***In vivo* anti-LAP mAb enhances IL-17/IFN- γ responses and abrogates anti-CD3-induced oral tolerance**

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Abstract

Regulatory T cells (Tregs) play a critical role in the maintenance of immunological tolerance. The best-characterized Tregs are those expressing the transcription factor Foxp3 and *in vivo* modulation of Foxp3 Tregs has been employed to study their role in immune homeostasis. Latency-associated peptide (LAP) is a membrane-bound TGF- β complex that has also been shown to play a role in Treg function and oral tolerance. We developed a novel anti-mouse LAP mAb that allowed us to investigate the effect of targeting LAP *in vivo* on immune function and on anti-CD3-induced oral tolerance. We found that *in vivo* anti-LAP mAb administration led to a decrease in the number of CD4+LAP+ Tregs in spleen and lymph nodes without affecting CD4+Foxp3+ Tregs. Spleen cells from anti-LAP-injected mice proliferated more *in vitro* and produced increased amounts of IL-2, IL-17 and IFN- γ . Moreover, injection of anti-LAP antibody abrogated the protective effect of oral anti-CD3 on experimental autoimmune encephalomyelitis (EAE). Finally, *in vivo* anti-LAP administration prior to myelin oligodendrocyte glycoprotein immunization resulted in severe EAE in the absence of pertussis toxin, which is used for EAE induction. Our findings demonstrate the importance of CD4+LAP+ T cells in the control of immune homeostasis and autoimmunity and provides a new tool for the *in vivo* investigation of murine LAP+ Tregs on immune function.

Keywords: experimental autoimmune encephalomyelitis, inflammation, latency-associated peptide, regulatory T cells, TGF-beta

Introduction

Regulatory T cells (Tregs) are key mediators of immunologic homeostasis and are essential to prevent autoimmunity and limit chronic inflammatory diseases. The transcription factor Foxp3 has been extensively studied and is considered to be a master regulator of Treg development and function (1–3). Additionally, the cytokine TGF- β has been shown to be involved in the induction of Foxp3 expression on CD4+ T cells (4, 5). Foxp3+ Tregs are an important source of TGF- β and TGF- β plays a crucial role in controlling autoimmune diseases (6).

We and others have demonstrated the importance of the membrane-bound latency-associated peptide (LAP)/TGF- β complex in Treg function (7–11). TGF- β is synthesized as pro-TGF- β and is then intracellularly processed by furin proprotein convertase to form a latent complex, consisting of TGF- β associated with LAP (12). Activated Tregs express LAP on the surface through the anchoring molecule glycoprotein A repetitions predominant (GARP) (13, 14) and it has been proposed that Tregs mediate their suppressive function by presenting active TGF- β to effector cells in a cell contact-dependent manner (7). LAP-expressing Tregs are also able

to confer infectious tolerance by inducing Foxp3 expression on naive CD4+ T cells in *in vitro* coculture experiments (11). It has been demonstrated that LAP+ T cells suppress disease in animal models of colitis (9, 15), lupus (16), atherosclerosis (17) and in experimental autoimmune encephalomyelitis (EAE) (18, 19). Furthermore, we have demonstrated a link between LAP+ T cells and oral tolerance as oral anti-CD3 induces CD4+LAP+ T cells that suppress EAE in a TGF- β -dependent mechanism (20). Tregs play an important role in oral tolerance (21–24) and orally administered antigen induces the secretion of TGF- β (25, 26) and TGF- β -secreting Treg cells termed T_H3 cells (27). Thus, LAP expression on the cell surface may be one of the features of T_H3-type Tregs induced by oral tolerance.

In vivo depletion of Tregs has been used as an important tool to investigate the role of Tregs in immune system homeostasis and control of autoimmunity. Treg depletion is usually accomplished either by the administration of anti-CD25 mAb (28) or by the use of Dereg (DEpletion of REGulatory T cells) mice carrying a DTR-eGFP transgene under the control of

an additional Foxp3 promoter, allowing specific depletion of Tregs by administration of diphtheria toxin (29). Both methods used to deplete Foxp3+ regulatory T cells demonstrate the important role Treg cells play in the balance between immunity and tolerance (30).

Given the importance of LAP+ T cells on immune regulation, we generated a murine-specific anti-LAP mAb (31) and in the present study we investigated for the first time the effect of *in vivo* administration of anti-LAP mAb on immune regulation and oral tolerance.

Methods

Mice

C57BL/6 and RAG-1 deficient (RAG-1^{-/-}) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Foxp3-GFP knock-in mice were obtained from Dr Vijay K. Kuchroo (Harvard Medical School, Cambridge, MA, USA). All mice were housed in a specific pathogen-free environment according to the animal protocol guidelines of the Committee on Animals of Harvard Medical School, which also approved the experiments.

Antibodies and reagents

Antibody specific to CD3 (145-2C11) (BD Biosciences, San Jose, CA, USA) was used to stimulate T cells *in vitro*. Fluorescent anti-mouse mAbs used in flow cytometry were CD4 (GK 1.5), CD8a (53-6.7) and LAP (TW7-16B4), purchased from Biolegend, San Diego, CA, USA, and Foxp3 (FJK-16s), IL-17 (eBio17B7), GARP (YGIC86), F4/80 (BM8) and IFN- γ (XMG1.2) purchased from eBiosciences, Santa Clara, CA, USA. Fixable Viability Dye eFluor 780 (FVD eFluor 780) from eBiosciences was used for cell viability studies. For Fc γ receptor blocking, we used CD16/CD32-specific antibody (BioXCell). Anti-LAP (clone TW7-16B4) mAb used for *in vivo* treatment was purified from hybridoma generated in our laboratory by Taka Oida (31) and the isotype control (IC; unspecific mouse IgG1 clone MOPC-21) was purchased from BioXcell. The P3U1 cell line expressing mouse TGF- β 1 (P3U1-mTGF- β 1) was generated in our laboratory by Taka Oida as previously described (31).

Oral administration of anti-CD3 antibody and *in vivo* anti-LAP treatment

In studies of oral tolerance induction, mice were orally treated with 5 μ g of hamster IgG anti-mouse CD3-specific antibody (clone 145-2C11) or hamster IgG control antibody (both from BioXCell) dissolved in 200 μ l of PBS by gastric intubation with an 18-gauge stainless steel feeding needle (Thomas Scientific) once a day for five consecutive days. In EAE experiments, we immunized mice 1 day after the last feeding. In other experiments, lymphoid organs were taken 1 day after the last oral treatment without subsequent immunization. For *in vivo* anti-LAP treatment, mice were injected intra-peritoneally (i.p.) with 50 μ g of anti-LAP-specific antibody (clone TW7-16B4) or mouse IgG1 IC antibody (BioXCell) dissolved in 200 μ l of PBS once a day for five consecutive days. In some experiments, EAE was induced in anti-LAP or IC-treated mice 1 day after the last antibody injection. In other experiments,

lymphoid organs were taken 1 day after the last antibody injection without subsequent immunization.

Induction and evaluation of EAE

Mice were injected subcutaneously (s.c.) in both flanks with 100 μ g myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) dissolved in PBS emulsified in an equal volume of CFA (Difco) supplemented with 5 mg ml⁻¹ *Mycobacterium tuberculosis* H37Ra. Pertussis toxin (PT; List Biological Laboratories) was injected i.p. (150 ng) on the day of immunization and 48 h later. Clinical assessment of EAE was performed daily after disease induction according to the following criteria: 0, no disease; 1, tail paralysis; 2, hindlimb weakness or partial paralysis; 3, complete hindlimb paralysis; 4, forelimb and hindlimb paralysis; and 5, moribund state. Mean clinical scores on separate days were calculated by adding scores of individual mice and dividing total number of mice in each group, including mice that did not develop signs of EAE.

Suppression assay

For the *in vitro* suppression assay, 1 $\times$ 10⁵ sorted CD4+ Foxp3+ T cells from anti-LAP or IC-treated mice cells were cultured at a 1:1 ratio with syngeneic naive responder CD4+ T cells (CD4+CD62L+CD44-Foxp3-) previously stained with CellTrace Violet according to the manufacturer's recommendation (CellTrace Violet proliferation kit; Invitrogen). Cells were stimulated with 1 μ g ml⁻¹ of soluble anti-CD3 in the presence of mitomycin-treated antigen-presenting cells (APCs) in 200 μ l of Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (FBS) in 96-well round-bottom plates. Proliferation was assessed 72 h later by flow cytometry based on the dilution of the CellTrace Violet dye on responder cells. In another *in vitro* suppression assay with similar conditions as described above, anti-LAP or IC antibodies were added (50 μ g ml⁻¹) directly to the coculture of nTregs (CD4+Foxp3+ T cells) and naive CD4+ T cells.

T-cell proliferation

Cells were cultured in quadruplicates at 2.0 $\times$ 10⁵ per well in the presence of various concentrations of anti-CD3 antibody or MOG₃₅₋₅₅ peptide in 96-well round-bottom microtiter plates (Corning, Tewksbury, MA, USA) for 72 h at 37°C with 5% CO₂ in a humid incubator. Cultures were pulsed with 0.25 μ Ci tritiated thymidine (3H-Thymidine; PerkinElmer, Waltham, MA, USA) for the last 12 h. 3H-Thymidine incorporation was measured (CPM) using a liquid scintillation beta counter (Wallac; PerkinElmer).

Cytokine analysis

Spleens and lymph nodes were harvested from mice, and single-cell suspensions were prepared. Cells were cultured at 2.0 $\times$ 10⁵ per well in 96-well U-bottom plates with 20 μ g ml⁻¹ MOG₃₅₋₅₅ peptide or 1 μ g ml⁻¹ of anti-CD3 in IMDM medium supplemented with 10% FBS. For TGF- β detection, cells were cultured in X-VIVO 15 serum-free medium (Lonza). Supernatants were harvested at 72 h of culture and the concentrations of indicated cytokines were measured by

quantitative capture ELISA according to the guidelines of the manufacturer (BD Biosciences).

Flow cytometry

Cells were washed (1700 r.p.m., 5 min at 4°C) with FACS buffer (Mg^{2+} and Ca^{2+} free HBSS with 2% FBS, 0.4% 0.5M EDTA and 2.5% 1M HEPES). $\text{Fc}\gamma$ receptors were blocked by incubation with anti-CD16/CD32 antibody for 10 min at 4°C. Cells were then stained with fluorescent anti-mouse cell surface molecule antibodies for 25 min at 4°C in dark. After staining, cells were washed again with FACS buffer before flow cytometry (BD™ LSR II; Becton Dickinson, Franklin Lakes, NJ, USA). For Foxp3 staining, cells were fixed and permeabilized after initial staining of surface antigens. After fixing/permeabilization, cells were incubated with anti-mouse Foxp3 antibody for 25 min at 4°C in the dark and then washed again in FACS buffer. For intracellular cytokine staining, cells were stimulated with PMA (50 ng ml^{-1}) and ionomycin (1000 ng ml^{-1}) in culture medium containing $1 \mu\text{l ml}^{-1}$ GolgiSTOP (BD Biosciences) for 4 h at 37°C with 5% CO_2 in a humid incubator. After incubation, cells were fixed and permeabilized before being stained. All FACS results show events gated on CD4+ T cells. All FACS data were analyzed using FlowJo software (TreeStar).

Adoptive transfer

Spleen cells from Foxp3-GFP knock-in mice were first enriched using CD4 microbeads isolation kit (Miltenyi Biotec). CD4+LAP–Foxp3– T cells were then sorted and injected (1×10^6 cells per mouse) intravenously into RAG1^{−/−} mice. Twenty-four hours later, recipient mice were treated daily, for 10 consecutive days, with 5 μg of oral anti-CD3 or IC.

Antibody-dependent cell-mediated phagocytosis assay

Thioglycollate-elicited peritoneal exudate cells (thio-PEC) were used as effector cells. To obtain thio-PEC, mice were injected i.p. with 1 ml of 3% thioglycollate (Sigma Aldrich, St Louis, MO, USA). Five days later, peritoneal cells were collected and the contaminating erythrocytes were lysed. The target cells, P3U1-mTGF β -1 cells, were first labeled with CellTrace violet (Invitrogen), incubated with $10 \mu\text{g ml}^{-1}$ of TW7-16B4 anti-LAP mAb or mouse IgG1 for 30 min on ice and washed. Thio-PEC at a target/effector ratio of 1:2 were added and incubated for 2 h at 37°C. The cells were then stained with anti-F4/80 Ab-allophycocyanin and analyzed by flow cytometry.

Complement-mediated cytotoxicity

Anti-LAP was added to P3U1-mTGF- β 1 cells ($1 \times 10^6 \text{ ml}^{-1}$) in complete medium supplemented by mouse serum, inactivated or not by incubation at 56°C for 30 min. Cells were incubated for 3 h at 37°C and cell lysis was determined by viability dye staining (FVD eFluor 780) according to the manufacturer's instructions and analyzed by flow cytometry.

Statistical analysis

GraphPad Prism 6.0 was used for statistical analysis (unpaired, two-tailed Student's *t*-test or one-way ANOVA,

followed by Tukey's multiple comparisons). Differences were considered statistically significant with a *P* value of <0.05.

Results

Effect of in vivo anti-LAP mAb administration on CD4+LAP+ T cells and immune function

Given the important regulatory activity of CD4+LAP+ T cells in experimental models, we generated a novel anti-mouse LAP mAb that binds to LAP on the surface of mouse cells as we found that monoclonal anti-human LAP antibody does not cross-react with mouse LAP (31). Our murine-specific anti-LAP mAb provided the opportunity to investigate the role of LAP+ Tregs *in vivo* and to determine whether targeting LAP+ Tregs *in vivo* affected Foxp3 Tregs. To study LAP+ cells *in vivo*, we treated mice daily for five consecutive days with anti-LAP (TW7-16B4 clone) i.p. (Fig. 1A). We found that anti-LAP administration significantly reduced CD4+LAP+ T cells in spleen and mesenteric lymph nodes (MLNs) (Fig. 1B) without affecting the CD4+Foxp3+ T-cell population percentage (Fig. 1C), although it decreased LAP expression mainly on CD4+Foxp3+ T cells (Fig. 1D). GARP (LRRC32) has been described as the anchoring molecule for surface LAP (13, 14). *In vivo* anti-LAP treatment also reduced the number of LAP+GARP+ double-positive CD4 T cells (Fig. 1E), and a reduction was also observed on the CD4+GARP+ T-cell total population (Supplementary Figure 1A, available at *International Immunology* Online). Anti-LAP administration did not affect LAP expression on CD8+ T cells (Supplementary Figure 1B, available at *International Immunology* Online), a previously described T-cell population with regulatory activity (32).

Although a reduction of LAP expression on Foxp3+ CD4 T cells was observed following *in vivo* anti-LAP treatment (Fig. 1D), the *in vitro*-suppressive function of these cells was not affected as Foxp3+ cells from anti-LAP or IC-treated mice were equally suppressive (Fig. 1F). The addition of anti-LAP directly to the *in vitro* suppression assay did not affect the regulatory function of Foxp3+ CD4 T cells either (Supplementary Figure 1C, available at *International Immunology* Online).

In addition, spleen and MLN cells from anti-LAP-treated mice proliferated more vigorously and produced more IFN- γ , IL-17 and IL-2 when activated with anti-CD3 *in vitro* for 72 h (Fig. 1G and H, respectively). These results indicate that reduction of CD4+LAP+ T cells by *in vivo* anti-LAP promoted a shift toward a pro-inflammatory immunological profile under steady-state conditions.

In studies of Foxp3+ Treg depletion, investigators asked whether phagocytosis could be demonstrated in an *in vitro* system (28). The TW7-16B4 anti-LAP mAb is a mouse IgG1 antibody and through its Fc domain, could potentially activate cellular effector mechanisms for antibody-dependent cell-mediated cytotoxicity or antibody-dependent cell-mediated phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). To investigate these mechanisms, we established an *in vitro* ADCP assay using thio-PEC from wild-type mice as effector cells; a flow cytometry profile of thio-PEC showed that >90% express F4/80, a pan macrophage marker (Supplementary Figure 2A, available at *International Immunology* Online). Although LAP+ Tregs could be used

Fig. 1. Anti-LAP *in vivo* administration induces a pro-inflammatory phenotype in naive mice. C57BL/6J mice were injected i.p. with 50 µg of anti-LAP (TW7-16B4 clone) or IC for 5 days and 24 h after the last injection, MLNs and spleen cells were harvested (A). FACS staining showing the percentages of CD4+LAP+ (B), CD4+Foxp3+ (C), CD4+Foxp3+LAP+ and CD4+GARP+LAP+ T cells (E). To access *in vitro*-suppressive function of Tregs, CD4+Foxp3+ T cells sorted from Foxp3-GFP knock-in mice treated with 50 µg of anti-LAP (TW7-16B4 clone) or IC daily for 5 days were cocultured for 3 days with CellTrace Violet-stained naive (CD4+CD62L+CD44–Foxp3–) T cells from Foxp3-GFP knock-in mice in the presence of mitomycin-treated APCs at a 1:1 ratio and stimulated with 1 µg ml⁻¹ of purified anti-CD3 (F). Spleen and MLN cells from anti-LAP or IC-treated mice were activated *in vitro* for 72 h with different concentrations of anti-CD3 for proliferation assay (G). IL-2, IL-17 and IFN-γ were measured in the supernatant of spleen and MLN cell cultures stimulated with 1 µg ml⁻¹ of anti-CD3 for 72 h (H).

as target cells, LAP+ Treg isolation requires antibody binding to LAP on the cell surface, which would interfere with the ADCP assay. Thus, we used P3U1-mTGF-β1 cells previously generated in our laboratory (31). This cell line expresses high levels of murine LAP on the surface and does not express F4/80 (Supplementary Figure 2B, available at *International Immunology Online*). As shown in Fig. 2(A), anti-LAP antibody significantly enhanced the percentage of CellTrace Violet-labeled P3U1-mTGF-β1 cells that were phagocytosed by F4/80+ macrophages. We also investigated complement-mediated lysis (CDC) and found that in the presence of anti-LAP, CDC (abrogated after heat inactivation of serum) caused an increase in P3U1-mTGF-β1 target cell death (Fig. 2B), indicating that lysis by

CDC is another mechanism through which anti-LAP mAb can affect LAP+ cells.

Effect of in vivo anti-LAP mAb administration on CD4+LAP+ T cells, GARP expression and IL-17/IFN-γ responses after active immunization

To investigate the effect of anti-LAP treatment during an inflammatory immunological response, mice treated with anti-LAP antibody were then immunized with MOG₃₅₋₅₅ in CFA. Two weeks after immunization, mice were sacrificed and spleen and inguinal lymph node (iLN) cells harvested for *in vitro* recall response (Fig. 3A). We found that the percentage of CD4+LAP+ T cells was decreased following anti-LAP

Fig. 2. LAP+ cell depletion is partially dependent on antibody-dependent macrophage phagocytosis and complement lysis. To confirm the role of macrophages on CD4+LAP+ T-cell depletion, the ADCP assay was performed using thio-PEC from wild-type mice as effectors and CellTrace Violet-labeled P3U1-mTGF- β 1 cells as targets. P3U1-mTGF- β 1 were incubated with 10 μ g ml⁻¹ of anti-LAP or IC and then cocultured for 2 h at 37°C with thio-PEC at a target/effector ratio of 1:2. Cells were then stained with anti-F4/80 antibody and analyzed by flow cytometry (A). Complement-mediated lysis of P3U1-mTGF- β 1 cells was determined by viability dye staining and flow cytometry analysis after incubation with anti-LAP and normal or heat-inactivated mouse serum for 3 h (B).

administration even 14 days after immunization and the last anti-LAP injection, whereas the number of CD4+Foxp3+ T cells was not affected (Fig. 3B), although LAP expression on Foxp3+ CD4 T cells was again reduced (Supplementary Figure 3A, available at *International Immunology* Online). Similarly to what was observed in unimmunized mice, GARP+LAP+ double-positive CD4 T cells were also reduced (Supplementary Figure 3B, available at *International Immunology* Online).

In vitro re-stimulation of spleen and iLN cells from anti-LAP-treated mice with MOG₃₅₋₅₅ peptide showed a marked increase in proliferation (Fig. 3C). Anti-LAP-treated mice also produced higher amounts of IL-17 and IFN- γ after *in vitro* re-stimulation with MOG₃₅₋₅₅ peptide (Fig. 3D). Thus, anti-LAP administration promotes a stronger MOG-specific inflammatory response after MOG₃₅₋₅₅ immunization.

In vivo anti-LAP administration abrogates the protective effect of oral anti-CD3 in EAE

We have shown that the induction of CD4+LAP+ T cells by oral anti-CD3 suppresses EAE (20). To test whether *in vivo* anti-LAP administration interferes with oral tolerance induced by anti-CD3 feeding, we treated C57BL/6 mice concomitantly with oral anti-CD3 and anti-LAP i.p. followed by immunization with MOG₃₅₋₅₅ in CFA for EAE induction. Mice also received PT injection i.p. on the day of the immunization and 48 h later (Fig. 4A). We found that anti-LAP administration reversed oral

tolerance induced by anti-CD3 (Fig. 4B). Mice treated orally with anti-CD3 showed an increase in CD4+LAP+ T cells in the spleen 20 days after EAE induction, which was reversed by anti-LAP administration (Fig. 4C). Additionally, spleen cells from oral anti-CD3-treated mice displayed reduced proliferation after MOG₃₅₋₅₅ re-stimulation and this reduction was abrogated by anti-LAP administration (Fig. 4D). These results are consistent with the effect on the EAE clinical score (Fig. 4B). Anti-LAP administration in IC-fed mice did not lead to a more severe EAE (Fig. 4B).

Anti-LAP mAb may affect oral tolerance by depleting LAP+ T cells and/or by preventing the induction of new LAP-expressing cells. To determine whether LAP+ T-cell expansion after oral anti-CD3 came from cells previously expressing LAP or from *de novo* induction of LAP expression on previously LAP- cells, we transferred CD4+LAP-Foxp3- T cells from Foxp3-GFP knock-in mice into Rag-1^{-/-} mice and fed them anti-CD3. We found that oral anti-CD3 induced LAP expression with no increase in Foxp3 expression on the transferred cells (Fig. 5A). MLN cells from anti-CD3-treated mice secreted more TGF- β upon *in vitro* activation with anti-CD3 (Fig. 5B), which is involved in the establishment of a tolerogenic milieu in the gut.

Effect of in vivo anti-LAP administration on EAE induced without PT

Our results indicate that *in vivo* anti-LAP administration induces a pro-inflammatory immunologic response with

Fig. 3. Anti-LAP administration enhances the pro-inflammatory immune response following MOG₃₅₋₅₅ immunization. C57BL/6J mice were injected i.p. with 50 μ g of anti-LAP (TW7-16B4 clone) or IC for 5 days and 24 h after the last injection, mice were immunized subcutaneously with MOG₃₅₋₅₅+CFA. Fourteen days later, iLNs and spleen cells were harvested (A). FACS staining showing the percentage of CD4⁺LAP⁺ and CD4⁺Foxp3⁺ T cells in spleen and lymph nodes of anti-LAP or IC-treated mice (B). Spleen and lymph node cells from anti-LAP or IC-treated mice were activated *in vitro* for 72 h with different concentrations of MOG₃₅₋₅₅ peptide for the proliferation assay (C). IFN- γ and IL-17 were measured in the supernatant of spleen and iLNs cell cultures stimulated with 100 μ g ml⁻¹ of MOG₃₅₋₅₅ peptide for 72 h (D).

Fig. 4. Anti-LAP administration abrogates oral tolerance induced by oral anti-CD3. C57BL/6J mice were orally treated with 5 μ g of anti-CD3 or its IC for five consecutive days and concomitantly injected i.p. with 50 μ g of anti-LAP (TW7-16B4 clone) or IC for 5 days. Twenty-four hours after the last injection and oral treatment, mice were immunized subcutaneously with MOG₃₅₋₅₅+CFA and also received PT i.p. injection that was repeated 48 h later for EAE induction (A). EAE progression was followed and the disease score was monitored from day 10–30 (B). Some mice were sacrificed 14 days after immunization and iLNs and spleen cells were harvested for FACS staining and proliferation. The percentage of CD4⁺LAP⁺ T cells is shown on gated CD4⁺ T cells (C). Spleen and iLN cells from anti-LAP or IC-treated mice were activated *in vitro* for 72 h with 100 μ g ml⁻¹ of MOG₃₅₋₅₅ peptide for the proliferation assay (D).

Fig. 5. Oral anti-CD3 induces LAP expression on T cells that were LAP⁺Foxp3⁺. CD4⁺LAP⁺Foxp3⁺ T cells were sorted from Foxp3-GFP knock-in mice and transferred into RAG-1^{-/-} recipient mice. Recipients were treated orally for 10 days with 5 μ g of anti-CD3 or IC and 24 h after the last treatment, MLN cells were harvested and stained for CD4 and LAP and analyzed by FACS (A). MLN cells of anti-CD3- or IC-treated recipient mice were activated *in vitro* with 1 μ g ml⁻¹ of anti-CD3 and TGF- β was measured in the supernatant after 72 h of cell culture (B).

increased proliferation and IL-17 and IFN- γ secretion, cytokines known to be important in the pathogenesis of EAE (33, 34). Although we did not observe enhancement of MOG-induced EAE in C57BL/6 mice given anti-LAP antibody (Fig. 4B), we asked whether *in vivo* administration of anti-LAP antibody would affect EAE if EAE was induced in a sub-optimal fashion. Thus, we investigated whether anti-LAP administration enhanced EAE in mice not given PT injection (Fig. 6A), a compound classically used to facilitate EAE induction (35, 36). Mice immunized with MOG₃₅₋₅₅ in CFA without PT only developed very mild EAE (Fig. 6B). We found, however, that anti-LAP administration prior to MOG₃₅₋₅₅ immunization in the absence of pertussis led to the development of severe EAE (Fig. 6B), accompanied by increased T_H1 and T_H17 infiltration in the central nervous system (CNS) (Fig. 6C). Spleen and iLN cells from anti-LAP-treated mice also displayed increased proliferation after *in vitro* activation with MOG₃₅₋₅₅ (Fig. 6D). No differences in the frequency of CD4⁺LAP⁺ T cells were observed at 30 days after immunization (Fig. 6E), suggesting that CD4⁺LAP⁺ T cells play an important role in the induction phase of EAE as opposed to after disease is established.

Discussion

The expression of TGF- β on the cell surface of Tregs has been described as playing an important role in Treg-mediated suppression (7, 11) and LAP⁺ Tregs play a key role in oral tolerance induction (37). Using a novel mouse-specific anti-LAP mAb we were able to investigate the importance of LAP⁺ cells *in vivo*. We found that *in vivo* administration of anti-LAP mAb to naive mice induced a pro-inflammatory phenotype associated with increased T-cell proliferation and increased IL-17 and IFN- γ secretion. These effects may in part be due to the depletion of LAP⁺ T cells since anti-LAP antibody binding

in vitro caused increased ADCP and CDC. Down-modulation (endocytosis) of surface LAP may also occur. Although LAP expression was decreased on Foxp3⁺ CD4⁺ T cells following anti-LAP administration, the number of Foxp3⁺ CD4⁺ T cells did not change and Foxp3 Tregs retained their suppressive properties. This may in part be because only a small percentage of Foxp3⁺ CD4⁺ T cells express LAP *in vivo* and overall, the activity of total Foxp3⁺ CD4⁺ T-cell population was not directly affected by anti-LAP administration.

The effect of *in vivo* anti-LAP was also evident in an autoimmune inflammatory scenario. In EAE, PT acts to increase permeabilization of the blood-brain barrier facilitating the migration of pathogenic T cells to the CNS (36, 38, 39) and also promotes development of encephalitogenic T_H17 cells (35, 40). Without PT, only mild EAE results. We found that MOG/CFA immunization in the absence of PT resulted in severe EAE when anti-LAP was administered. This was associated with increased T-cell proliferation and infiltration of T_H1 and T_H17 cells into the CNS. Thus, it appears that the high incidence and severity of EAE in the absence of PT injection is due to enhanced T-cell activation and induction of T_H1 and T_H17 cells secondary to the loss of LAP⁺ Treg activity. Interestingly, we did not observe worsening of EAE by anti-LAP administration in animals given PT, suggesting that LAP⁺ Tregs may not be effective when there is maximal induction of EAE.

We have previously reported the importance of LAP⁺ T cells in oral tolerance and we found here that *in vivo* anti-LAP administration completely abolished the tolerogenic effect of oral anti-CD3 by affecting CD4⁺LAP⁺ T cells. Moreover, we found that surface LAP induction following oral anti-CD3 occurs in a population of T cells without prior regulatory function. Thus, oral anti-CD3 may act not only by expanding an existing population of regulatory T cells but also by *de novo* induction of CD4⁺LAP⁺ Tregs. In this context, LAP expression appears to

Fig. 6. Anti-LAP *in vivo* administration exacerbates EAE. C57BL/6J mice were injected i.p. with 50 µg of anti-LAP (TW7-16B4 clone) or IC for 5 days and 24 h after last injection, mice were immunized s.c. with MOG₃₅₋₅₅+CFA without PT i.p. injection for EAE induction (A). EAE progression was followed and disease score was monitored from day 10–30 (B). Mice were sacrificed at day 30 after immunization and iLNs, spleen and CNS-infiltrating cells were harvested for FACS staining and proliferation. The percentage and total number of CD4+IL-17+ or CD4+IFN-γ+ in CNS are shown (C). Spleen and lymph node cells from anti-LAP or IC-treated mice were activated *in vitro* for 72 h with different concentrations of MOG₃₅₋₅₅ peptide for the proliferation assay (D). FACS staining with the percentage of CD4+LAP+ T cells in spleen, iLNs and CNS is shown (E). FACS results show events gated on CD4+ T cells.

be a key marker of T_h3-type Tregs induced by oral anti-CD3 (20). Our group has also shown that CD4+LAP+ Tregs play an important role in mucosal tolerance induced by nasal anti-CD3 administration. In this instance, regulatory activity was dependent on IL-10 secretion (16, 41). Thus, LAP+ expression on Tregs is not only related to TGF-β-dependent Tregs but may also be related to Tr1-type Treg cells. The induction of TGF-β versus IL-10-dependent Tregs appears to be dependent on the mucosal site of induction of the Tregs population (42). In the nasal anti-CD3 model, we have shown that the induction of CD4+LAP+IL-10+ T cells is dependent on IL-27 produced by upper airway resident dendritic cells and is controlled by the transcription factors aryl hydrocarbon receptor and c-Maf (41).

Tregs not only serve to modulate autoimmunity, but enhanced Treg activity can promote malignancy (43, 44). It has been shown that membrane-bound TGF-β is expressed on the surface of malignant B cells in B-cell non-Hodgkin

lymphoma and this correlates with the inhibition of proliferation and cytokine production of intratumoral T cells (45). Furthermore, studies of colorectal cancer in humans have shown that CD4+LAP+ T cells that accumulate in tumor sites are more suppressive than Foxp3+ Tregs and are also associated with disease progression (46, 47). Thus, it is possible that anti-LAP antibody administration could have a role in the therapy of diseases where excessive immune regulation contributes to the disease process.

In summary, our findings demonstrate the importance of CD4+LAP+ T cells on the control of immune homeostasis and autoimmunity and provides a new tool for the *in vivo* investigation of murine LAP+ Tregs on immune function.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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