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Targeted inhibition of IL-10-secreting CD25$^-$ Treg *via* p38 MAPK suppression in cancer immunotherapy

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Cancer-induced immunotolerance mediated by inducible Treg (iTreg) is a major obstacle to cancer immunotherapy. In a basic study of immunotolerance, injection of an endogenous superantigen, *i.e.* the minor lymphocyte stimulatory (Mls)-1^a, into specific TCR Vβ8.1-Tg mice enabled generation of anergic CD25$^-$ iTreg, the immunosuppressive function of which was maintained by IL-10 production *via* p38-MAPK activation. Interestingly, although p38-chemical inhibitor (p38-inhibitor) is capable of breaking CD25$^-$ iTreg-induced immunotolerance, the p38-inhibitor had hardly any immunotolerance breaking effect when CD25$^+$ Treg were present, suggesting that depletion of CD25$^+$ Treg is necessary for p38-inhibitor to be effective. Peptide OVA$_{323–339}$ *iv.*-injection into its specific TCR-Tg (OT-II) mice also induced adaptive tolerance by iTreg. Peptide immunotherapy with p38-inhibitor after CD25$^+$ Treg-depletion was performed in an OVA-expressing lymphoma E.G7-bearing tolerant model established by adoptive transfer of OT-II CD25$^-$ iTreg, which resulted in suppression of tumor growth. Similarly, the antitumor immunity induced by peptide immunotherapy in colon carcinoma CT26-bearing mice, in which the number of IL-10-secreting iTreg is increased, was augmented by treatment with p38-inhibitor after CD25$^+$ Treg-depletion and resulted in inhibition of tumor progression. These results suggest that simultaneous inhibition of two distinct Treg-functions may be important to the success of cancer immunotherapy.

Key words: Anergy · Immunotherapy · Tolerance · Treg · Tumor immunology

Supporting Information available online

Introduction

Anergic CD4$^+$ treg, which function as immunosuppressors, are essential to the negative regulation of autoimmune or excessive inflammatory responses [1]. Two subpopulations are known to exist: CD25$^+$ natural treg (CD25$^+$ nTreg), which develop naturally in the thymus, and inducible Treg (iTreg), which are generated by peripheral lymphoid tissue in response to exposure to a specific exogenous Ag [2, 3]. Recent evidence indicates that iTreg include not only the CD25$^+$CD4$^+$ population, but also the CD25$^-$CD4$^+$ population.

The CD25$^-$CD4$^+$ population, which include IL-10-secreting Treg type 1 (Tr1) cells, are generated by peripheral CD4$^+$ T cells under the influence of IL-10 or tolerogenic

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DC (tDC) [4–6], and they are also found in melanoma patients [7].

Recent cancer-immunotherapy strategies attempt to overcome the immunotolerance associated with cancer [8, 9]. Anti-CD25 Ab have been used to deplete $CD25^+$ nTreg populations, since they have been shown to suppress antitumor immunity [10, 11]. However, these approaches alone may hinder active immunotherapy or the generation of tumor-specific T cells, because CD25 is up-regulated on effector T cells, and not expressed on all Treg [12, 13].

We have previously shown that the p38 MAPK pathway in IL-10-producing anergic $CD4^+$ T cells is dominant and a target for inhibition of both IL-10 production and unresponsiveness [14]. In the present study we found that depletion of $CD25^+$ Treg in combination with treatment with a p38 chemical inhibitor (p38-inhibitor) is necessary to completely block the immunosuppressive function of IL-10-producing anergic $CD25^-$ iTreg, which suggested that a combination of treatment by both methods may be important to the success of peptide immunotherapy in cancer patients.

Results

p38 chemical inhibitor breaks the immunotolerance of $CD25^-$ iTreg in the absence of $CD25^+$ Treg

Anergic $CD4^+$ T cells can be induced *in vivo* from the majority of $CD4^+$ T cells in TCR Vβ8.1-Tg mice (CBA/Ca strain) by adoptive transfer of super-Ag minor lymphocyte stimulatory (Mls)-1^a-expressing B cells purified from CBA/j strain, as previously described [15]. The unresponsiveness maintained by iTreg is also called adaptive tolerance [2]. We previously reported that Mls-1^a-induced immunotolerance was maintained by IL-10 production, and that the immunotolerance was mediated by autophosphorylation of p38 in anergic $CD4^+$ T cells. Proliferation of and IL-2 production by $CD4^+$ T cells is restored by treating the cells with the p38-inhibitor SB203580 together with TCR stimulation *via* Mls-1^a [15] (Supporting Information Fig. 1). In the present study we examined the effect of p38 inhibition *in vitro* on anergic $CD4^+$ T cells that included iTreg from which the $CD25^+$ Treg had or had not been depleted. Interestingly, after depletion of $CD25^+$ Treg proliferation of anergic $CD25^-$ T cells significantly recovered from unresponsiveness, and an approximately 3.5-fold increase was observed when compared with the response of anergic $CD4^+$ T cells (of which 13–16% were $CD25^+$ T cells) in the absence of depletion of the $CD25^+$ Treg by SB203580 treatment and TCR stimulation ($p<0.005$; *t*-test) (Fig. 1A). However, there was little recovery of the TCR response by anergic $CD25^-$ T cells after SB203580 treatment in co-culture with a 20% addition of $CD25^+$ cells ($p<0.05$, *t*-test) (Fig. 1B). By contrast, the proliferative response of naïve T cells was unchanged by SB203580 treatment ($p = 0.13$; *t*-test), and it decreased with a 20% addition of $CD25^+$ nTreg ($p = 0.067$; *t*-test) (Fig. 1B). These results suggested that in the absence of $CD25^+$ Treg, the immunotolerance of $CD25^-$ iTreg was effectively eliminated by treatment with SB203580 and TCR-stimulation.

Foxp3 and *TAB1* gene expression by anergic $CD25^+$ and $CD25^-CD4^+$ T cells

$CD25^+$ nTreg occur naturally in the thymus and express the transcription factor Foxp3 [16]. $Foxp3^+$ T cells can also be generated from the peripheral immune compartment [3]. We used the RT-PCR method to compare expression of the *TGF-β-activated kinase-1-binding protein-1 (TAB1)* gene [17] and *Foxp3* gene in a population of $CD25^+$ and a population of $CD25^-$ cells containing iTreg, because our previous data indicated an increase in the *TAB1* gene in Mls-1^a-induced anergic $CD4^+$ T cells, whereas there was no difference between naïve and anergic $CD4^+$ T cells in the level of ubiquitous TAB1 protein [18]. Although the function of the increase in the *TAB1* gene is unclear, our data suggest that the almost ubiquitous TAB1 protein (72 kDa) may be present in a glycosylated and partially phosphorylated form in the membrane and cytosol fractions of naïve $CD4^+$ T cell lysates, whereas native TAB1 protein (56 kDa) increased in anergic $CD4^+$ T cells lysates. Interestingly, deglyosylation by treatment with a peptide:*N*-glycanase (PNGase) shifted the molecular weight of TAB1 from 72 to 56 kDa, and up-regulated the ability of TAB1 to activate p38 in a kinase assay, suggesting that TAB1 (56 kDa)

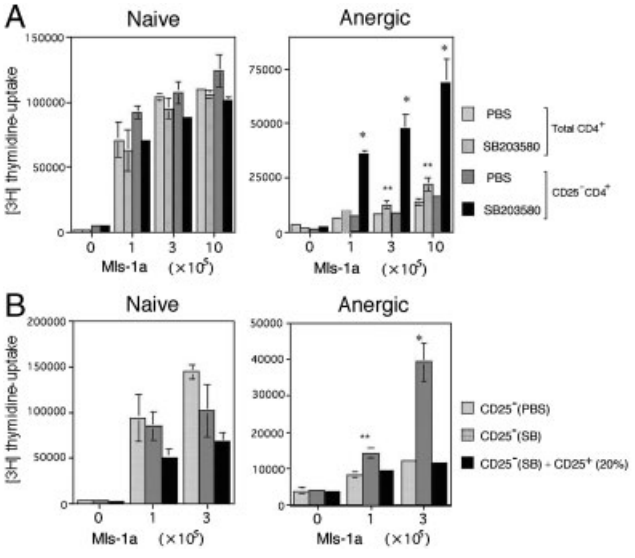

Figure 1. p38-inhibitor is capable of breaking the immunotolerance of $CD25^-$ iTreg in the absence of $CD25^+$ Treg. Spleens were harvested from Mls-1^a-treated and untreated Vβ8.1-Tg mice and purified into $CD4^+$, $CD25^-CD4^+$, and $CD25^+CD4^+$ populations as described in the *Materials and methods* section. (A) The effect of SB203580 (2 μM) treatment on the proliferative TCR response mediated by Mls-1^a of all $CD4^+$ and $CD25^-CD4^+$ populations of naïve and anergic T cells was analyzed by [^{3}H]-TdR assay. (B) The proliferative response to PBS and SB203580 (2 μM) by Mls-1^a-stimulated naïve $CD25^-$ T cells and anergic $CD25^-$ T cells (5×10^5/well) with or without 20% addition (1.2×10^5/well) of $CD25^+$ nTreg or anergic $CD25^+$ T cells was analyzed by [^{3}H]-TdR assay. $^*p<0.005$, $^{**}p<0.05$; Student's *t*-test. Data show mean ± SD. Data are representative of three independent experiments.

expression, which is dependent on increase of *TAB1* gene, may activate p38 less efficiently than ubiquitous TAB1 (72 kDa) (Supporting Information Fig. 2). In addition, our previous data indicated that there was no expression of the *TAB1* gene or 56 kDa TAB1 protein in Th1 (expressing *T-bet* gene)-polarized cells, Th2 (expressing *GATA3* gene)-polarized cells, and $CD4^+$ T cells activated by PMA plus Ca^{2+}-ionophore *in vitro* [14]. At least, expression of both the *TAB1* and *Foxp3* gene was detected in anergic $CD25^-$ T cells and anergic $CD25^+$ T cells, but not in naïve $CD4^+$ T cells. By contrast, $CD25^+$ nTreg expressed *Foxp3* but not *TAB1* (Fig. 2A). These results suggest that *TAB1* gene increase in populations ($CD25^-$ and $CD25^+$) of both anergic $CD4^+$ T cells can be showed as the characteristics in profiling of iTreg.

Difference between the immunosuppressive functions of $CD25^-$ iTreg and $CD25^+$ nTreg

The distinct immunosuppressive activity of anergic $CD25^-$, $CD25^+$ iTreg, and $CD25^+$ nTreg against naïve $CD4^+$ T cells was evaluated in a T-cell suppression assay. Both the anergic $CD25^-$ T cells ($CD25^-$ iTreg) and anergic $CD25^+$ T cells ($CD25^+$ iTreg) significantly inhibited proliferation by naïve $CD4^+$ T cells ($p<0.05$; *t*-test) (Fig. 2B). Immunosuppression was significantly induced by the 20% addition of anergic $CD25^-$ or anergic $CD25^+$ iTreg, but 20% addition of $CD25^+$ nTreg had hardly any effect (Fig. 2B). Therefore, we further evaluated the response with 33% addition of each cell population. CFSE-labeled naïve $CD4^+$ T cells were used as a monitor in the FACS analysis. Similar T-cell suppression assays of $CD25^+$ nTreg and anergic $CD25^-$ iTreg were performed, and p38-inhibitor sensitivity and IL-10 dependency were assessed in order to analyze the differences between the immunosuppressive functions of $CD25^-$ iTreg and $CD25^+$ nTreg. The immunosuppressive response induced by the 33% addition of anergic $CD25^-$ iTreg was eliminated by treatment with SB203580 or neutralization of IL-10, but neither had any effect on the suppressive capacity of $CD25^+$ nTreg (Fig. 2C), thereby demonstrating that the immunosuppressive activity of IL-10-producing $CD25^-$ iTreg, but not $CD25^+$ nTreg can be selectively blocked by SB203580 treatment.

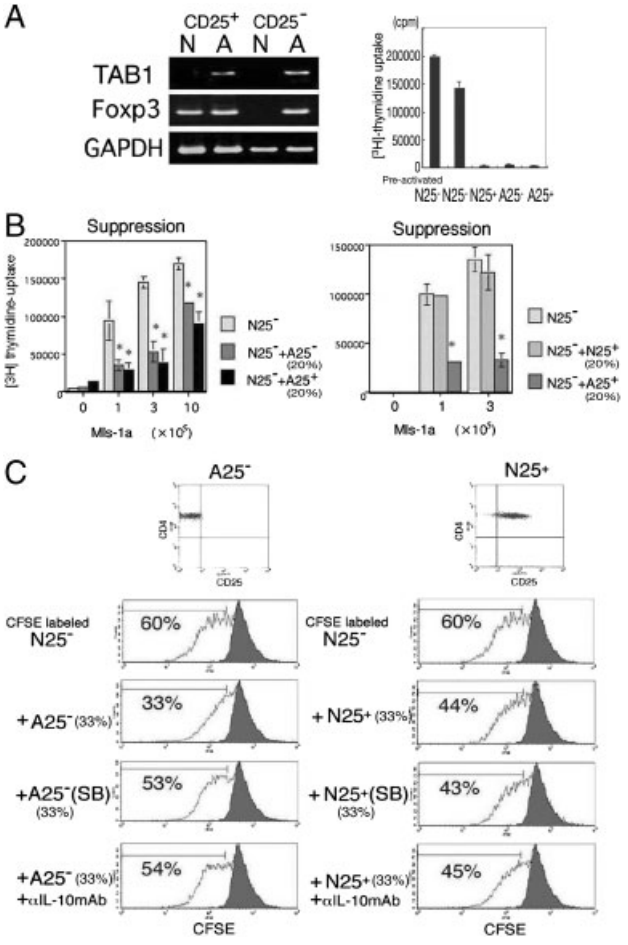

Figure 2. Difference between the immunosuppressive functions of $CD25^-$ iTreg and $CD25^+$ nTreg. (A) TAB1 and Foxp3 gene expression in naïve T cells ($N25^-$), $CD25^+$ nTreg ($N25^+$), anergic $CD25^-CD4^+$ T cells ($A25^-$), and anergic $CD25^+CD4^+$ T cells ($A25^+$) from Vβ8.1 Tg mice was analyzed by RT-PCR. The pre-activated $N25^-$ is naïve $CD25^-CD4^+$ T cells activated by treatment for 4 h with PMA (10 ng/mL) plus ionomycin (1 μM). Proliferation by $CD4^+$ (pre-activated $N25^-$, $N25^-$, $N25^+$, $A25^-$, and $A25^+$) T cells was analyzed by $[^3H]$-TdR uptake. Data show mean ± SD. Data are representative of two independent experiments. (B) The immunosuppressive activity by 20% addition of $CD25^+$ nTreg ($N25^+$), anergic $CD25^-$ T cells ($A25^-$), and anergic $CD25^+$ T cells ($A25^+$) was analyzed by T-cell suppression assay, as described in the *Materials and methods* section. (C) The immunosuppressive activity by 33% addition of $CD25^+$ nTreg ($N25^+$) or anergic $CD25^-CD4^+$ T cells ($A25^-$) on Mls-1^a-stimulated proliferation (cell division) by CFSE-labeled naïve T cells ($N25^-$) was analyzed by FACS. PBS, SB203580 (2 μM), or anti-IL-10 (NA/LE) mAb (20 μg/mL) was added to the cell-culture for 72 h. $^*p<0.05$; Student's *t*-test. Data are representative of two independent experiments.

Characteristics of anergic OT-II $CD25^-$ iTreg induced by i.v.-injection of $pOVA_{323-339}$

Since $pOVA_{323-339}$ (5 μg, i.v.) induces peripheral tolerance in DO11.10 mice [19, 20], we investigated whether adaptive tolerance would be induced when $pOVA_{323-339}$ was injected i.v. into $I\text{-}A^b$ restricted $OVA_{323-339}$-specific TCR-transgenic (OT-II Tg) mice. The $pOVA_{323-339}$-specific $CD25^-CD4^+$ T cells that were freshly purified from OT-II Tg mice on day 7 after injection of a dose of 0, 10, 50, or 150 μg of $pOVA_{323-339}$ showed unresponsiveness of proliferation (anergic state) in a dose-dependent manner (Fig.3A and B). Interestingly, expression of TAB1 (56 kDa) and phospho-p38 in $CD25^-CD4^+$ T cells increased in response to $pOVA_{323-339}$ in a dose-dependent manner after i.v. injection into OT-II mice (Fig. 3A). We therefore used the 50 μg dose of $pOVA_{323-339}$ i.v. to induce a sufficiently anergic state as determined by unresponsiveness with regard to IL-2 production and proliferation (Fig. 4C). Characteristics of the anergic OT-II iTreg were an increase in the $ICOS^+CD25^-GITR^-$ population from 2.8 to 29.3% (data not shown), and clearly expression of TAB1 (56 kD) and the genes of *TAB1, Foxp3, IL-10,* and *TLR9* in the $CD25^-CD4^+$ population (Fig. 3C and 4B). Interestingly, a

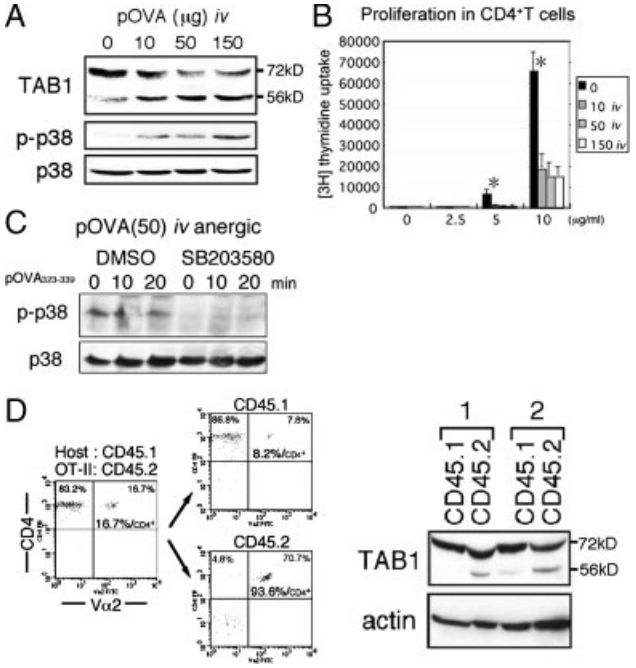

Figure 3. Increase in native TAB1 (56 kD) in OVA-specific OT-II anergic $CD25^-$ iTreg. (A) OVA-specific $CD25^-CD4^+$ T cells were freshly purified from OT-II Tg mice on day 7 after injection of 0, 10, 50, or 150 μg of $pOVA_{323–339}$. Expression of TAB1, p-p38, and total p38 in anergic OT-II $CD25^-$ iTreg was analyzed by Western blot. Data are representative of two independent experiments. (B) Proliferation in response to $pOVA_{323–339}$ (0–10 μg/mL) by anergic OT-II $CD25^-$ iTreg was analyzed by $[^3H]$-TdR uptake. $^*p<0.05$, Student's *t*-test. Data are representative of two independent experiments. (C) p38 autophosphorylation in anergic OT-II $CD25^-$ iTreg was analyzed by Western blot. SB203580 (2 μM) was used as the target inhibitor of p38 MAPK substrate phosphorylation. (D) On day 7 after injection of Ly5.1-B6 mice (CD45.1) with 50 μg $pOVA_{323–339}$ following adoptive transfer of OT-II $CD4^+$ T cells (CD45.2), $CD4^+$ T cells were purified and separated into CD45.1 cells and CD45.2 cells by streptavidin-BD IMag DM after staining with biotin-conjugated anti-CD45.2 mAb. These cell populations are shown in the left panel after staining with anti-CD4/anti-Vα2 (OT-II). Western blot shows the pattern of TAB1 expression in two independent experiments.

recent study showed that IL-10-producing $ICOS^+$ Treg, but not TGF-β-producing $ICOS^-$ Treg, are capable of suppressing functions of both DC and T cells [21]. These findings suggest that the characteristics of anergic OT-II $CD25^-$ iTreg are similar to those of iTreg induced by IL-10 or tDC. Next, since the p38-activation in anergic OT-II $CD25^-$ iTreg was strongly induced in 15 min by $pOVA_{323–339}$–pulsed DC (irradiated at 50 Gy) compared with OT-II naïve $CD25^-$ T cells (Fig. 4A), we investigated the effect of the p38 inhibitor in the anergic state and on the characteristics of OT-II $CD25^-$ iTreg (Fig. 4C and D). Expression of the *TAB1*, *TLR9*, and *Foxp3* genes, but not of the *IL-10* gene, was unchanged by the p38 inhibitor (data not shown). IL-2 production and proliferation by anergic OT-II $CD25^-$ iTreg were restored from unresponsiveness by treatment with the p38 inhibitor and TCR stimulation, the same as Mls-1^a-induced tolerance, and the unresponsiveness of anergic OT-II $CD25^-$ iTreg was restored by two different p38-inhibitors, SB203580 and SB239063 (Fig. 4D). Interestingly, SB239063 as compared with SB203580 did not inhibit naïve OT-II $CD25^-$ T cells, and it induced a strong proliferative response in anergic OT-II $CD25^-$ iTreg.

The effect of p38 inhibition following $CD25^+$ Treg depletion in E.G7 tumor-bearing OVA-tolerant murine model

First, we established a tumor Ag-tolerant murine model that was tolerant to the tumor Ag OVA. The *i.d.*-inoculated lymphoma E.G7 (OVA-expressing EL4) cells grew in C57BL/6 mice after adoptive transfer of anergic OT-II $CD25^-$ iTreg when tumor diameter was approximately 6–8 mm on day 9 after E.G7 inoculation, but they hardly grew at all in non-adoptive transfer C57BL/6 mice (data not shown). The effect of the p38-inhibitor after $CD25^+$ Treg-depletion in the tolerant model established was evaluated by tumor size after footpad injection of a $pOVA_{257-264}$ / $pOVA_{323–339}$ / incomplete Freund's adjuvant (IFA) mixture (Fig. 4E). On day 17 after E.G7 inoculation, a 73.6% reduction in tumor size was observed in the p38 inhibitor group (tumor size: 246 ± 129 (SE) mm^3) ($n = 6$, $p<0.0002$, *t*-test) in comparison with the control group (tumor size: $929 \pm 466\ mm^3$). Thus, in the control group of the established tolerant model, the effect of $CD25^+$ Treg-depletion alone in peptide-immunization could not repress E.G7 tumor growth. This finding suggested that treatment with the p38-inhibitor after $CD25^+$ Treg-depletion, but not $CD25^+$ Treg-depletion alone, enhanced the effect of peptide-immunization by breaking the immunotolerant function of iTreg and strongly repressed E.G7 tumor growth. We propose that p38-inhibitor treatment after $CD25^+$ Treg-depletion be referred to as the "p38-inhibition program" of peptide immunotherapy.

The effect of p38 inhibition following $CD25^+$ Treg depletion in CT26 tumor-bearing murine model

We tried immunizing CT26 tumor-bearing mice with CTL-epitope peptides 138-147 (AH1) and helper-epitope peptides 320-333 ($gp70_{320–333}$) plus IFA after the "p38-inhibition program" in order to investigate the efficacy of the program as a means of inducing antitumor immunity in a therapy model after tumor challenge. The peptide immunotherapy was started on day 5 after tumor challenge (when tumor diameter was approximately 5 mm; 62.5 mm^3). The mice ($n = 4$) were primarily immunized with both AH1 and gp70 peptides after administering the "p38-inhibition program". A week later, the mice were secondarily boosted in a similar manner. Evaluation of the efficacy of the "p38-inhibition program" in relation to immunization by tumor size on day 21 after CT26 challenge revealed significant inhibition of tumor growth on days 18 and 21 by immunization with the Ag or the interaction between immunization with the Ag and "p38-inhibition program" ($p<0.05$, *two-way ANOVA*). Furthermore, tumor growth on days 16–22 in the AH1 and gp70 peptide-immunized groups was significantly inhibited by SB239063 ($p<0.05$, two-way ANOVA). However, the interaction effect of anti-CD25 mAb and

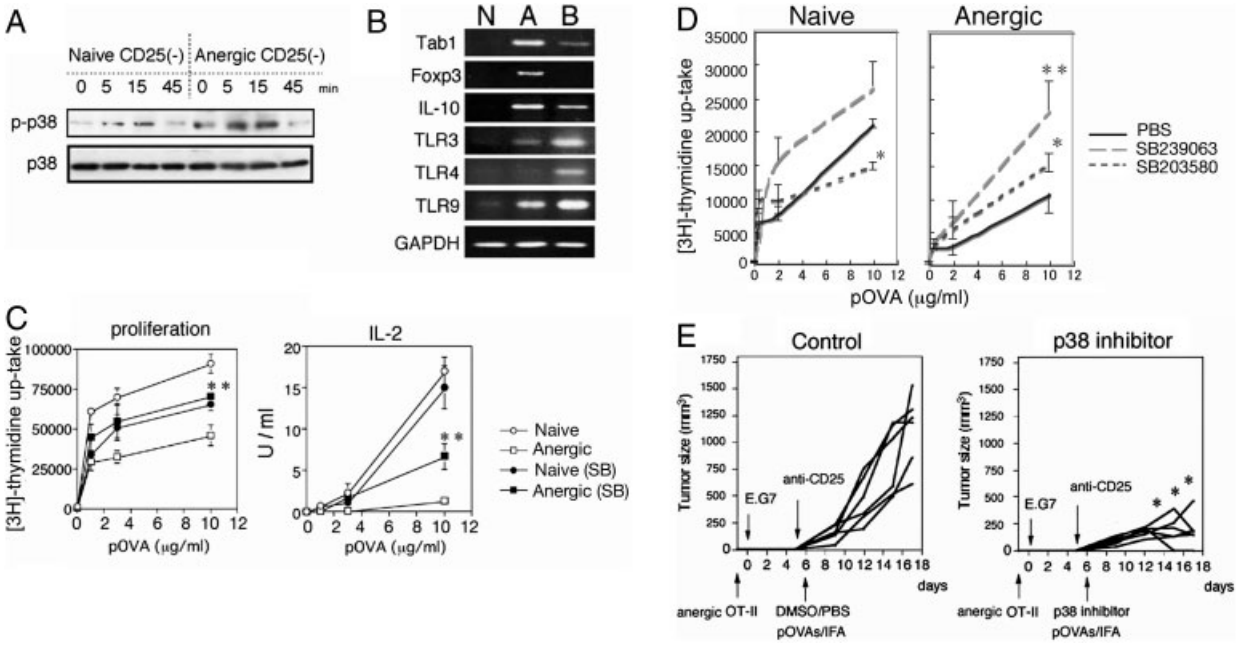

Figure 4. Characterization of $CD25^-$ iTreg and efficacy of p38-inhibitor for immunotherapy in OVA-tolerant model established by adoptive transfer of $CD25^-$ iTreg. (A) Naive OT-II $CD25^-CD4^+$ T cells and anergic OT-II $CD25^-$ iTreg were stimulated for the times indicated by co-culture with $pOVA_{323–339}$-pulsed DC (irradiated at 50 Gy), $CD4^+$ cells were separated with anti-CD4 BD IMag magnetic particles, and analyzed for p38-activity by Western blot. (B) Gene expression of *TAB1*, *Foxp3*, *IL-10* *TLR3*, *TLR4*, and *TLR9* in naïve OT-II $CD25^-CD4^+$ T cells (N), anergic OT-II $CD25^-$ iTreg (A), and naïve OT-II B cells (B) was analyzed by RT-PCR. (C) The effect of SB203580 (2 μM) on proliferation and IL-2 production by naïve OT-II $CD25^-CD4^+$ T cells and anergic OT-II $CD25^-$ iTreg was assessed by $[^3H]$-TdR uptake and ELISA, respectively. A statistically significant difference between control medium and treatment of SB203580 was found (**p<0.01, Students *t*-test). (D) The effect of different p38-inhibitors, *i.e.* SB203580 (2 μM) and SB239063 (2 μM) on proliferation by naïve OT-II $CD25^-CD4^+$ T cells and anergic OT-II $CD25^-$ iTreg was assessed by $[^3H]$-TdR uptake. (*p<0.05, **p<0.01, Student's *t*-test) (E) OVA Ag-tolerant mice were challenged by injection of E.G7 tumor cells ($3x10^6$, *i.d.*) following adoptive transfer of anergic OT-II $CD25^-$ iTreg ($3x10^6$). On day 6 after i.p.-injection of anti-CD25 mAb (anti-CD25) on day 5 after E.G7 inoculation, the mixture of $pOVA_{257–264}$ (2 μg) and $pOVA_{323–339}$ (2 μg) were injected into footpads with i.p.-injection of 0.1 % DMSO/PBS or SB203580 (0.2 mL of a 200 μM solution/body). Tumor diameter was measured at various time points and calculated as described in the *Materials and methods* section. (*$p<0.05$ at days 13, 15, 17, $n = 6$ *one-way ANOVA*). Data are representative of two independent experiments.

SB239063 reduced tumor size and incidence, although the difference in size was not significant ($p = 0.2651$, two-way ANOVA) (Fig. 5A). These results suggested that immunization with a mixture of peptides by the "p38-inhibition program" strongly repressed malignant tumor outgrowth, whereas the tumors in the groups that were not immunized with the peptides were hardly affected. To analyze the effect of a p38 inhibitor against non-immune cells, we used CT26 tumor-bearing immunocompetent mice (NOD/SCID). SB239063 had no direct effect on CT26 tumor growth, because SB239063-treatment alone or immunization according to the "p38-inhibition program" was not effective against tumor outgrowth in immunocompetent mice (data not shown). FACS analysis data indicated that the $CD25^+$ T-cell population was transiently depleted for 2–3 days following i.v.-inoculation with the anti-CD25 mAb (data not shown). These results suggested that p38-inhibitor treatment during transient depletion of $CD25^+$ Treg for 2–3 days may provide an opportunity to induce adaptive immunity specific to tumor Ag by immunization strong enough to inhibit tumor growth.

We performed a ^{51}Cr-release assay to investigate killing specific to the CT26 tumors. MHC class I ($H\text{-}2L^d$)-restricted CTL-killing specific to the CT26 tumors was significantly higher in $CD8^+$ T cells purified from the AH1/gp70/IFA (αCD25mAb/SB) group than from the DMSO/IFA, DMSO/IFA (αCD25mAb/SB), or AH1/gp70/IFA (PBS) groups (E/T ratio 20, $p<0.02$, E/T ratio 40, $p<0.05$, *two-way ANOVA*) (Fig. 5B), and IFN-γ production by $CD8^+$ T cells purified from the AH1/gp70/IFA (αCD25mAb/SB) group was significantly greater than by $CD8^+$ cells purified from the other groups ($p<0.05$, *two-way ANOVA*) (Fig. 5C). These results suggested that the "p38-inhibition program" is capable of enhancing the effects of peptide immunotherapy by increasing CTL-killing and IFN-γ production.

Increase in IL-10-secreting iTreg in tumor-bearing mice

Next, to determine whether there is an increase in IL-10-secreting iTreg in tumor-bearing mice, we performed a FACS analysis to assess expression of IL-10 and ICOS by p-p38(±) and Foxp3(±) cells in tumor-bearing mice 8 wk after CT26 inoculation. The percentage of $p\text{-}p38^+$ cells among freshly isolated $CD4^+$ T cells from tumor-bearing mice (28.7±10.9%) was significantly higher than in naïve mice (3.47±1.28%) ($p<0.05$, *t*-test), half of whose cells produced IL-10 (Fig. 6A). $CD4^+$ T cells purified from naïve, tumor-bearing, and tumor-bearing (SB *ip*) mice treated with the

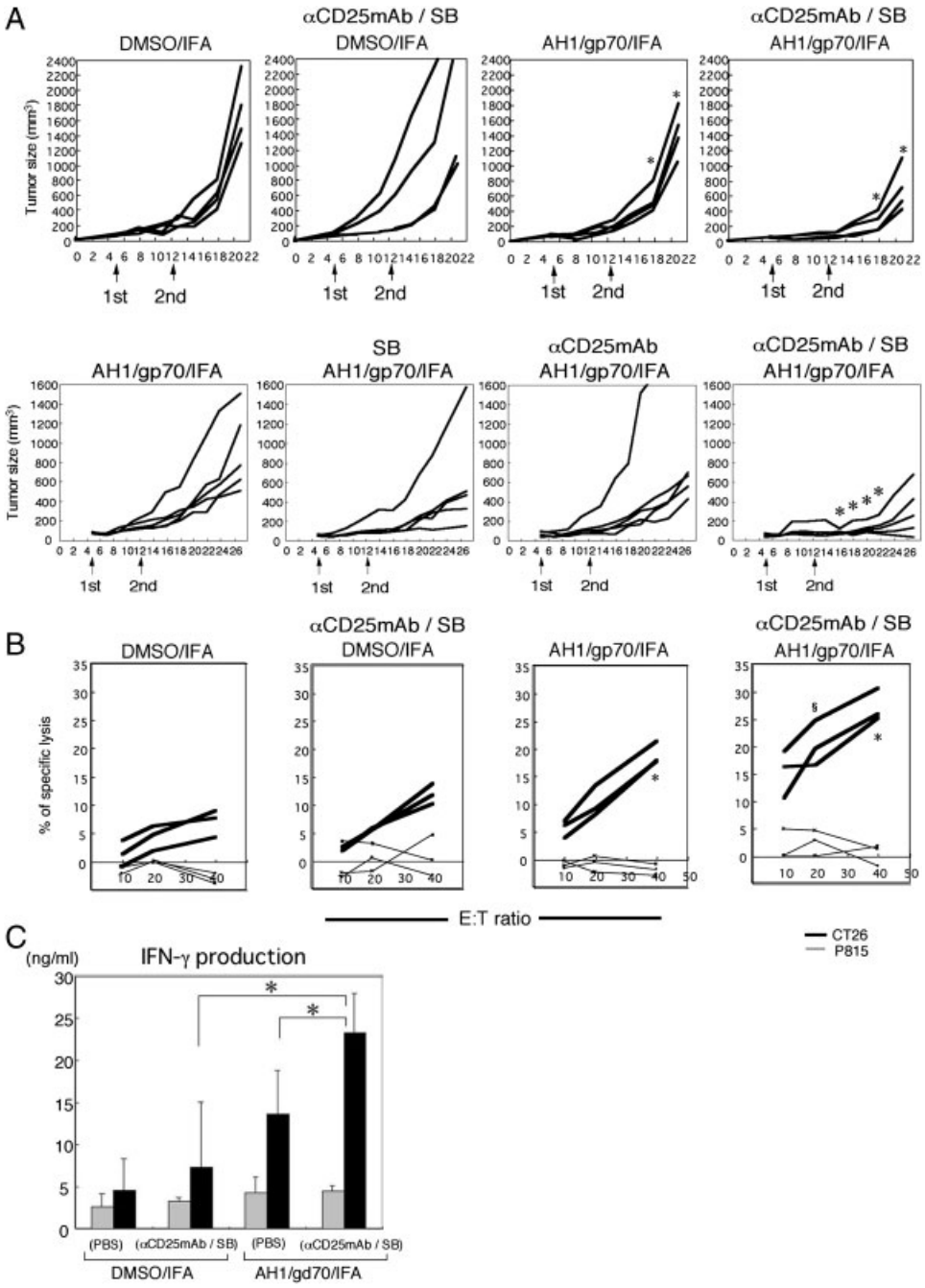

Figure 5. Ag-specific CTL-activation and inhibition of tumor growth by peptide immunotherapy with a p38-inhibitor following depletion of $CD25^+$ Treg. BALB/c mice were *i.d.*-inoculated in the left flank with CT26 tumor cells. The mice were immunized as described in the *Materials and methods* section. The treatments were as follows: DMSO/IFA (PBS), DMSO/IFA (αCD25mAb/SB), AH1/gp70/IFA (PBS), and AH1/gp70/IFA (αCD25mAb/SB) as described in the figure. (A) Tumor size was measured for 21 days after the CT26 tumor challenge ($n = 4$). In addition, to analyze the effect of p38-inhibitor SB239063 (SB), αCD25mAb, and both of them in AH1/gp70/IFA-immunized mice, tumor size was measured for 27 days after CT26 tumor challenge ($n = 5$). Tumor volume was calculated as described in the *Materials and methods* section (*$p<0.05$; *two-way ANOVA*). (B) $CD8^+$ T cells were purified from mice ($n = 3$) on day 21 after CT26 challenge. After AH1-specific $CD8^+$ T cells were expanded *in vitro* by co-culture for 5 days with AH1 peptide-pulsed DC (irradiated at 50 Gy), a standard 4 h ^{51}Cr-release assay for AH1-specific CTL was performed. CT26 or P815 was used as the target tumor or the negative control target. Data show mean ± SE (*$p<0.05$, E/T ratio = 40, the effect of factor [AH1/gp70/IFA]; §$p < 0.05$, E/T ratio = 20, the effect of factors [AH1/gp70/IFA] and [αCD25mAb/SB]. *Two-way ANOVA*). (C) After co-culture for 5 days with AH1 peptide-pulsed DC (irradiated at 50 Gy), the IFN-γ in culture supernatant for 12 h of AH1-specific $CD8^+$ T cells against CT26 (black) and P815 (gray) was detected by ELISA. Data show mean+SE (*$p<0.05$, Student's *t*-test). Data are representative of two independent experiments.

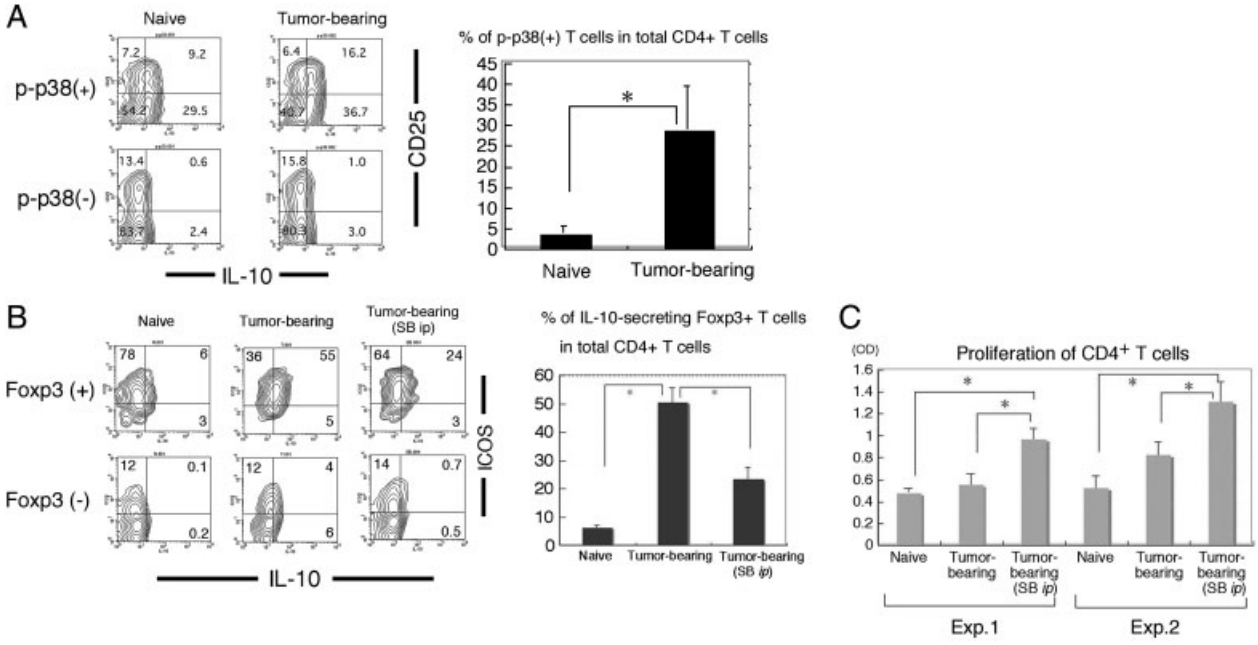

Figure 6. Increase in IL-10-secreting iTreg in tumor-bearing mice. (A) CT26 tumor cells were inoculated *i.d.* into the left flank of BALB/c mice. Fresh $CD4^+$ T cells purified from naïve mice or CT26 tumor-bearing mice 8 wk after tumor inoculation were analyzed by FACS after staining for CD25, and intracellular IL-10, p-p38. The right panel shows the $p\text{-}p38^+$ T cells as a percentage of total $CD4^+$ T cells in naïve and tumor-bearing mice. Data show mean+SE (*$p<0.05$, Student's *t*-test, $n = 4$). (B) $CD4^+$ T cells were purified from naïve mice, CT26 tumor-bearing mice, and CT26 tumor-bearing mice after i.p.-treatment with SB239063 (SB) (0.2 mL of a 200 μM solution / body) for 4 h. After $CD4^+$ T cells were cocultured for 8 h with peptide gp70-pulsed DC (irradiated with 50 Gy) from naïve mice, ICOS and IL-10 expression in $Foxp3^+$ and $Foxp3^-$ cells were analyzed by FACS. The right panel shows the IL-10-secreting $Foxp3^+$ T cells as a percentage of total $CD4^+$ T cells in naïve, tumor-bearing, and SB239063-treated tumor-bearing mice. Data show mean+SE (*$p<0.05$, Student's *t*-test, $n = 4$). (C) After $CD4^+$ T cells were cocultured for 48 h with peptide gp70-pulsed DC (irradiated with 50 Gy) from naïve mice, proliferation by $CD4^+$ T cells was assessed by MTT assay in two independent experiments. The *y*-axis indicates the optical density (OD). Data show mean+SE of triplicates for each experiment (*$p<0.05$, Student's *t*-test, $n = 3$).

p38 inhibitor SB239063 *in vivo* were then stimulated with peptide gp70-pulsed DC. IL-10 production by $Foxp3^+ICOS^+CD4^+$ T cells that had expanded in tumor-bearing mice was significantly inhibited by *in vivo* pretreatment with the p38 inhibitor (Fig. 6B). Total proliferation by $CD4^+$ T cells purified from tumor-bearing (SB *ip*) mice was significantly greater than by $CD4^+$ T cells purified from naïve and tumor-bearing mice ($p<0.05$, *t*-test) (Fig. 6C). These results suggest that p38 inhibition *in vivo* functionally inhibits IL-10 production by $Foxp3^+$ $ICOS^+$ iTreg but not generation of these cells in the immune microenvironment of tumor, thereby resulting in recovery of the proliferative response by all $CD4^+$ T cells.

Discussion

In this study we focused on the selective effect of a p38-inhibitor in the peripheral tolerance of $CD25^-$ iTreg but not $CD25^+$ nTreg. IL-10-producing $CD25^-$ iTreg exhibited suppressive activity in a p38-dependent manner, and it was possible to break their immunotolerance by treatment with the p38-inhibitor in the absence of $CD25^+$ Treg. It was recently shown in humans that p38 activation of iTreg induced *in vitro* by tDC was essential for cell cycle arrest and immunosuppressive function [22]. However, the suppressor function that is not mediated by IL-10 in the human iTreg may be distinguished to that of *in vivo* Ag-induced mouse iTreg. We now suspect that the dominant p38 activation in response to other MAPK (ERK and JNK) in *in vivo* Ag-induced iTreg is related to both the suppressor function *via* IL-10 production and the anergic state *via* cell cycle arrest and p27 expression and not *via* IL-10 [14]. Recent studies have indicated that p38 activation is required for TGF-β-induced *in vitro* conversion to iTreg [23, 24] and is involved in IL-10 production *via* innate TLR2 signaling of HSP60 in iTreg [25] or in the induction to iTreg through tDC treated with CpG [26]. We suspect that p38-dependent IL-10 production is mediated by TAB1. Non-classical p38 autophosphorylation *via* a p38 scaffold protein such as TAB1 [14], Dlgh1 [27, 28], and so on that is a novel functional modulator of the p38 pathway negatively regulates the proliferative response of $CD4^+$ T cells. We showed an increase in the *TAB1* gene and p38 autophosphorylation in anergic $CD4^+$ T cells, but there was no difference between the levels of ubiquitous TAB1 protein (72 kD) in naïve and anergic $CD4^+$ T cells. However, there was an increase in low molecular weight TAB1 (56 kD) in anergic $CD4^+$ T cells (Fig. 3). Interestingly, almost all of the ubiquitous TAB1 protein (72 kD) shifted to 56 kD in response to treatment with a PNGase, and the shift resulted in down-regulation of ERK activity following p38 activation through increase of the TAB1 (56 kD) (Supporting Information Fig. 2). Although these results suggest a balance

between IL-2 production mediated by ERK and IL-10 production mediated by p38, the mechanisms underlying the effects of the PNGase are still unclear.

Next, adaptive tolerance by iTreg was induced by i.v.-injection of super-Ag or peptide $OVA_{323–339}$ into its specific TCR Tg (Vβ8.1 or OT-II) mice, although several types of iTreg have been described with a unique mechanism of action that varies depending on the experimental model. A recent study demonstrated that an *in vivo* switch in Ag (allergen)-specific Th1 and Th2 cells toward IL-10-producing iTreg by high-dose allergen exposure to mice [29], and that characterization of IL-10/IFN-γ-producing human Tr1-like cells showed that they are IL-7R$^-$ cells [30]. A recent review states that tDC may induce Th0, Th1, Th2, and Th17 cells to differentiate into IL-10-producing suppressive T cells exhibiting each characteristic through the influence of IL-27 [31] *via* IL-21 [32], suggesting that *in vivo* Ag stimulation may induce both effector Th types and iTreg by way of a negative feedback system, as also demonstrated in a recent study [33].

Two recent clinical trials of a cancer vaccine containing depletion of CD25$^+$ nTreg with a recombinant IL-2 diphtheria toxin conjugate denileukin diftitox (DAB_{389}IL-2) [34, 35] or a CD25-directed immunotoxin LMB-2 [36] was performed in patients with renal cell carcinoma or melanoma. Tumor-infiltrating CD25$^+$ Treg and tumor-associated Ag-specific immunological enhancement were induced in these clinical trials, but most cases of which could hardly block the progressive tumor growth. A recent study showed that tumor-associated Ag-specific IL-10-producing CD25$^-$ iTreg, not CD25$^+$ nTreg, were mainly induced in patients with metastatic melanoma [7] and in CT26-inoculated BALB/c mice (Fig. 6), suggesting the need to overcome cancer immunotolerance maintained by IL-10-producing CD25$^-$ iTreg and not by CD25$^+$ nTreg. The results of our study suggest that the effect of CD25$^+$ Treg-depletion with DAB_{389}IL-2 or LMB-2 in cancer patients may be enhanced by using a p38-inhibitor in the existing immunotherapy.

Materials and methods

Peptides

L^d-restricted gp70-peptide (AH1) comprising amino acids 138–147 (SPSYVYHQF), I-A^d-restricted gp70-peptide ($gp70_{320–333}$) comprising amino acids 320–333 (LVQFIKDRISVVQA) [37], K^b-restricted OVA-peptide ($pOVA_{257-264}$) comprising amino acids 257–264 (SIINFEKL), and I-A^b restricted OVA-peptide ($pOVA_{323–339}$) comprising amino acids 323–339 (ISQAVHAAHAEINEAGR) were synthesized by American Peptides (Sunnyvale, CA, USA).

Mice

TCR Vβ8.1-Tg mice backcrossed with CBA/Ca mice (H-2^k, Mls-1^b) for more than ten generations were provided by Dr. K. Yui [15]. OT-II Tg mice were provided by Dr. W. R. Heath [38]. CD45.1 mice (C57BL/6) were provided by Dr. H. Nakauchi. CBA/j (H-2^k, Mls-1^a), C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from Charles River (Yokohama, Japan), and 8–12-wk-old mice were used in the study. All animal procedures were conducted with the approval of the Animal Care and Use Committee Nippon Veterinary and Life-science University (NVLU).

Induction of anergic iTreg with super-Ag Mls-1^a

The super-Ag Mls-1^a, which is endogenously expressed on B cells purified from CBA/j mice, is a highly specific stimulator of TCRVβ6, 7, 8.1, and 9 CD4$^+$ T cells. Anergy (unresponsiveness) was induced in the majority of CD4$^+$ T cells by inoculating Vβ8.1-Tg mice with Mls-1^a-expressing B cells (CBA/j strain) (1.5×10^7/body), as previously described [15]. The anergic iTreg were prepared from lymph nodes of Vβ8.1-Tg mice 12–18 days after inoculation with Mls-1^a-expressing B cells (CBA/j strain).

Induction of anergic iTreg with peptide $OVA_{323–339}$

OVA-specific T-cell anergy was induced in the majority of CD4$^+$ T cells by inoculating OT-II Tg mice with peptide $OVA_{323–339}$ (50 μg). CD4$^+$ T cells prepared from lymph nodes and the spleen of the OT-II Tg mice on day 7 or 8 after inoculation were used as OT-II iTreg. In addition, CD25$^-$ OT-II iTreg were purified with BD IMag-anti-mouse CD4 DM (IMag-CD4) (BD Biosciences, San Diego, CA, USA) following depletion of the CD25$^+$ T cells with the anti-CD25 mAb (ascitic fluid from nu/nu mice inoculated with PC61 cells) and a rabbit complement.

Immunization in CT26 tumor-inoculated mice model

BALB/c mice were *i.d.*-inoculated in the left flank with CT26 tumor cells. The mice were primarily immunized by footpad injection of an AH1/gp70/IFA mixture and i.p.-injection of SB239063 (0.2 mL of a 200 μM solution/ body) on day 1 after i.v.-injection of anti-CD25 mAb. A week later, the mice were secondarily boosted in a similar manner.

Cell purification

CD25$^-$CD4$^+$ T cells (>98%) were purified with BD IMag-anti-mouse CD4 DM (IMag-CD4) (BD Biosciences) after CD25$^+$ T cells depletion with the anti-CD25 mAb (ascitic fluid; PC61) and a rabbit complement. CD25$^+$CD4$^+$ T cells were purified with streptavidin-IMag (BD Biosciences) after staining with a mixture of FITC-conjugated and biotin-conjugated anti-CD25 mAb (3C7) (eBioscience, San Diego, CA, USA), and checked the purity by FACS (>97%).

Proliferation assay

[^{3}H]-thymidine (TdR) uptake

Naive or anergic CD4$^+$ T cells (5×10^5) purified from Vβ8.1-Tg mice were TCR-stimulated for 72 h in round 96-well plates (Nalge Nunc International, NY, USA) by co-culture with mitomycin C (Kyowa Pharmaceutical, Tokyo, Japan)-treated Mls-1^a-expressing B cells ($0–10 \times 10^5$) purified from the spleen of CBA/j mice, and PBS or the p38-inhibitor SB203580 or SB239063 (2 μM; Merck Biosciences, UK) was added to the assay medium. Cell proliferation was assayed on the basis of [^{3}H]-TdR (Perkin Elmer, Branchburg, NJ, USA) uptake. Each well was incubated for 8 h with [^{3}H]-TdR 18.5 kBq/well, and the cells were harvested onto filter mats (UniFilter plate-96; Perkin Elmer). Incorporated radioactivity was measured by scintillation counting with a Packed Top Counter (Perkin Elmer).

MTT assay

Cell proliferation was assayed based on uptake of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (CCK-8 solution; Dojindo Molecular Technologies, Kumamoto, Japan). Each well was incubated for 4 h with 10 μL of CCK-8 solution, and absorbance was measured at 450 nm with a microplate reader (Bio-RAD Labs, Hercules, CA, USA).

T-cell suppression assay

Analysis based on [^{3}H]-TdR uptake

The suppressive effect on proliferation of naïve CD4$^+$ T cells (5×10^5/well) was analyzed by co-culture with CD25$^+$ nTreg, anergic CD25$^+$ T cells, or anergic CD25$^-$ T cells (1.2×10^5/well) purified from Vβ8.1-Tg mice. Mitomycin C (Kyowa Pharmaceutical)-treated Mls-1^a-expressing B cells from CBA/j mice were used as APC for TCR-stimulation. PBS or the p38-inhibitor SB203580 (2 μM) was then added to the assay medium, and proliferation was assayed by using the [^{3}H]-TdR uptake assay.

CFSE dilution assay

Suppressive effects on the proliferation of naïve CD4$^+$ T cells (5×10^5/well) labeled by incubation with 2.5 μM CFSE (Molecular Probes, Eugene, OR, USA) for 10 min was analyzed by co-culture with CD25$^+$ nTreg or anergic CD25$^-$ T cells (2.4×10^5/well) purified from Vβ8.1-Tg mice. Mitomycin C (Kyowa Pharmaceutical)-treated Mls-1^a-expressing B cells from CBA/j mice were used as APC for TCR-stimulation, and PBS, SB203580 (1 μM), anti-IL-10 (NA/LE) mAb (20 μg/mL; BD Biosciences), or monoclonal isotype control IgG (NA/LE) (20 μg/mL; BD Biosciences) was added to the assay medium. Cell division by CFSE-labeled naïve CD4$^+$ T cells was detected by FACS analysis (FACSCaliber; BD Biosciences).

RT-PCR

Total cellular RNA was extracted with an ISOGEN kit (Nippon-gene, Toyama, Japan). The RNA (0.5 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany). Aliquots of the mixture were then amplified with Taq DNA polymerase (Promega). The following primer pairs were used for the PCR: IL-2 forward, 5′-AACAGCGCACCCACTT-CAA-3′; IL-2 reverse, 5′-TTGAGATGATGCTTTGACA-3′; TAB1 forward, 5′-TGGGGAGCGTGCCAAGTTCTG-3′; TAB1 reverse, 5′-TTGGCACTGGCCCTCTGTGTC-3′; Foxp3 forward, 5′-CAATGTGGC-CAGTCTGGAATGG-3′; Foxp3 reverse, 5′-GTTGGCTCCT-CTTCTTGCGA-3′; IL-10 forward, 5′-TCAAACAAAGGACCAGCTG-GACAACATACTG-3′; IL-10 reverse, 5′-CTGTCTAGGTCCTGGAGTC-CAGCAGACTCAA-3′; TLR3 forward, 5′-TTGTCTTCTGCAC-GAACCTG-3′; TLR3 reverse, 5′-CGCAACGCAAGGATTTTATT-3′; TLR4 forward, 5′-CAAGAACATAGATCTGAGCTTCAACCC-3′; TLR4 reverse, 5′-GCTGTCCAATAGGGAAGCTTTCTAGAG-3′; TLR9 forward, 5′-CCGCAAGACTCTATTTGTGCTGG-3′; TLR9 reverse, 5′-TGTCCCTAGTCAGGGCTGTACTCAG-3′; GAPDH forward, 5′-ACCA-CAGTCCATGCCATCAC-3′; and GAPDH reverse, 5′-TCCAC-CACCCTGTGCTGTA-3′. Samples were amplified by 32 cycles for GAPDH probes (94°C for 60 s, 58°C for 60 s, and 72°C for 60 s), 36 cycles for IL-2, TAB1, Foxp3, IL-10 probes (94°C for 30 s, 57°C for 50 s, and 72°C for 90 s), and 28 cycles for TLR3, 4, 9 (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s). The PCR products were separated by electrophoresis on 2% agarose gel.

Immunization

BALB/c mice were challenged by *i.d.*-inoculation of CT26 tumor cells (1×10^5). On day 5, the footpads of the mice were injected with AH1 and gp70 peptides (100 μg/body each) and IFA (Difco, Detroit, MI, USA). The mice were treated with the p38-inhibitor SB239063 (0.1 mL of a 200 μM solution, i.p.) after pre-treatment with anti-CD25 mAb (ascitic fluid; PC61) [i.p.]. After 7 days, the mice were secondarily boosted in a similar manner.

Tumor volume was calculated by using the formula: tumor volume = length × (width)^2 × 0.5 [39].

Western blot analysis

Naive (1×10^6) or anergic CD25$^-$CD4$^+$ T cells (1×10^6) were re-suspended in 50 μL of lysis buffer (1 % Triton X-100, 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM PMSF). The lysates for deglycosylation were pre-treated for 6 h at 37°C with PNGase F (0–500 U/50 μL) (New England Biolabs, Beverly, MA, USA). The lysates were then size-fractionated by SDS-12.5% PAGE and electrotransferred to a polyvinylidene difluoride membrane. The membrane was incubated in blocking buffer (Tris-buffered saline containing 5% skim milk and 0.1% Tween 20) and then probed with anti-p38 mAb, anti-phospho-p38 mAb, anti-ERK mAb, anti-phospho-ERK mAb (New England Biolabs),

anti-TAB1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO, USA). The polyvinylidene difluoride membrane was then washed with washing buffer (Tris-buffered saline and 0.1% Tween 20) and incubated with HRP-conjugated anti-rabbit IgG Ab or HRP-conjugated anti-mouse IgG Ab (MBL, Medical & Biological Laboratories, Nagoya, Japan). Proteins were visualized by using an ECL kit according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA).

^{51}Cr-release assay

CTL activity was measured by a standard 4 h ^{51}Cr-release assay. Target cells were labeled with 1.85 MBq sodium chromate ^{51}Cr (Amersham Biosciences), and the ^{51}Cr-labeled target cells (5×10^3/well) were co-cultured for 4 h with T cells in 96-well U-bottom plates (Nalge Nunc International). The supernatant of each well was then harvested with harvesting frames (Nihon Molecular Devices, Tokyo, Japan), and the ^{51}Cr-release activity was measured with a Wallac 1480 Wizard 3 scintillation counter (Perkin Elmer). ^{51}Cr-release was calculated as follows: [(sample count−spontaneous count)]/[(maximum count−spontaneous count)] × 100%.

ELISA

IFN-γ and IL-2 in the culture supernatant were assayed by using the BD OptEIA mouse IFN-γ (BD Biosciences) and BD OptEIA mouse IL-2 ELISA kits (BD Biosciences) according to the manufacturer's instructions. Plates were read at 450 nm in an automated microplate reader model 680 (Bio-RAD Labs).

Statistical analysis

Comparisons between two groups were performed by Student's *t*-test with Microsoft Excel software. Comparisons among more than two groups were performed by one-way or two-way factorial ANOVA followed by the Bonferroni *t*-test ($p = 0.05$). ANOVA was performed by using ANOVA4 available on a website (http://www.hju.ac.jp/~kiriki/anova4/).

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Abbreviations: **$CD25^+$ nTreg:** $CD25^+CD4^+$ natural treg · **IFA:** incomplete Freund's adjuvant · **iTreg:** inducible Treg · **Mls:** minor lymphocyte stimulatory · **OT-II Tg:** $I\text{-}A^b$ restricted $OVA_{323\text{-}339}$-specific TCR Tg mice · **PNGase:** peptide:*N*-glycanase · **p38-inhibition program:** the program of peptide-immunization plus treatment with a p38-inhibitor after $CD25^+$ Treg-depletion · **p38-inhibitor:** p38-chemical inhibitor · **TAB1:** TGF-β-activated kinase-1-binding protein-1 · **tDC:** tolerogenic DC · **Tr1:** Treg type 1

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