivo studies with in vitro-generated LAP^+ Tregs

In vitro-generated LAP^+ Tregs ($Thy1.2^+$) were adoptively transferred into Thy1.1 recipient mice. The recipient mice were then immunized and challenged with OVA, as described. Mice were either assessed for lung inflammation or sacrificed on day 2 or 6 after transfer, and pooled LN and spleen cells were harvested to track the transferred cells by flow cytometry, as before.

Multiplex cytokine assays

Sorted T cell populations were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 24 h. Cytokine secretion was measured using BioPlex Pro mouse cytokine assay kits (Bio-Rad, Hercules, CA). All assays were carried out directly in a 96-well filtration plate (Millipore, Billerica, MA) at room temperature and protected from light. Briefly, wells were prewet with 100 μl assay buffer (PBS containing 1% BSA) and then magnetic beads, together with a standard, sample, and blank, were added in a final volume of 100 μl and incubated at room temperature for 30 min with continuous shaking at 500 rpm. Beads were washed three times with 100 μl wash buffer (PBS containing 1% BSA and 0.05% Tween 20). A mixture of biotinylated detection Abs (25 μl/well) was added to the beads for an additional 30-min incubation, with continuous shaking at 500 rpm. Beads were washed three times and then streptavidin-PE was added for 10 min. Beads were again washed three times and resuspended in 125 μl assay buffer (PBS containing 1% BSA and 0.05% Tween 20). The fluorescence intensity of the beads was measured using a BioPlex array reader. BioPlex Manager 4.0 software with five-parametric-curve fitting was used for data analysis.

Reverse-transcription and real-time PCR

FACS-sorted cells were lysed using TRIzol reagent (Invitrogen). An aliquot of total RNA (5 μg) was reverse transcribed to cDNA using SuperScript III (Invitrogen). The oligonucleotide primer sequences of Foxp3 were forward primer 5′-GGC CCT TCT CCA GGA CAG A-3′ and reverse primer 5′-GCT GAT CAT GGC TGG GTT GT-3′. Real-time PCR assay was carried out with LightCycler using LightCycler 480 SYBR Green I master (both from Roche Diagnostics, Indianapolis, IN). Data are presented as normalized to ribosomal protein housekeeping gene *L32*.

Statistical analysis

Where appropriate, data were analyzed using the Student *t* test. Unless otherwise indicated, data represent the mean ± SEM. A p value < 0.05 was considered significant.

Results

Inhaled Ag induces LAP$^+$Foxp3$^-$CD4$^+$ T cells

Inhalation of soluble Ag induces airway tolerance and prevents susceptibility to developing Th2-driven allergic inflammation in the lung (6, 7, 20). We previously reported the induction of Ag-specific Foxp3$^+$ Tregs during the initial 5 d of exposure to Ag in the lungs (7) but were interested in whether other types of Tregs might also develop. To visualize and track the response of Ag-reactive T cells, we transferred naive (CD25$^-$, Foxp3$^-$, LAP$^-$) OVA-specific CD4 T cells from OT-II TCR transgenic Foxp3/GFP reporter mice (Thy1.2) into Thy1.1 recipients. These Thy1.1 mice were then treated with soluble OVA given i.n. once a day for 3 d, a protocol that efficiently promotes airway tolerance (7). Flow cytometry analysis of lung-draining LNs from individual mice, examined 2 d after the last OVA challenge, demonstrated that 3–5% of OT-II CD4$^+$ cells were induced by Ag to express membrane LAP (Fig. 1*A*, 1*B*). In comparison, 4–6% of OT-II CD4$^+$ cells were induced to express Foxp3, in line with our previous studies (7). These responses appeared to be systemic, because OVA-specific LAP$^+$ and Foxp3$^+$ T cells were also found in the spleen and lungs (Supplemental Fig. 1). Most interestingly, we found that the majority of LAP$^+$ cells did not express Foxp3 and, conversely, the majority of Foxp3$^+$ cells did not express LAP (Fig. 1*B*). A minor population expressed both molecules. The kinetics of induction of LAP$^+$CD4$^+$ T cells in the draining LN was similar to that of Foxp3$^+$ Tregs, peaking between days 2 and 5 after the initial exposure to soluble Ag (Fig. 1*C*).

We previously showed that TGF-β plays an important role in the induction of airway tolerance. Neutralizing TGF-β at the time of inhalation of soluble Ag allowed the development of Th2-driven eosinophilia in the airway, and this corresponded with blocking the generation of Ag-specific Foxp3$^+$ Tregs (7). Of possible significance to our visualization of LAP$^+$Foxp3$^-$ T cells, two recent studies found that forced expression of the TGF-β gene into CD4$^+$CD25$^-$ T cells promoted surface LAP (18) and, importantly, that exogenous TGF-β induced LAP expression in Foxp3$^-$ T cells (19). Correspondingly, we found that neutralizing TGF-β in vivo when OVA was inhaled strongly reduced the generation of OVA-specific LAP$^+$Foxp3$^-$CD4$^+$ cells by ~70%, similar to the reduction seen in OVA-specific LAP$^-$Foxp3$^+$CD4$^+$ cells (Fig. 2*A*, 2*B*, Supplemental Fig. 1*B*). Not surprisingly, because the LAP$^+$ and Foxp3$^+$ population were only a fraction of the total OVA-reactive CD4 T cells, neutralizing TGF-β did not significantly alter the overall number of CD4$^+$ OT-II cells visualized, also showing specificity for TGF-β activity (Fig. 2*B*). Together with our previous results (7), this implied that LAP$^+$ cells, induced from naive CD4$^+$ T cells by Ag and endogenously produced TGF-β, may also be a key contributor to airway tolerance along with Foxp3$^+$ Tregs.

IL-10 is another inhibitory cytokine implicated in the induction of airway tolerance (21). It was reported to promote both IL-10$^+$Foxp3$^-$ Tregs and TGF-β–induced Foxp3$^+$ Tregs (22, 23). We did not visualize any IL-10–producing CD4$^+$ T cells in the lungs or lung-draining LNs (W. Duan and M. Croft, unpublished observations) in this tolerance model, but we did find that blocking IL-10R at the time of exposure to i.n. OVA strongly prevented the induction of tolerance (Supplemental Fig. 2), similar to our prior observation of neutralizing TGF-β (7). Blocking IL-10R concomitantly decreased the generation of both LAP$^+$ T cells and Foxp3$^+$ T cells (Fig. 2*A*, 2*C*, Supplemental Fig. 1*A*). This did not significantly affect the total number of OVA-specific CD4$^+$ cells visualized, also showing specificity (Fig. 2*C*). These data again suggested that the LAP$^+$ T cells might represent an important regulatory population. This is in line with a study showing that LAP$^+$ T cells generated in the gut after probiotic administration were also IL-10 dependent (24).

LAP$^+$ T cells display regulatory activity

Because the frequency of LAP$^+$ T cells induced in vivo was too low to be characterized effectively, we sought to generate an equivalent population in vitro. We isolated naive CD4$^+$CD25$^-$ T cells from GFP/Foxp3 reporter/OT-II TCR transgenic mice and cultured them with T-depleted splenocytes and OVA peptide, in the presence of exogenous TGF-β. We found that these culture conditions, which are normally used to generate Foxp3$^+$ Tregs, also resulted in the generation of LAP$^+$Foxp3$^-$ cells (LAP$^+$ cells) over 3–5 d, similar to the population induced in vivo under tolerogenic conditions (Fig. 3*A*). Using cells from GFP/Foxp3 re-

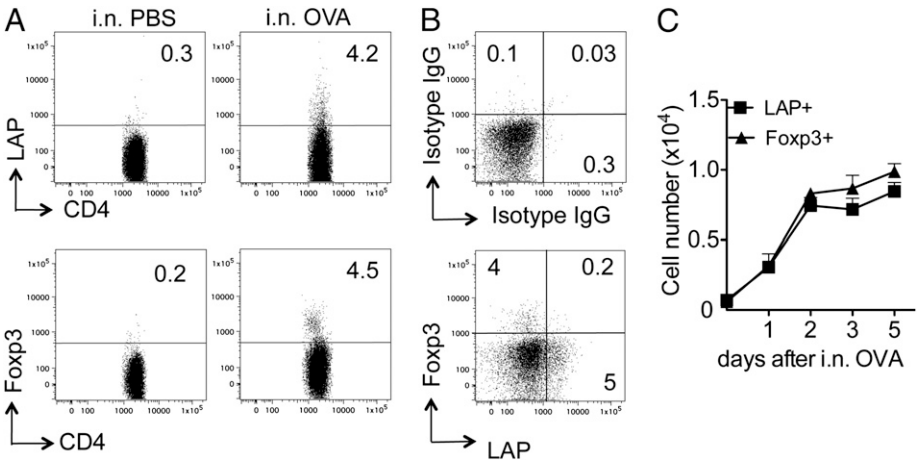

FIGURE 1. Inhalation of soluble Ag induces LAP$^+$Foxp3$^-$ CD4$^+$ T cells. Naive CD4$^+$CD25$^-$ T cells isolated from Thy1.2 OT-II TCR transgenic Foxp3/GFP reporter mice were transferred into Thy1.1 recipient mice. Recipient mice were then tolerized by exposure to soluble OVA (100 μg) in PBS given i.n. for three consecutive days. Control (nontolerized) mice were exposed to PBS without OVA. *A*, Representative flow dot plot of LAP and Foxp3 (GFP) expression on gated Thy1.2$^+$ OT-II CD4$^+$ T cells in lung-draining LN from an individual mouse at day 5 after the initial exposure to OVA. *B*, Representative flow dot plot of LAP and Foxp3 (GFP) costaining (*bottom panel*) and isotype IgG (*top panel*) on gated Thy1.2$^+$ OT-II CD4$^+$ T cells in pooled lung-draining LN from an individual mouse at day 5 after the initial exposure to OVA. *C*, Total numbers of LAP$^+$Foxp3$^-$ and Foxp3$^+$LAP$^-$ OT-II CD4$^+$ T cells in lung draining LN populations on different days after the initial exposure to OVA. Data are mean ± SEM from four individual mice/group and are representative of three independent experiments.

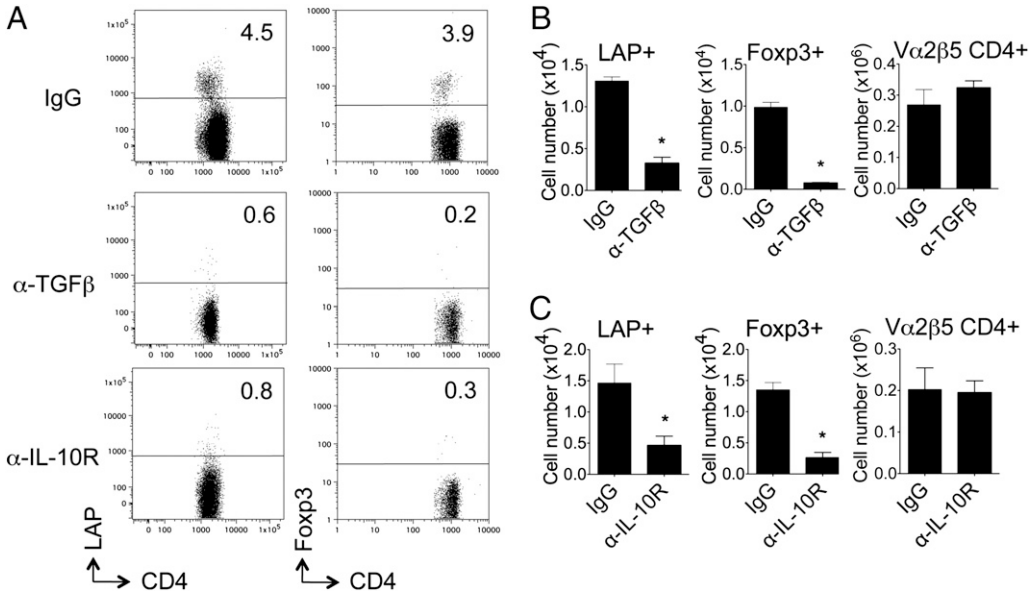

FIGURE 2. TGF-β and IL-10 are required for induction of LAP$^+$ T cells in vivo. Thy1.2 OT-II LAP$^+$ and Foxp3$^+$ T cells were tracked in vivo, as described in Fig. 1. A single dose of anti–IL-10R (200 μg), anti–TGF-β (200 μg), or control IgG was given i.p. at the time of initial exposure to i.n. OVA. *A*, Representative flow cytometry dot plots of LAP and Foxp3 (GFP) expression on gated Thy1.2$^+$ OT-II CD4$^+$ T cells in lung-draining LN from individual mice. *B* and *C*, Numbers of LAP$^+$Foxp3$^-$ and Foxp3$^+$LAP$^-$ OT-II CD4$^+$ T cells and total OT-II CD4$^+$ cells were calculated in lung-draining LNs on day 5. All results are mean ± SEM from four individual mice/group. Data are representative of three independent experiments. *$p < 0.05$.

porter mice allowed us to sort the two distinct populations. The purity of sorted LAP$^+$Foxp3$^-$ cells was ≥89%, and the purity of sorted LAP$^-$Foxp3$^+$ cells was >97% (Fig. 3*A*). Contamination of Foxp3$^+$ cells in the LAP$^+$ fraction was ≤0.2%. Purity was confirmed by PCR, with sorted LAP$^+$Foxp3$^-$ populations expressing minimal Foxp3 mRNA. As a control, Foxp3$^-$LAP$^-$ (double-

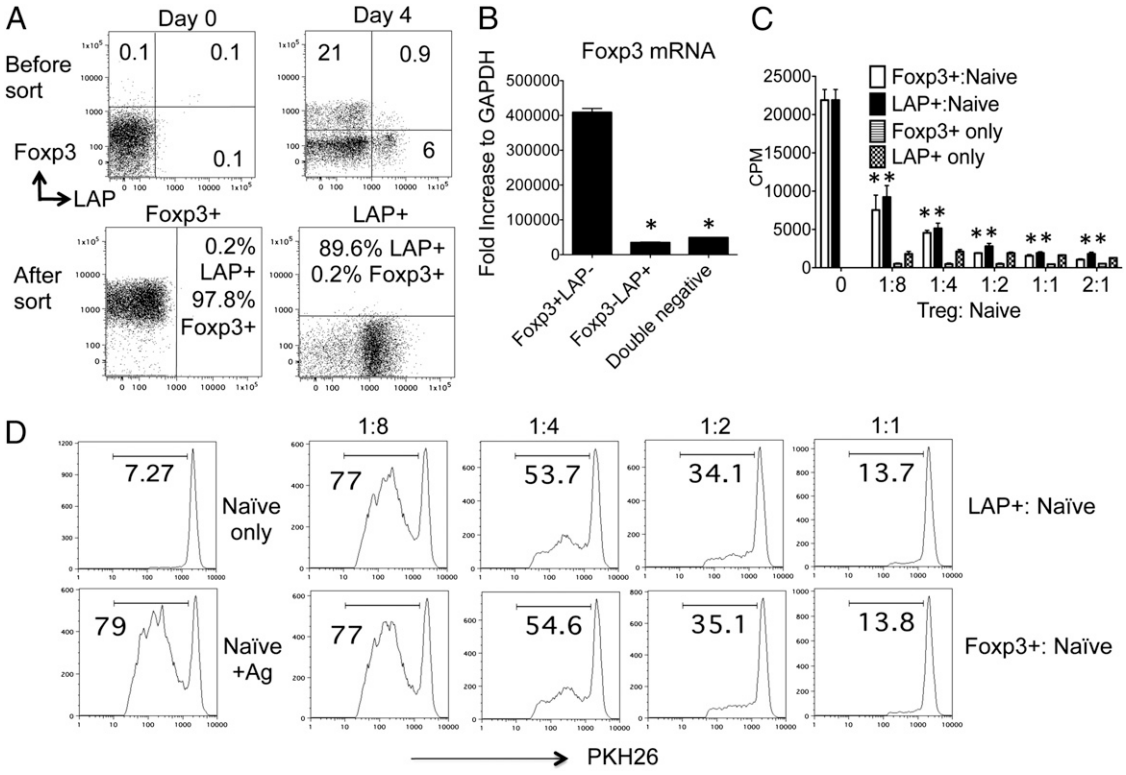

FIGURE 3. LAP$^+$Foxp3$^-$ T cells inhibit naive CD4$^+$ T cell proliferation in vitro. Naive CD4$^+$ T cells from OT-II TCR transgenic GFP/Foxp3 reporter mice were cultured with T-depleted splenocytes and OVA peptide (1 μM) in the presence of exogenous TGF-β (10 ng/ml). Cells were analyzed and sorted on day 4. *A*, *Top row*: Foxp3 versus LAP staining on CD4 T cells before and after culture. *Bottom row*: Purity of the sorted LAP$^+$Foxp3$^-$ and LAP$^-$Foxp3$^+$ CD4$^+$ T cell populations. *B*, Foxp3 mRNA levels in sorted LAP$^+$Foxp3$^-$ and LAP$^-$Foxp3$^+$ populations, analyzed using real-time PCR. Data are normalized to the housekeeping gene GAPDH. *C* and *D*, FACS-sorted LAP$^+$Foxp3$^-$ and LAP$^-$Foxp3$^+$ OT-II T cells (1×10^5) were cultured, alone or at varying ratios with naive CD4$^+$CD25$^-$ OT-II T cells, together with irradiated (3000 rad) syngeneic splenocytes in the presence of 0.5 μM OVA peptide. *C*, Proliferation was assessed after a pulse with [^{3}H]thymidine for the last 16 h of a 72-h incubation period. Data are mean ± SEM. *D*, Responder naive T cells were labeled with PKH26 dye, and division was assessed by loss of the dye after 3 d. Data represent one of three independent experiments. *$p < 0.05$.

negative) T cells from the same cultures (Fig. 3*A*) were also sorted and found to express minimal Foxp3 mRNA (Fig. *3B*). This data further confirmed that LAP$^+$Foxp3$^-$ and LAP$^-$Foxp3$^+$ T cells are two distinct populations.

To test whether in vitro-generated OVA-specific LAP$^+$ T cells could act as Tregs, we cocultured the sorted LAP$^+$ or Foxp3$^+$ T cells with naive OT-II T cells, T-depleted APCs, and OVA peptide and measured proliferation either by tritiated thymidine incorporation or after PKH26 labeling of the responder T cells (Fig. 3*C*, 3*D*). LAP$^+$ T cells were hypoproliferative to Ag in vitro, similar to Foxp3$^+$ Tregs compared with naive CD4$^+$ T cells (Fig. 3*C*). However, LAP$^+$ T cells strongly inhibited the division of naive T cells in a dose-dependent manner (Fig. 3*C*, 3*D*). Importantly, the potency of suppression was similar to that of Foxp3$^+$ Tregs, showing that the activity could not derive from contaminating Foxp3$^+$ cells. These data confirmed that TGF-β–induced LAP$^+$ (Foxp3$^-$) T cells could display regulatory activity, in line with recently published results (17, 19).

Phenotype of in vitro-generated LAP$^+$ Tregs

To further pursue similarities or differences between LAP$^+$ Tregs and Foxp3$^+$ Tregs, we assessed a number of molecules reported to be associated with Tregs (Fig. 4*A*, Supplemental Fig. 3). When LAP$^+$ Tregs were first visualized on day 2 of the TGF-β–induced cultures, CD25 was quickly upregulated on a small portion (18%) of Foxp3$^+$ cells but not on LAP$^+$ cells (data not shown). By day 4, the majority of the LAP$^+$ and Foxp3$^+$ cells expressed high levels of CD25 (Fig. 4*A*), which was maintained on day 6, albeit at a lower level on LAP$^+$ cells (Supplemental Fig. 3*A*). GITR (TNFRSF18), a receptor belonging to the TNFR superfamily that has also been associated with Foxp3$^+$ Tregs, was also rapidly expressed and maintained at high levels on both LAP$^+$ and Foxp3$^+$ cells. CD103, the integrin $\alpha_E\beta_7$, was additionally reported to be expressed on Foxp3$^+$ Tregs (25, 26) and, again, was markedly upregulated on both LAP$^+$ and Foxp3$^+$ cells to a similar extent. We also examined the Treg-associated suppressive molecules granzyme B and CTLA-4. Granzyme B, a serine protease, which is responsible for the induction of apoptosis in target cells, was shown to be highly upregulated in CD4$^+$CD25$^+$ (Foxp3$^+$) Tregs and served as one mechanism for cell contact-mediated suppression (27, 28). Granzyme B was upregulated to high levels on the majority of LAP$^+$ and Foxp3$^+$ cells on day 4 (Fig. 4*A*). Expression was maintained over 6 d (Supplemental Fig. 3*A*) but at lower levels in LAP$^+$ cells, suggesting that the expression of intracellular granzyme B might be transient. CTLA-4, another suppressive molecule expressed by Foxp3$^+$ Tregs, can attenuate T cell activation either directly or indirectly through interaction with CD80 and CD86 (B7) molecules on APCs. Again, intracellular CTLA-4 was strongly expressed by both LAP$^+$ and Foxp3$^+$ T cells on day 4 (Fig. 4*A*) and was largely maintained at day 6 (Supplemental Fig. 3*A*). We also assessed expression of CD44, CD69, and TGF-βRII (Supplemental Fig. 3*B*). In addition to its important role in T cell activation, migration, and apoptosis (29, 30), CD44 has been associated with Foxp3 expression and Treg function (31, 32) and promoting the surface expression of TGF-β (33). We found that CD44 was upregulated on 46% of LAP$^+$ cells and 65% of Foxp3$^+$

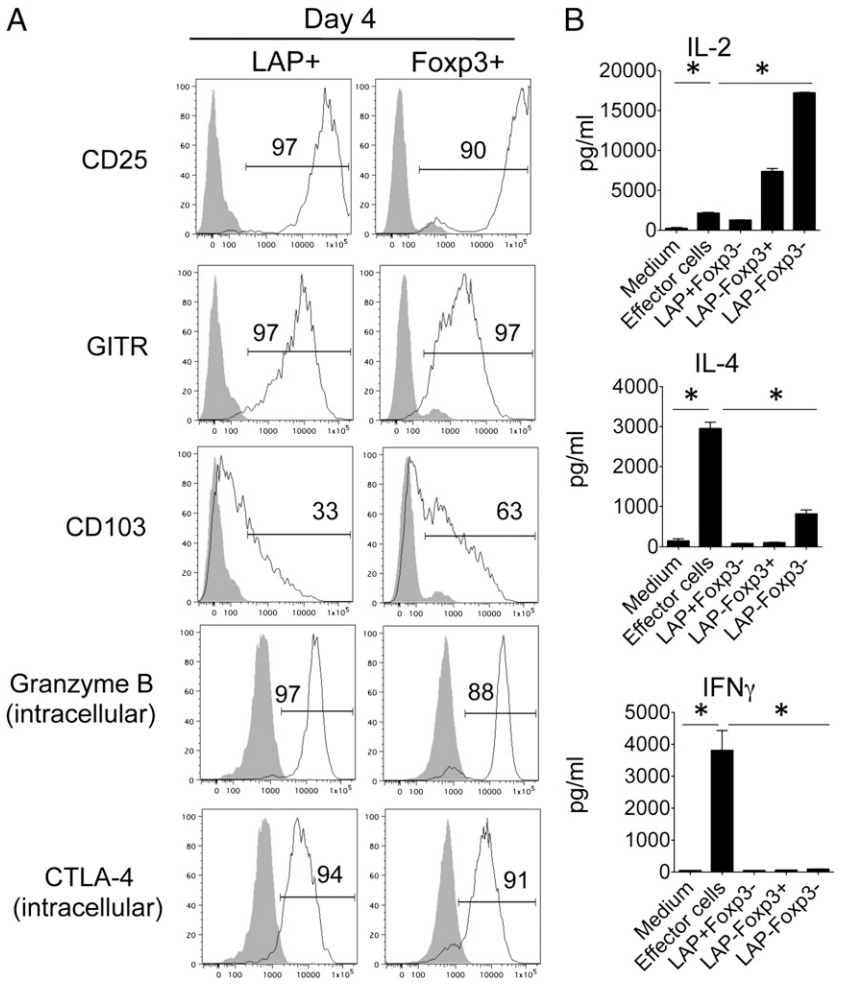

FIGURE 4. Phenotype of TGF-β–induced LAP$^+$ Foxp3$^-$ T cells. LAP$^+$Foxp3$^-$ and LAP$^-$Foxp3$^+$ OT-II T cells were generated, as described in Fig. 3. *A*, Representative expression of CD25, GITR, CD103, granzyme B, and CTLA-4 (black line) with isotype controls (shaded graph) at day 4 on gated LAP$^+$Foxp3$^-$ and LAP$^-$Foxp3$^+$ CD4$^+$ T cells. *B*, Cytokine-secretion profiles of TGF-β–induced sorted LAP$^+$Foxp3$^-$, LAP$^-$Foxp3$^+$, and LAP$^-$Foxp3$^-$ T cells. Positive control populations (effector cells) were from cultures of OT-II splenocytes stimulated with OVA peptide in nonskewing conditions for 4 d. T cells were restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 24 h. IL-2, IL-4, and IFN-γ levels were measured using mouse cytokine multiplex kits. Similar data were obtained in three independent experiments. *$p < 0.05$.

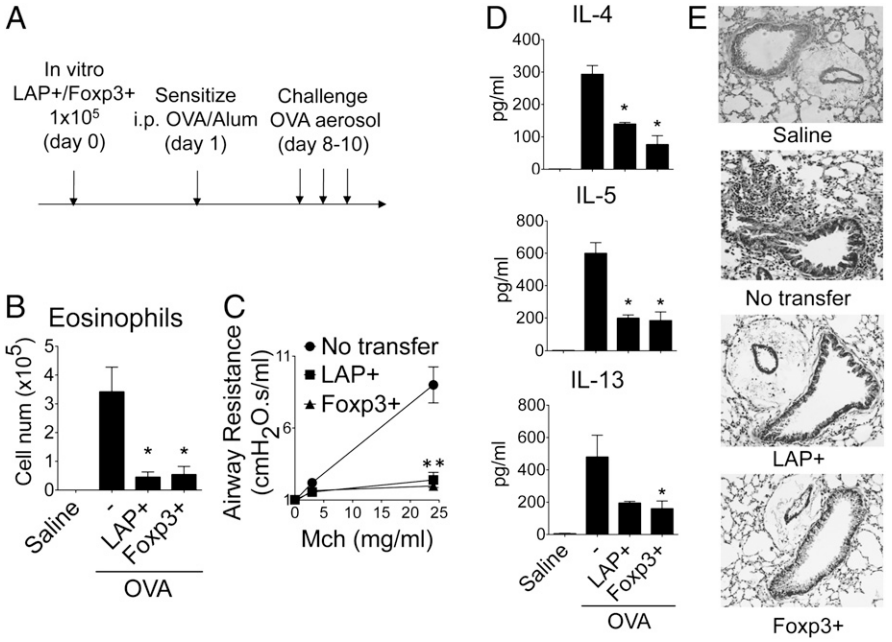

FIGURE 5. LAP+ T cells prevent induction of asthmatic lung inflammation in vivo. LAP+ Foxp3− or Foxp3+LAP− OT-II T cells, generated in vitro as described in Fig. 3, were sorted and transferred into naive BL/6 recipient mice. One day later, the mice were sensitized with OVA (20 μg) in Alum (4 mg) and subsequently challenged with OVA aerosol to induce lung inflammation. *A*, Protocol timeline. *B*, Eosinophils in BALF. *C*, AHR to methacholine, assessed using a FlexiVent. *D*, Cytokines in BALF by ELISA. *E*, Representative H&E staining of lung sections. Original magnification ×400. Data are mean ± SEM from three mice/group. Similar data were generated from three independent experiments. $*p < 0.05$.

cells (Supplemental Fig. 3*B*). Signaling from the T cell early activation marker CD69 can induce the synthesis of TGF-β (34), and CD69+Foxp3−CD4+ Tregs were shown to suppress T cell proliferation through membrane-bound TGF-β (35). We found that more LAP+ cells expressed higher levels of CD69 (Supplemental Fig. 3*B*). TGF-βRII expression was also reported to be important for Treg maintenance (36, 37), and the induced LAP+ T cells expressed higher levels compared with Foxp3+ cells (Supplemental Fig. 3*B*). These data are in line with reports showing that murine CD4+CD25−LAP+ and human CD4+LAP+ cells express CD69 and TGF-βRII (17, 38).

Next, we assessed the ability of Tregs to secrete cytokines (Fig. 4*B*, Supplemental Fig. 4). We sorted LAP+Foxp3− and LAP− Foxp3+ OT-II T cells and, as a control, LAP−Foxp3− T cells, from 4-d TGF-β–induced cultures, as described in Fig. 3*A*. These cells were restimulated with PMA and ionomycin for 24 h. Splenocytes from OT-II mice were cultured with OVA peptide under neutral (nonskewing) conditions for 4 d and then also restimulated with PMA and ionomycin as an additional positive control (labeled effector cells). LAP+Foxp3− Tregs displayed a largely anergic-type phenotype compared with the unskewed effector cell population, producing little/no inflammatory cytokines or chemokines, including IL-2, IL-4, and IFN-γ (Fig. 4*B*, Supplemental Fig. 4). This profile was again similar to Foxp3+ Tregs, although Foxp3+ cells, and not LAP+ cells, produced significant levels of MCP-1α and β (Supplemental Fig. 4). In contrast, the LAP− Foxp3− T cells isolated from the same TGF-β–induced cultures produced a substantial amount of IL-2, some IL-4 and IL-17, and

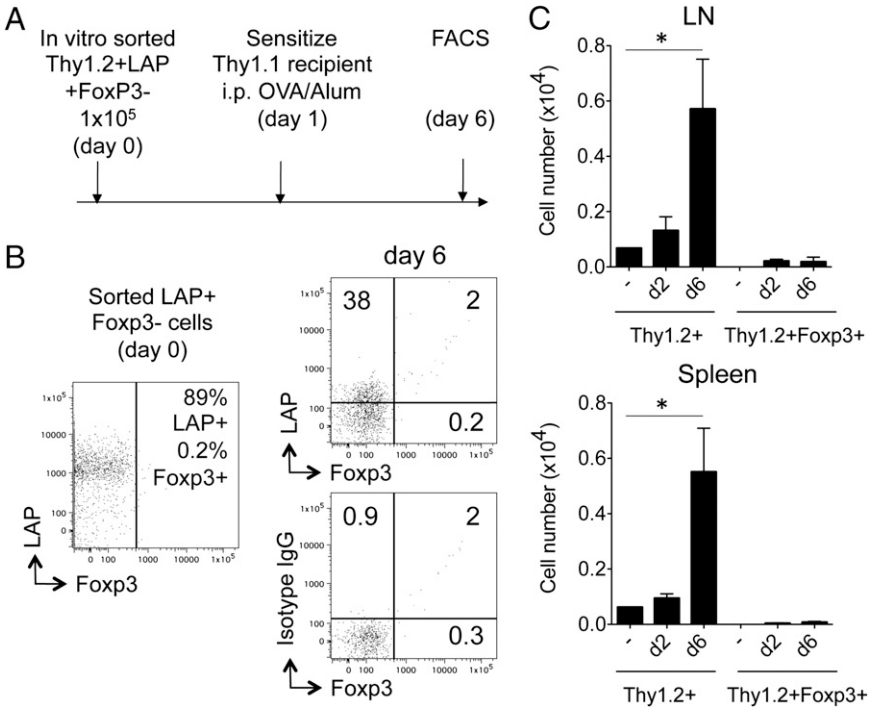

FIGURE 6. LAP+ T cells do not convert to Foxp3+ T cells in vivo. Sorted Thy1.2+LAP+ Foxp3− T cells, generated in vitro as described in Figs. 3 and 5, were transferred into Thy1.1 recipient mice. One day later, the recipient mice were immunized with OVA (20 μg) and Alum (4 mg). *A*, Protocol timeline. *B*, The expression of surface LAP and intracellular Foxp3 on gated Thy1.2+ T cells was analyzed in pooled LN and spleen on day 6 after the immunization. Representative dot plot of T cells before and 6 d after the transfer. Isotype control staining for LAP is shown in *lower right panel*. *C*, Total numbers of recovered Thy1.2+ T cells in pooled LNs and spleens on days 2 and 6 compared with recovered Thy1.2+Foxp3+ T cells. All results are mean ± SEM from four individual mice/group. Data are representative of three independent experiments. $*p < 0.05$.

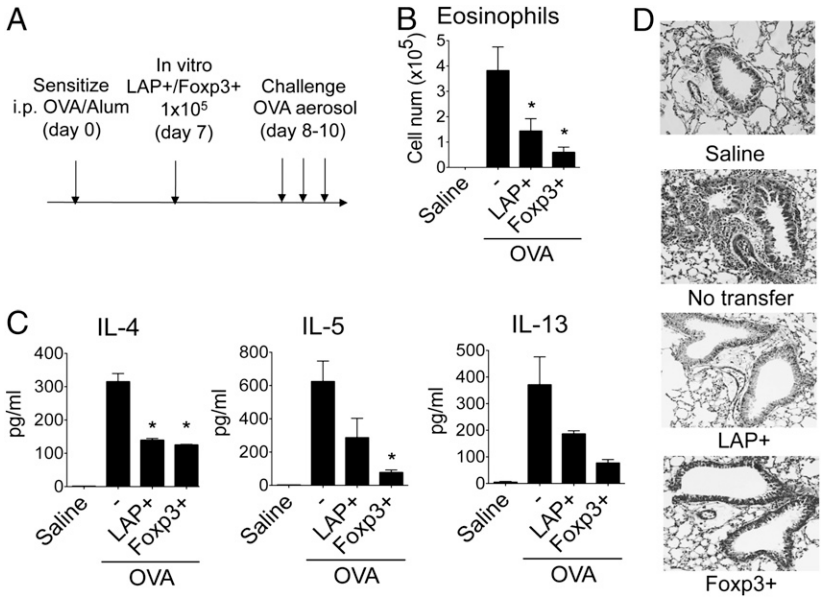

FIGURE 7. LAP$^+$ T cells suppress asthmatic lung inflammation in sensitized mice. FACS-sorted LAP$^+$ Foxp3$^-$ or Foxp3$^+$LAP$^-$ OT-II T cells, generated as described in Fig. 3, were transferred into BL/6 mice that had been sensitized with OVA and Alum for 7 d. The recipients were then challenged with OVA aerosol to induce lung inflammation starting 1 d later. *A*, Protocol timeline. *B*, Eosinophils in BALF. *C*, Cytokines in BALF by ELISA. *D*, Representative H&E staining of lung sections. Original magnification ×400. Data are mean ± SEM from three mice/group and are representative of three independent experiments. $*p < 0.05$.

MCP-1α and β, suggesting that they were largely undifferentiated (Fig. 4*B*, Supplemental Fig. 4). Neither LAP$^+$ nor Foxp3$^+$ Tregs produced substantial levels of IL-10 (Supplemental Fig. 4). Taken together, these results showed that Ag-induced LAP$^+$ Tregs were phenotypically very similar to Ag-induced Foxp3$^+$ Tregs.

LAP$^+$ Tregs suppress the onset of asthmatic lung inflammation in vivo

To investigate the in vivo suppressive function of the induced LAP$^+$ Tregs, we sorted LAP$^+$ and Foxp3$^+$ OT-II T cells as in Fig. 3 and adoptively transferred them into syngeneic BL/6 host mice. The recipients were then sensitized with OVA in Alum and subsequently challenged with OVA via the airways to induce lung inflammation (Fig. 5*A*). A total of 1×10^5 LAP$^+$ Tregs substantially inhibited the induction of eosinophilia in the airways (Fig. 5*B*), AHR to methacholine (Fig. 5*C*), Th2 cytokines in BALF (Fig. 5*D*), and peribronchial cell infiltration in lung parenchyma (Fig. 5*E*). Adoptively transferred Foxp3$^+$ Tregs (1×10^5) also strongly prevented the development of the Th2 inflammatory response, suggesting that LAP$^+$ Tregs and Foxp3$^+$ Tregs are equally potent as regulatory populations that can promote airway tolerance. To eliminate the possibility that the observed suppression by sorted LAP$^+$ Tregs was due to the minor contamination of Foxp3$^+$ Tregs (0.2%, Fig. 3*A*), we repeated the experiment using 1×10^3 sorted Foxp3$^+$ Tregs, which is 1% of the 10^5 Tregs that were able to strongly inhibit the asthmatic response. A total of 10^3 Foxp3$^+$ Tregs failed to suppress the OVA-induced eosinophilia and lung inflammation (Supplemental Fig. 3*C*, 3*D*, data not shown), confirming that the observed suppression by sorted LAP$^+$ Tregs was not due to contamination of Foxp3$^+$ Tregs.

To investigate whether the LAP$^+$ Tregs converted to Foxp3$^+$ Tregs upon Ag encounter in vivo, we sorted Thy1.2$^+$LAP$^+$ T cells, as described in Fig. 3*A*, and transferred them into Thy1.1 recipient mice. The mice were then immunized with OVA in Alum, as above, and the transferred Thy1.2$^+$ cells were visualized after immunization (Fig. 6). The expression of LAP was downregulated on the majority of cells (6 ± 1% positive) on day 2 (data not shown), whereas 38 ± 8% were positive on day 6 (Fig. 6*B*). Importantly, there was no significant expression of Foxp3 detected in the majority of T cells, indicating that the transferred LAP$^+$ Tregs did not convert to Foxp3$^+$ Tregs. Enumerating the total number of Thy1.2$^+$ T cells in pooled LN and spleen showed strong expansion of the transferred population between 2 and 6 d, but only a fraction were Foxp3$^+$ cells throughout this time period, confirming minimal conversion and accumulation of Foxp3$^+$ cells (Fig. 6*C*).

LAP$^+$ Tregs suppress asthmatic lung inflammation induced by primed T cells

We further tested the in vivo suppressive ability of LAP$^+$ Tregs injected after the initial generation of pathogenic T cells. Sorted OVA-specific CD4$^+$LAP$^+$ T cells, as described in Fig. 3*A*, were transferred into recipient mice sensitized 1 wk earlier with OVA/Alum. The recipients were subsequently challenged with OVA aerosol to induce lung inflammation (Fig. 7*A*). A total of 1×10^5 LAP$^+$ T cells again markedly inhibited OVA-induced eosinophilia in the airways (Fig. 7*B*), Th2 cytokines in BALF (Fig. 7*C*), and peribronchial cell infiltration in lung parenchyma (Fig. 7*D*). This indicated that LAP$^+$ Tregs are able to suppress effector T cell activation during the peak of inflammatory responses. In com-

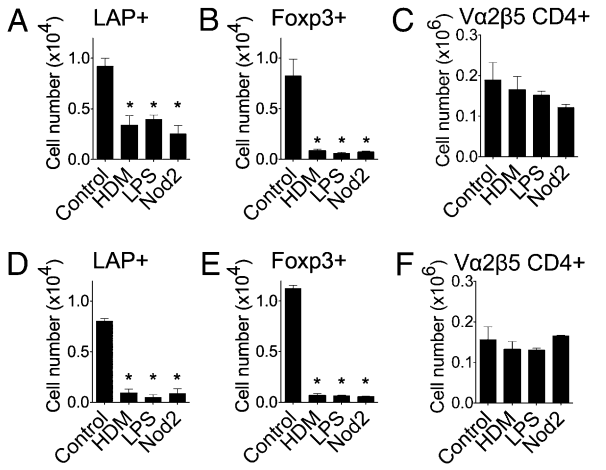

FIGURE 8. Allergens and proinflammatory MAMPs suppress the generation of LAP$^+$ Tregs. The generation of LAP$^+$Foxp3$^-$ and Foxp3$^+$LAP$^-$ OT-II T cells was tracked in vivo, as described in Fig. 1. HDM extract, MDP, or LPS was given i.n. concurrently with soluble OVA. Numbers of LAP$^+$Foxp3$^-$, LAP$^-$Foxp3$^+$, and total OT-II CD4$^+$ T cells in lung-draining LNs (*A–C*) and lung tissue (*D–F*). Data are mean ± SEM from three mice/group and are representative of three independent experiments. $*p < 0.05$.

parison, Foxp3+ Tregs displayed a slightly stronger inhibitory activity in suppressing effector T cell-driven lung inflammation (Fig. 7*B–D*). Collectively, these data showed that LAP+Foxp3− T cells induced with Ag are a bona fide regulatory population that can suppress asthmatic lung inflammation with activity similar to conventional Foxp3+ Tregs.

Allergens and proinflammatory microbial-associated molecular patterns suppress the generation of LAP+ Tregs

We and other investigators previously showed that allergens, or isolated microbial-associated molecular patterns (MAMPs), such as LPS that targets TLR4, and peptidoglycan derivatives that target the intracellular Nod2 receptor, can prevent the induction of airway tolerance and lead to susceptibility to development of Th2-driven lung inflammation (7, 39, 40). Although this activity occurs, at least in part, through antagonizing the generation of Foxp3+ Tregs, our data suggested that LAP+ Tregs may also be controlled by these stimuli. HDM extract, the Nod2 ligand MDP, and LPS were given concurrently with soluble OVA i.n., and the induction of LAP+ Tregs was tracked, as described in Fig. 1. HDM, LPS, and Nod2 ligand strongly inhibited the generation of Ag-specific LAP+ T cells in the lung-draining LNs (Fig. 8*A*), lung tissue (Fig. 8*D*), and spleen (Supplemental Fig. 7*A*), in addition to blocking the induction of Foxp3+ T cells (Fig. 8*B*, 8*E*, Supplemental Fig. 1*C*). HDM, LPS, and Nod2 ligand did not significantly affect the total number of OVA-specific CD4+ T cells visualized, again showing specificity (Fig. 8*C*, 8*F*). Therefore, allergens and associated MAMPs likely antagonize airway tolerance by preventing the generation of both LAP+ Tregs and Foxp3+ Tregs.

Discussion

Tregs have been demonstrated to maintain airway tolerance in mouse models of asthma, and increased numbers of Tregs or Treg activity has been associated with current treatments that reduce allergic and asthmatic symptoms. CD4+CD25+Foxp3+ Treg numbers were observed to increase in patients undergoing allergen immunotherapy, for example after exposure to escalating doses of HDM (41), grass pollen (42), and venom (43). Enhanced numbers of Foxp3+ Tregs have also been found in glucocorticoid-treated asthmatic patients (44), and histamine receptor (H4R) agonists were shown to enrich the Foxp3+ Treg population in a mouse model of asthma (45). These data strongly support the idea that methods to induce Tregs, or the adoptive transfer of Tregs, would be efficacious in the prevention and treatment of lung inflammatory disease. In this article, we report a novel Treg population bearing surface LAP that does not express Foxp3 but expresses Treg-associated suppressive molecules, including granzyme B and CTLA-4. These cells were equally potent at suppressing asthmatic lung inflammation in the mouse, suggesting that they are another population of Tregs that should be considered a target of analysis or therapy for allergic disease.

Interestingly, a recent report found that a small percentage (1.32%) of CD4+ cells in human peripheral blood expressed LAP (17). These LAP+ cells did not express Foxp3, were hypoproliferative, and exhibited suppressive activity in vitro. Therefore, it is highly likely that the LAP+ Tregs that we describe are similar to these human peripheral LAP+ Tregs, although there was an apparent discrepancy between the cytokine-secretion profiles; our cells made little/no inflammatory cytokines, whereas the human cells made IL-8, IL-9, IL-10, and IFN-γ. However, collectively the data suggested that CD4+LAP+ Tregs may have potential in cell-based immunotherapy for treating asthma, as well as being another Treg population that can be used to track the activity of alternate forms of therapy for asthma.

The Ag-induced CD4+LAP+ Tregs that we visualized in vivo and in vitro lacked Foxp3, even after in vivo reactivation with Ag, whereas the majority of Foxp3+ Tregs generated concomitantly did not express LAP. This is in contrast to some reports that found activated Foxp3+ Tregs can also express surface LAP. Our in vivo and in vitro analyses showed that there was a small proportion of Foxp3+LAP+ cells generated in response to TGF-β, but they were strongly outnumbered by single-positive cells. The discrepancy between our observations and those of other investigators that visualized LAP on Foxp3+ T cells might due to different environments to which the T cells were exposed, variations in stimulation conditions, and/or differences in the T cells that were analyzed. We used naive T cells from a TCR transgenic mouse stimulated with Ag to become adaptive inducible Tregs, whereas, for example, one study observed LAP expression on mouse CD4+ CD25+ naturally occurring Tregs that were restimulated with anti-CD3 and IL-2 for 3 d (46). Another report observed 50–70% human Foxp3+ cells expressing LAP (15), which were likely largely naturally occurring Tregs, obtained from peripheral blood and expanded for 14–21 d before restimulation with anti-CD3/CD28. Oida and Weiner (19) used conditions most similar to our systems, stimulating BALB/c CD4+CD25− T cells, which would have been a mixture of naive and memory T cells, with plate-bound anti-CD3/anti-CD28 and 10 ng/ml TGF-β. They found LAP expression in Foxp3− cells, as well as the majority of Foxp3+ cells. It is not clear whether they would have seen the same result with naive T cells stimulated with Ag.

Oral anti-CD3 administration was shown to induce a population of CD4+LAP+ regulatory cells (47), which were associated with suppressive activity in a variety of autoimmune disease models, including autoimmune encephalomyelitis (16, 38), diabetes (48, 49), colitis (50), and arthritis (51). At least in one case, when analyzed, these CD4+LAP+ cells coexpressed Foxp3 (16). However, given our data and others mentioned above, it is likely that some Tregs were LAP+Foxp3−, because oral anti-CD3 is most similar to mucosal immunization with soluble Ag, as we used in this study. It was proposed that LAP could be used for isolating Foxp3+ Tregs (15). We suggest that isolation of Tregs exclusively based on LAP expression might also include the Foxp3− cells, which would be advantageous because these LAP+Foxp3− T cells are potent suppressors and likely cooperate with Foxp3+ Tregs to limit autoreactivity and inadvertent inflammatory responses, such as those induced by allergens.

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Disclosures

The authors have no financial conflicts of interest.

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