

**Inhibition of T cell activation through down-regulation of TCR-CD3 expression
mediated by an anti-CD90 Ab**

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Abstract

We are trying to develop new Abs that can manipulate CD4 T cell responses and are usable as immunosuppressive agents. To this end, we performed functional screening, in which we examined the effect of an Ab on the proliferation of mouse CD4 T cells upon activation. The Ab, LP5, inhibited the activation of CD4 T cells stimulated with an anti-CD3 Ab or peptide antigen. The Ab alone had no stimulatory effect on CD4 T cells. Biochemical experiments demonstrated that LP5 recognized the Thy-1 (CD90) molecule. Interestingly, the treatment of CD4 T cells with LP5 *in vitro* induced a temporary down-regulation of CD3 expression at the cell surface. TCR molecules were also affected. Other anti-CD90 Abs not inhibitory to CD4 T cell activation failed to induce a reduction in CD3. Experiments *in vitro* revealed that the down-regulation caused by LP5 is due to an accelerated endocytosis of cell surface CD3. In addition, it was shown that CD3 down-regulation before or in the early stages of T cell activation is critical for the induction of hyporesponsiveness. Experiments *in vivo* showed that pre-treatment of CD4 T cells with LP5 inhibited the rejection of semi-allogeneic bone marrow transplants. Based on these observations, we propose that CD3 down-regulation without any stimulatory activity against T cells could be one approach to inhibiting T cell activation, and CD90 would be an appropriate target.

Key words: CD3, CD90, T cell activation

Introduction

CD4⁺Foxp3⁺ regulatory T cells (Treg cells) have suppressive functions and play important roles in the maintenance of immune homeostasis [1,2]. It has been demonstrated that the manipulation of Treg cells in terms of number and function is useful in the prevention of autoimmune, inflammatory, and graft-versus-host diseases. To increase the number of Treg cells, purified Treg cells have been cultured with IL-2, dendritic cells (DCs) as APCs, and so on [3,4]. In some cases, to increase the suppressive function of Treg cells, it was required to pre-stimulate Treg cells with allogeneic cells before the in vivo transfer of Treg cells since freshly isolated Treg cells did not induce tolerance to bone marrow allografts [5-8]. It was also reported that very large numbers of non-specifically expanded Treg cells were required to inhibit bone marrow allograft rejection [9-11]. In other reports, Treg cells induced from non-Treg cells, so-called iTreg cells, were utilized in manipulating immune responses in vivo [12], although it remained unsolved how long iTreg cells are stable in their suppressive activity. Thus, although Treg cells are an attractive cell population for regulating and manipulating immune responses, there are many complicated procedures involved in manipulating their numbers and functions.

As one approach to manipulating immune responses, we have tried to establish monoclonal Abs exhibiting Treg cell-like suppressive activities. Treg cells can suppress the activation of CD4 T cells stimulated with anti-CD3 Ab and/or Concanavalin A along with APC, allogeneic cells, and anti-CD3/anti-CD28 Ab-coated beads [3,13,14]. However, in the presence of anti-GITR Ab, IL-4, or supernatant (SN) from DCs stimulated with LPS, Treg cells did not exhibit suppressive effects [15-17]. Based on these observations, we tried to establish mAbs that suppress the activation of CD4 T

cells in cultures in which Treg cells can exhibit suppressive activity, and at the same time, mAbs that cannot inhibit the activation of CD4 T cells in cultures in which Treg cells cannot exert suppressive activity. Through the functional screenings of hybridomas, we have established some mAbs.

In the present study, we have examined one of those mAbs, LP5, that had almost Treg cell-like properties under the conditions examined. Biochemical experiments revealed that LP5 recognizes Thy-1 (CD90), a small GPI-anchored glycoprotein [18]. In the mouse, CD90 is present on a variety of cell types including thymocytes and peripheral T cells. More recently, CD90 has been used as a marker for lipid rafts in murine T cells [19]. Both stimulatory and negative regulatory roles for CD90 during T cell activation were suggested from studies using CD90-deficient mice or anti-CD90 Abs [20-25]. In the process of analyzing the functions of LP5, we found that the Ab induces a temporary down-regulation of CD3/TCR expression. It has been shown that TCR/CD3 complexes in T cells are internalized and recycled constitutively [26]. Furthermore, once T cells are activated by an antigen, down-regulation of TCR/CD3 expression at the cell surface is induced [27]. In contrast with this naturally occurring decrease, in this study, we demonstrated that the active down-regulation of CD3 is inducible by an anti-CD90 Ab, and the suppressive effect of LP5 against T cell-activation is dependent on this decrease.

Materials and Methods

Animals.

BALB/c (H-2^d), CB17.scid, and CBF1 (H-2^{dx^b}) mice and Wistar rats were purchased from Japan SLC Inc. (Shizuoka, Japan) or Clea Japan (Tokyo, Japan).

DO11.10 mice, OVA peptide (323-339)-specific, MHC class II-restricted, TCR transgenic mice, and DO11.10-RAG2^{-/-} mice, with an RAG2^{-/-} background, were obtained from Taconic Farms (Germantown, NY). All mice were maintained in a specific pathogen-free animal facility and treated in accordance with institutional guidelines for animal care.

Tumors.

Meth A, EL-4, and RLM1 [BALB/c-derived radiation leukaemia, a gift from E. Nakayama (Okayama University, Japan)] [28] were used for the preparation of cell lysates.

Cell preparation.

Splenic cells were incubated at 5×10^7 /ml for 45 min at 37°C with the culture supernatant of hybridoma cells secreting anti-CD25 Ab (7D4), and rabbit complement diluted to a final concentration of 1:10 (Cedarlane Lab., Canada). The treatment was repeated twice, and the resulting CD25⁺ cell-depleted cells were then incubated with magnetic beads conjugated with anti-CD4 (GK1.5) Ab (Miltenyi Biotec, Bergisch Gladbach, Germany), washed, and passed through a magnetic column to purify CD4⁺CD25⁻ T cells (positive selection). The cells that passed through the magnetic column as non-binding cells were treated with mitomycin C, washed, and used as splenic APCs. In some experiments, CD4 T cells were purified negatively with the use of a CD4⁺ T cell isolation kit (Miltenyi Biotec) (negative selection).

Cell culture.

CD4 T cells (1×10^4 /well) were stimulated with 5% SN of anti-CD3 Ab (145-2C11), which induces maximum proliferation, in the presence of splenic APCs (3×10^4 /well), in 96-well round-bottomed plates in DMEM containing 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μ M 2-ME. In some experiments, instead of anti-CD3 Ab SN plus APC, anti-CD3 Ab- and anti-CD28 Ab-conjugated beads (1×10^4 /well, Dynal Biotech ASA, Oslo, Norway) were used to stimulate CD4 T cells. The proliferation of T cells (triplicate cultures) was assessed by measuring the incorporation of [3 H]TdR (37kBq/well) for the final 4 h of a 3-day culture.

Abs and reagents.

The following Abs were used: anti-CD90 Abs (53-2.1, FF-10 and 30-H12), anti-CD3 (145-2C11 or M-20), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD45 (30-F11), anti-TCR β (H57-597), anti-H-2K^b (AF6-88.5), anti-H-2D^d (34-2-12), anti-mouse IgM and anti-rat IgG, all purchased from BD Pharmingen, Santa Cruz Biotechnology, eBioscience, Cell Signaling, or Abcam, and anti-CD90 Ab (HO-13-4) purchased from American Type Culture Collection. The intracellular staining of mouse splenocytes with anti-Foxp3 Ab was performed with an anti-mouse Foxp3 staining set (eBioscience), according to the manufacturer's directions. After staining, cells were analyzed with FACSCanto II (BD Biosciences). All Abs used for western-blotting experiments were purchased from Cell Signaling and Santa Cruz Biotechnology. In some experiments, cells were treated with methyl- β -cyclodextrin (M β CD), acetic acid, or paraformaldehyde.

Preparation of mAbs.

Wistar rats were i.p. immunized three times every 2 weeks with CD4⁺CD25⁻ T cells prepared from BALB/c mice, then i.v. injected with CD4⁺CD25⁻ T cells 1 month later. Spleen cells were fused with P3X63Ag8.653 myeloma cells (from American Type Culture Collection) 3 days after the final immunization. For details on the screening of the mAbs, see Results.

Immunoprecipitation and Western blotting.

Cells were washed and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (nacalai tesque, Japan). After a 20-min incubation on ice and following centrifugation, the cell lysate was recovered and incubated with Ab-coupled Sepharose 4FF (Amersham Biosciences) for 100 min at 4°C. After washing with lysis buffer three times, bound proteins were subjected to SDS-PAGE, blotted onto a membrane, and immunoblotted.

Bone marrow chimeras.

Bone marrow from femurs and tibias was collected in DMEM, and single cell suspensions were prepared. T cells and NK cells were eliminated using anti-CD4 (RL172.4), anti-CD8 (3.155), and anti-NK1.1 (PK136) Abs and rabbit complement. Cells (2×10^6) were then injected intravenously into γ -irradiated BALB/c mice (8.0 Gy, Gamma Cell 40 Exactor). In some experimental groups, CD4 T cells (2×10^5) negatively purified from BALB/c mice were co-injected with bone marrow cells.

Results

LP5 Ab can inhibit the activation of CD4 T cells.

To develop new mAbs for use as immunosuppressive agents, we first tried to establish mAbs having a suppressive effect on CD4 T cells through the functional screening of hybridoma SN. Rats were immunized with CD4⁺CD25⁻ T cells of BALB/c mice, and the immunized spleen cells were fused with P3X63Ag8.653 myeloma cells. SN from the resulting hybridomas were screened for the ability to inhibit the activation of CD4⁺CD25⁻ T cells. Hybridomas with stable anti-proliferative activity were subjected to subsequent cloning. Finally, one clone [LP5 (rat IgM)] was established. LP5 Ab could inhibit the polyclonal and/or Ag-specific activation of CD4 T cells (Fig.1A and B), and the inhibition was LP5 Ab dose-dependent (Fig. 1C). LP5 Ab itself had no stimulatory activity even in the presence of APCs. Furthermore, it was indicated that LP5 Ab directly acted on CD4 T cells since CD4 T cells stimulated with anti-CD3/CD28-coated beads were also inhibited by LP5 Ab (Fig. 1D). It was also observed that LP5 inhibits a signaling cascade leading to T-cell activation (Fig. S1).

LP5 Ab recognizes CD90.

Next, we examined the distribution of target molecules recognized by LP5 Ab. Whole spleen cells were stained with anti-CD4 or anti-CD8 Ab, and LP5 Ab (Fig. S2A). Almost all CD4 T cells and all CD8 T cells were positive for LP5, and the staining intensity of LP5 Ab was slightly decreased on the activated T cells. In addition, both Treg and non-Treg cells were positive for LP5 (Fig. S2B). Mouse T cell leukemia cell lines (EL-4 and RLM1) were stained positively with LP5 Ab, and Meth A (mouse fibrosarcoma) was not (data not shown). To identify the targets of LP5 Ab, first, cell-

lysate was prepared from these three tumor cells, subjected to SDS-PAGE under non-reducing conditions, transferred onto a membrane, and blotted with LP5 Ab. Western blot analysis revealed that LP5 Ab recognized molecules of approximately 25 kDa (Fig. 2A). Under reducing conditions, LP5 Ab failed to detect the proteins. Next, the lysate prepared from LP5-positive tumor cells was immunoprecipitated with LP5 Ab-beads, and the beads-bound proteins were analyzed. As shown in Fig. 2B, a band of the same size was immunoprecipitated with LP5 Ab. Then the single 25-kDa band in the silver-stained gel was excised, treated with trypsin, and subjected to MALDI-TOF/mass spectrometry. A database search revealed that this band contains a small GPI-anchored protein, Thy-1 (CD90). To confirm whether LP5 Ab recognizes CD90, the proteins immunoprecipitated with LP5 Ab-beads were blotted with two different anti-CD90 Abs (FF-10 and 30-H12). As shown in Fig. 2C and 2D, both anti-CD90 Abs detected the 25 kDa molecule immunoprecipitated with LP5 Ab. Furthermore, we performed a binding competition assay (Fig. 2E). Staining of CD90-positive EL-4 tumor cells with anti-CD90 Abs (53-2.1, 30-H12, and FF-10) was inhibited in the presence of the LP5 Ab with a somewhat different competitive efficiency. Taken together, these results indicate that LP5 Ab recognizes the CD90 molecule.

LP5 Ab induces the down-modulation of CD3 molecules.

It is reported that CD90 is involved in T cell activation [18]; some anti-CD90 Abs in the context of co-stimulation cause the activation of mouse T cells. For example, an anti-CD90 Ab, FF-10, can augment the activation of CD4 T cells stimulated with anti-CD3/CD28-beads or with anti-CD3 Ab in the presence of APCs, with some differences in efficiency (Fig. S3, A and B). Another anti-CD90 Ab, HO-13-4, exhibits

a slightly suppressive effect, or almost no effect, on the activation of CD4 T cells (Fig. S3, C and D). On comparing LP5 with these anti-CD90 Abs, we found that LP5 caused the down-regulation of CD3 expression at the cell surface. By culturing whole spleen cells with LP5 for 1 h at 37°C, the majority of CD4 T cells lost their CD3 surface expression (Fig. 3A). In contrast, the stimulatory anti-CD90 Ab, FF-10, had no effect on the staining intensity with anti-CD3 Ab. The HO-13-4 Ab slightly reduced the staining with the anti-CD3 Ab. In all cases with three different Abs, the staining with each anti-CD90 Ab itself exhibited no difference even after 1 h of culture (Fig. 3B). These results suggest that LP5 has no influence on the expression of CD90 molecules themselves, however, it induces the down-regulation of CD3, and that only a suppressive anti-CD90 Ab, LP5, induces this phenomenon.

Since the culture of purified CD4 T cells with LP5 resulted in the same decrease in CD3 (Fig. S4A), we confirmed that the direct action of the Ab on CD4 T cells is enough for this effect, with no requirement for other cells in the culture. Furthermore, it was observed that the reduction in CD3 is caused by LP5, on the other hand, the anti-CD3 Ab had no influence on the cell surface expression of CD90 (Fig. S4B). In addition, the fixed cells retained CD3 on their surface even in the presence of LP5 (Fig. S4C). Taken together, these results suggest that the diminished staining with the anti-CD3 Ab after LP5 pre-treatment is not due to the competition between LP5 and the anti-CD3 Ab. This unique property of LP5 was observed not only on splenic CD4 T cells but also on other tissue (mesenteric, inguinal, axillary lymph nodes and Peyer's patch)-derived CD4 T cells (data not shown).

To investigate the kinetics of the reduction in CD3, splenic cells were cultured in the presence or absence of LP5 for various periods, washed, and stained with the anti-

CD3 Ab (Fig. S5A). The amount of CD3 decreased after just 15 min, with a peak reduction at 1-2 h, and then recovered almost to the basal level at 24 h. The expression of CD90 was constant throughout the culture. Biochemical experiments revealed the total amount of CD3 to be constant throughout the period of LP5 treatment, with some decrease after 0.5 hr (Fig. S5B). We also examined the influence of the Ab on the expression of other T cell surface molecules (Fig. 3C). In addition to CD3, TCR β was affected by LP5. Other molecules including CD4 and CD45 remained largely unchanged in their cell surface expression.

Next, we investigated whether the LP5-induced decrease in CD3 was associated with lipid rafts, where CD90 is reportedly located [19]. T cells were treated with LP5 in the presence or absence of methyl- β -cyclodextrin (M β CD), which causes a depletion of membrane cholesterol [29,30]. Thirty minutes after the treatment, the decrease in CD3 reached a maximum in the absence of M β CD. In contrast, in the presence of M β CD, the effect of the Ab was inhibited significantly, and the majority of cells maintained an intermediate level of CD3 on the cell surface (Fig. 4A). The presence of M β CD had no influence on the staining with the anti-CD3 and LP5 Abs (data not shown). These results suggest that the effects of LP5 on CD3 expression depend on the construction of lipid rafts.

We have shown that there is no competition between LP5 and the anti-CD3 Ab in staining (Fig. S4, B and C). Some molecules expressed on the cell-surface are continuously recycled through endocytosis and exocytosis [26]. Therefore, we next examined whether LP5 has any effect on the recycling of CD3. Cells were treated with LP5 under neutral or acidic conditions, which can inhibit endocytosis [29]. As shown in Fig. 4B, in the acidic culture, the reduction in CD3 caused by LP5 was dramatically

inhibited. We confirmed that even in acidic conditions, the Ab could bind to CD90 molecules without any loss of staining intensity (data not shown). The results indicate that the LP5-mediated down-regulation is caused through accelerated endocytosis of CD3 molecules at the cell surface.

Down-regulation of CD3 expression in the early stages of T cell-activation is critical to hyporesponsiveness.

We have shown that LP5 added to a culture continuously inhibited the activation of CD4 T cells (Fig. 1) and induced a temporal reduction in the expression of CD3 (Fig. S5A). Then, we next investigated the relationship between the suppressive effect and decrease in CD3. First, we examined whether the pre-treatment of CD4 T cells with LP5 is enough, or the continuous presence of the Ab is required, for the induction of hyporesponsiveness. CD4 T cells were pre-treated with LP5 at 37°C for 90 min, washed three times, and stimulated with the anti-CD3 Ab along with APCs. As shown in Fig. 5A and 5B, pre-treatment with LP5 for 90 min was enough to cause hyporesponsiveness in CD4 T cells. In contrast, CD4 T cells pre-treated with LP5 for 90 min, washed, then cultured in normal medium for an additional 28 h, or CD4 T cells pre-treated with LP5 for 28 h and washed, showed normal proliferation in the stimulation culture (Fig. 5C). Furthermore, LP5 added in the late stages of a 3-day culture, but not in the initial stages, had no suppressive effect on CD4 T cell activation (Fig. 5D). Taken together, these results indicate that a down-regulation of CD3 expression before or in the early stages of T cell activation is required for the induction of hyporesponsiveness.

The pre-treatment of CD4 T cells with LP5 Ab prolonged the survival of allogeneic grafts.

Pre-treatment of CD4 T cells with LP5 was effective in inducing hyporesponsiveness in vitro (Fig. 5). Next, we investigated whether it is effective in vivo. First, we examined whether the Ab bound to CD4 T cells is toxic in vivo. To examine this possibility, BALB/c CD4 T cells pre-treated with LP5 were transferred into CB17.scid mice. Two weeks later, splenic cells were harvested, and examined for the presence of CD4 T cells. As shown in Fig. S6, the pre-treatment with LP5 had no significant toxic effect on CD4 T cells. Then we investigated whether this pre-treatment is effective in prolonging the graft's survival. To this end, we used a mouse model for bone marrow transplantation in which BALB/c hosts are lethally irradiated and reconstituted with a 1:1 mixture of BALB/c (H-2^d) and CBF1 (H-2^{dx^b}) bone marrow [8]. Two weeks after the transfer, bone marrow cells were examined for the presence of syngeneic and semi-allogeneic cells. When bone marrow cells alone were transferred, semi-allogeneic cells were detected (Fig. 6, circles). However, when BALB/c CD4 T cells were co-transferred, the semi-allogeneic cells were eliminated, and some of the recipient mice died probably due to the reaction of co-transferred BALB/c CD4 T cells with CBF1 bone marrow cells (squares). In contrast, when the co-transferred BALB/c CD4 T cells were pre-treated with LP5, we detected almost the same percentage of CBF1 bone marrow cells, and all recipient mice survived (triangles). In another set of experiments, mice were monitored until day 38 after cell-transfer. At this time point, we observed a decrease in the percentage of CBF1 cells, indicating that the pre-treatment of CD4 T cells with LP5 is not enough for the complete survival of semi-allogeneic cells, but enough for the prolongation of their survival. Collectively, these

results suggest that CD3 down-modulation induced by in vitro pre-treatment with LP5 is effective in preventing CD4 T cell activation in vivo.

Discussion

CD90 expressed by mouse T cells has signal transduction properties [18,31]. Studies using anti-CD90 Ab or CD90-deficient mice have proposed both stimulatory and inhibitory roles for CD90 [20-25]. For example, thymocytes from CD90-deficient mice were hyper-responsive to TCR-stimulation as demonstrated by cell proliferation [24]. CD90 cross-linking on T cells by an anti-CD90 Ab (21F10) inhibited T cell proliferation [32]. These reports suggest that CD90 functions as a negative regulator of TCR-mediated signaling. In contrast, the majority of studies using anti-CD90 Abs demonstrated a mitogenic role for CD90 in T cell activation [22,25,31,33]. One anti-Thy-1 Ab, clone G7, augmented TCR/CD28-driven T cell proliferation [33]. Another anti-CD90 Ab, 30-H12, had no potential to activate T cells, but, was able to block CD90 signaling as a soluble form, resulting in inhibition of the anti-CD3-induced proliferation of CD4 T cells [33], suggesting an important co-stimulatory role for CD90 signaling in mouse T cell activation. In this paper, we reported a new anti-CD90 Ab, LP5, which was established based on its suppressive activity against CD4 T cell activation. This anti-CD90 Ab had the potential to prevent activation of CD4 T cells through a temporal down-regulation of CD3/TCR expression, which has not been reported previously for an anti-CD90 Ab. Furthermore, we demonstrated that the down-regulation of TCR/CD3 complexes prior to, or during the early stages of, T cell activation results in T cell hypo-responsiveness, and effective in prolonging graft survival in vivo. Moreover, LP5 caused this down-modulation without any induction of T cell activation.

It is known that TCR/CD3 complexes in resting T cells are internalized and recycled constitutively [26]. Once T cells are activated by antigens, down-modulation of TCR/CD3 complexes from the cell surface is induced [27]. In contrast to this naturally occurring reduction in TCR/CD3 upon T cell activation [27], the active down-regulation of TCR/CD3 with the use of an anti-CD3 Ab is useful in regulating immune responses in mice and humans. For example, it has been demonstrated that anti-CD3 mAb therapy promotes remission of established type 1 diabetes mellitus [34,35]. As one mechanism leading to remission, it was proposed that the anti-CD3 Ab induces a capping of TCR/CD3 complexes at the cell surface and their internalization and/or shedding from the cell; the resulting T cells (TCR⁻CD3⁻ T cells) become transiently 'blind' to antigens and unresponsive to stimulation. However, at the same time, inoculation of the anti-CD3 Ab accompanied flu-like symptoms, linked to limited cytokine release in the early stages after treatment [36,37]. Because these side effects were associated with cross-linking of CD3 via an Fc-dependent mechanism, many kinds of anti-CD3 Abs were developed by modifying the Fc portion of the Abs to reduce FcR binding, which is critical to the stimulatory activity. Nevertheless, they still promoted some degree of T cell activation and cytokine release [38,39]. Therefore, Abs, such as LP5, having the potential to down-modulate TCR/CD3 complex, and having no stimulatory properties regarding T cells, would be useful in regulating immune responses.

We showed that an anti-CD90 Ab, LP5 (rat μ), could down-regulate the expression of CD3/TCR complexes. As shown in Fig. 3A, this property was not observed among other anti-CD90 Abs, FF-10 (rat γ_{2c}) and HO-13-4 (mouse μ). In another experiment, we also investigated whether other anti-CD90 Abs have this

potential; 30-H12 (rat γ_{2b}) and 53-2.1 (rat γ_{2a}) (both used at 1 $\mu\text{g/ml}$) had no effect on CD3 expression; J1j.10 (rat μ) and M5/49.4.1 (rat γ_{2a}) (both at 50% SN) induced a reduction in CD3 but less effectively than LP5 (rat μ). These results suggest that the properties of LP5 are not dependent on its Ig-subclass. LP5 could compete with other anti-CD90 Abs in staining (Fig. 2E), however, it might recognize a unique determinant on the CD90 molecule, which would be critical for the induction of CD3/TCR down-modulation. Taken together, LP5 would be appropriate for controlling TCR/CD3 complexes without any stimulatory effect on CD4 T cells. However, we also need to consider that a ligand or counter-receptor for mouse CD90 has not yet been identified [18]. In addition, CD90 is expressed on almost all peripheral T cells in mice, but in humans, it was expressed only on a small percentage of thymocytes, and $<1\%$ of CD3⁺ lymphocytes in the peripheral circulation. Therefore, seeking a new Ab against target molecules on the cell-surface, which induces a reduction in TCR/CD3 and has no stimulatory activity, would lead to the development of a better tool to manipulate immune systems.

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Figure legends

Figure 1 **The Ab LP5 can inhibit the activation of CD4 T cells.** (A, B)

CD4⁺CD25⁻ T cells from DO11.10 mice were cultured with APCs plus the titrated amount of anti-CD3 Ab (A) or OVA₃₂₃₋₃₃₉ peptide (B) in the presence (open symbol) or absence (closed) of LP5 (25 % SN). Three days later, cell proliferation was measured. (C, D) CD4⁺CD25⁻ T cells from BALB/c mice were cultured with anti-CD3 Ab (5% SN) plus APCs (C), or anti-CD3 Ab/anti-CD28 Ab-coated beads (D) in the presence (open) or absence (closed) of the titrated amount of LP5 Ab. Data are representative of more than five independent experiments.

Figure 2 **LP5 recognizes CD90.** (A) Total cell lysate from the indicated tumor cells was subjected to SDS-PAGE under non-reducing conditions, transferred onto a membrane, blotted with LP5, and visualized. (B) The lysate was immunoprecipitated (IP) with LP5 Ab- or Rat IgG-coated beads. The beads-bound proteins were subjected to SDS-PAGE under non-reducing conditions and western-blotted with LP5. (C and D)

The proteins immunoprecipitated by LP5-beads were treated as in (A), then blotted with anti-CD90 Abs [FF-10 (C) or 30-H12 (D)]. (A-D) The arrow indicates a molecular weight of 25 kDa. (E) EL-4 tumor cells were pre-treated with (green) or without (red) LP5, and then stained with the indicated anti-CD90 Abs. Negative control staining is shown as a black line. Data are representative of more than three (A-E) independent experiments.

Figure 3 LP5 induces down-regulation of CD3 expression. (A and B) BALB/c whole spleen cells were cultured at 37 °C in the presence (green line) or absence (red) of the Ab LP5 (rat μ , 50% SN), FF-10 (rat γ_{2c} , 10 μ g/ml), or HO-13-4 (mouse μ , 50% SN) as indicated. Sixty minutes later, cells were harvested, washed, and stained with anti-CD4 and anti-CD3 (A) or anti-rat or mouse Ig Ab (B). All CD4⁺ cells were analyzed for CD3 (A) or CD90 (B) staining. (C) Spleen cells were pre-treated with LP5 (bottom panels) or medium (top) for 60 min at 37°C, washed, and stained with the indicated Abs (green) and anti-CD4 Ab. Negative control staining is shown as a red line. The expression of the indicated molecules on CD4 T cells is shown as a histogram. Data are representative of more than two (A and B) or three (C) independent experiments.

Figure 4 LP5 can influence the internalization of CD3. (A) BALB/c spleen cells were washed with FCS-free medium, and pre-cultured with or without M β CD (1 mM) at 37°C. Ten minutes later, the cells were cultured in the presence (green lines) or absence (red) of LP5 for 30 min, washed, and stained with anti-CD3 and anti-CD4 Ab. All CD4⁺ T cells were analyzed for CD3 staining. (B) Splenic cells were suspended in neutral or acidic medium (40 mM acetic acid) for 5 min at 37°C, treated with (green) or

without (red) LP5 for 30 min, washed, stained and analyzed as in (A). We confirmed that even in the presence of M β CD (A), or under acidic conditions (B), LP5 could bind to CD90 without any decrease in staining intensity (data not shown). Data are representative of more than three independent experiments.

Figure 5 Pre-treatment of CD4 T cells with LP5 is enough for

hyporesponsiveness. (A) CD4 T cells were pre-treated with LP5 Ab for 90 min, washed three times, and then cultured with the anti-CD3 Ab along with APCs. Three days later, cell proliferation was measured. The proliferation of the pre-treated T cells was compared with that of untreated T cells, and expressed as % response. Each symbol represents individual preparations of pre-treated T cells. (B) CD4 T cells untreated (circle) or pre-treated with LP5 (square), as in (A), were cultured with the titrated amount of anti-CD3 Ab SN along with APCs. (C) CD4 T cells cultured in medium (circle) or LP5 (triangle) for 28 h, or CD4 T cells pre-treated with LP5 for 90 min, washed, and cultured in medium for an additional 26 h (square), were washed, and cultured with the titrated amount of anti-CD3 Ab along with APCs. (D) CD4 T cells were stimulated with the anti-CD3 Ab in the presence of APCs, and at the indicated time points during a 3-day culture, LP5 (25% SN) or medium was added to the culture. Three days later, cell proliferation was measured. Data are representative of two to three independent experiments (B-D).

Figure 6 Pre-treatment of CD4 T cells with LP5 prolonged the survival of

allogeneic grafts. Both bone marrow cells (1:1 ratio) from BALB/c and CBF1 mice were transferred into γ -irradiated BALB/c mice (circle). At the same time, untreated

(square) or LP5-treated CD4 T cells (triangle) from BALB/c mice were co-transferred. The survival of each recipient mouse was monitored (lower panels). At the end of the observation period, splenic cells from surviving mice were stained with anti-CD4, anti-H-2K^b, and anti-H-2D^d. The relative percentage of H-2K^bD^d-positive cells (semi-allogeneic cells) among all H-2D^{d+}CD4⁻ cells was calculated as follows: (% of semi-allogeneic cells in experimental mouse / mean % of semi-allogeneic cells in mice injected with bone marrow cells only) x 100. Each symbol represents an individual mouse. Combined data from two independent experiments are shown in left panels, whereas right panels represent a single experiment.

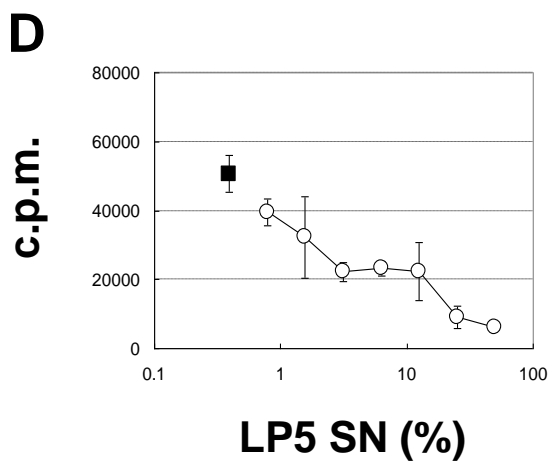
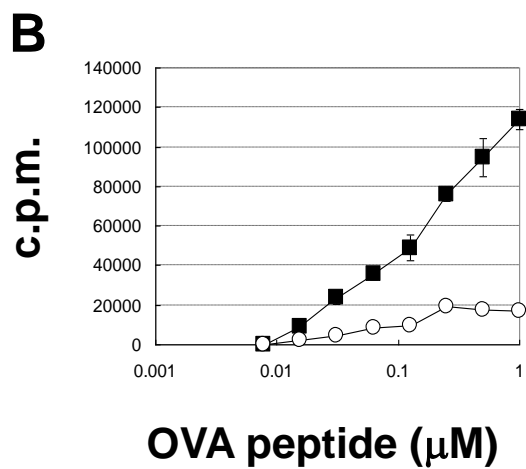
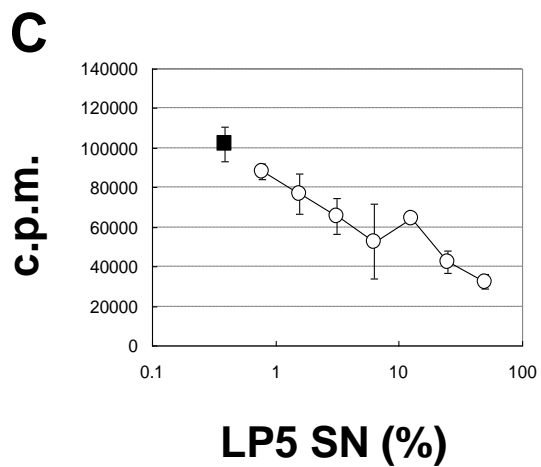
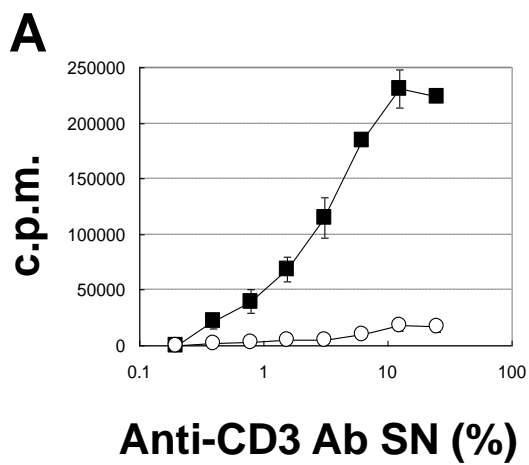
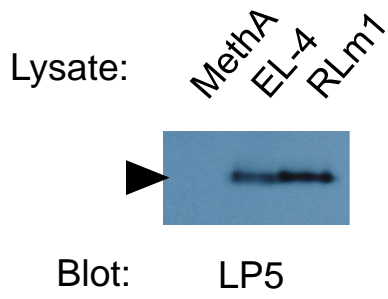
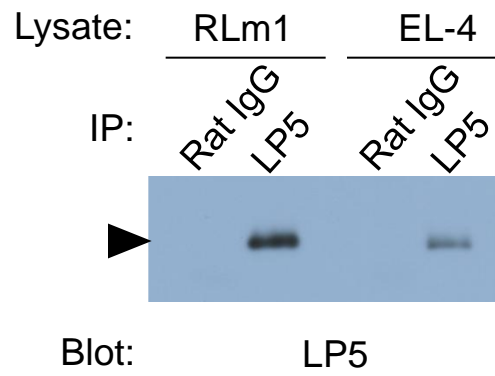
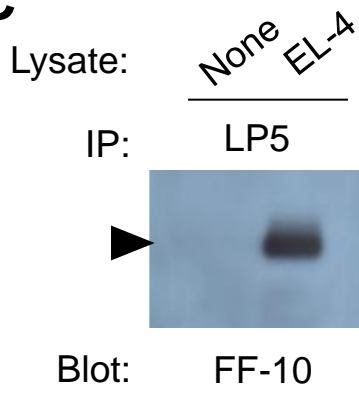
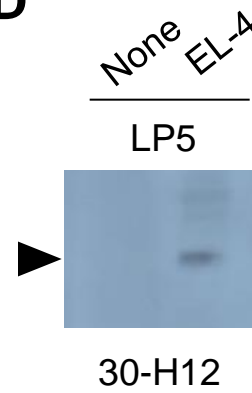
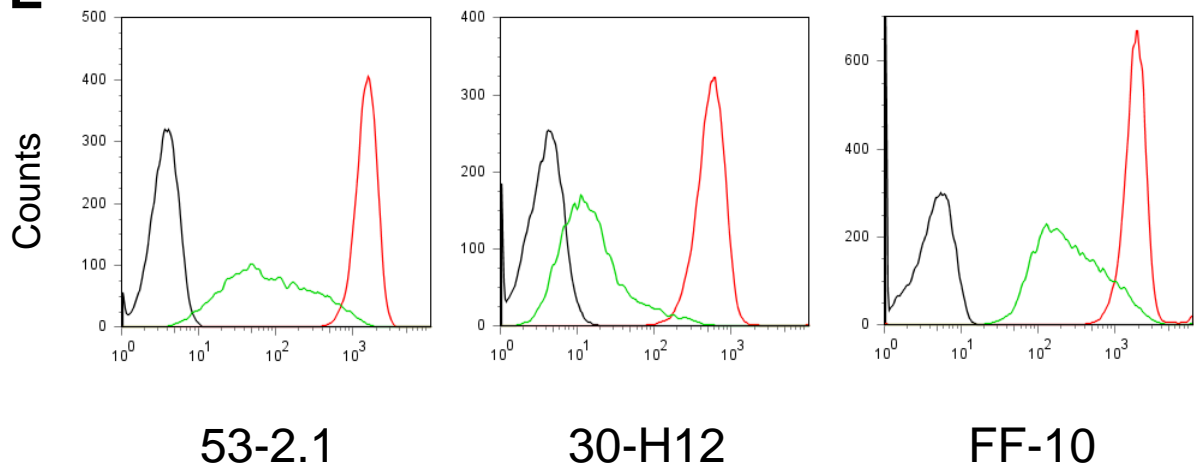


Fig. 1

A**B****C****D****E****Fig. 2**

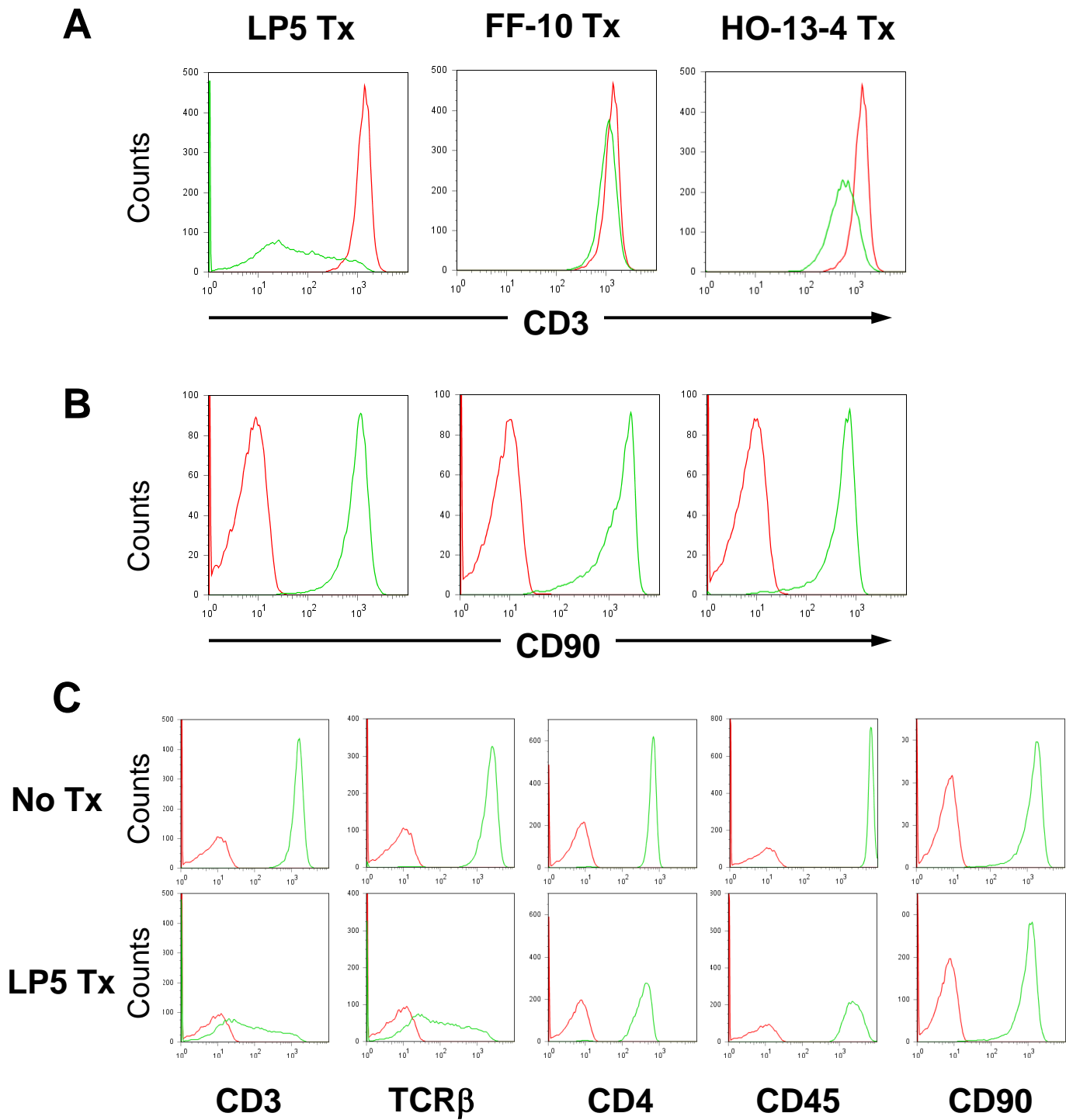


Fig. 3

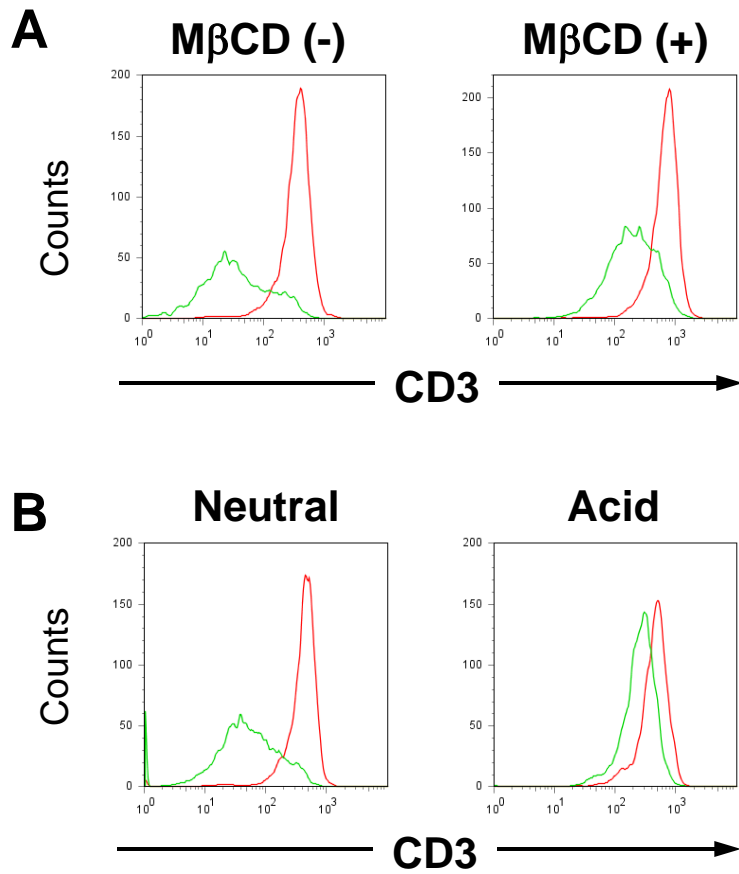
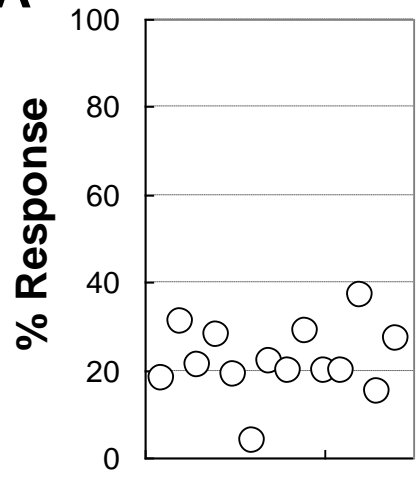
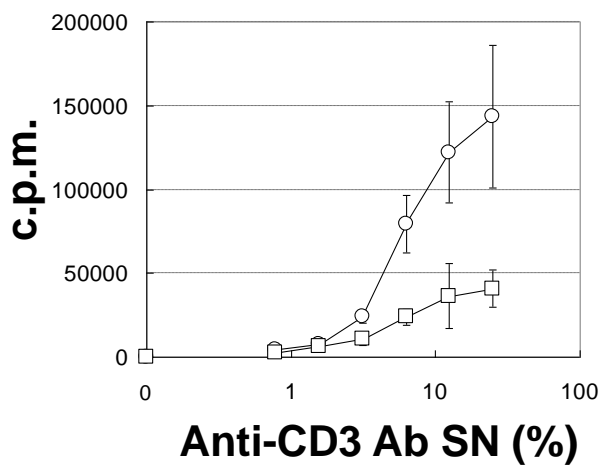
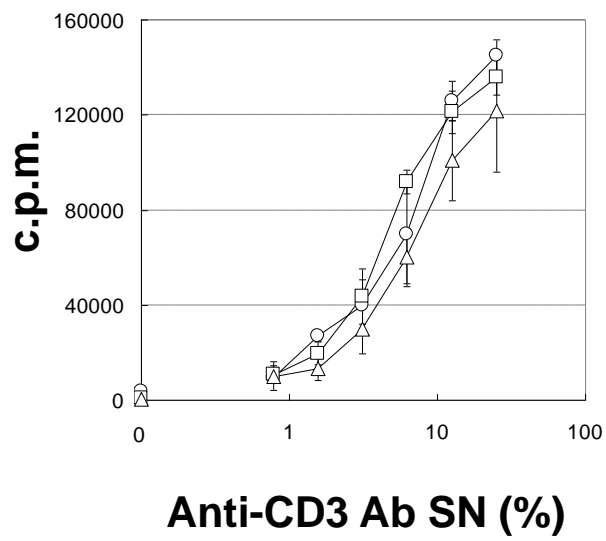
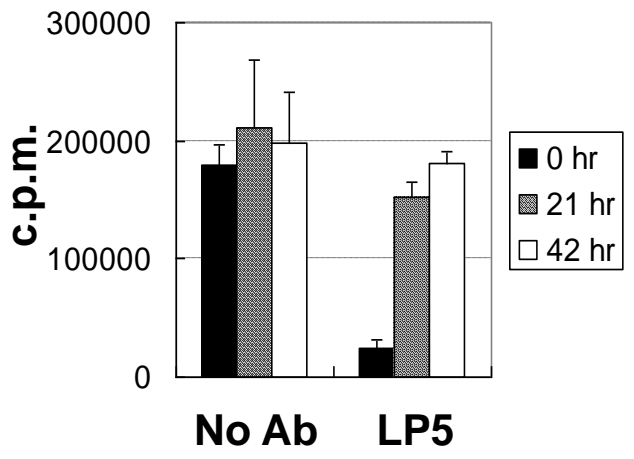


Fig. 4

A**B****C****D****Fig. 5**

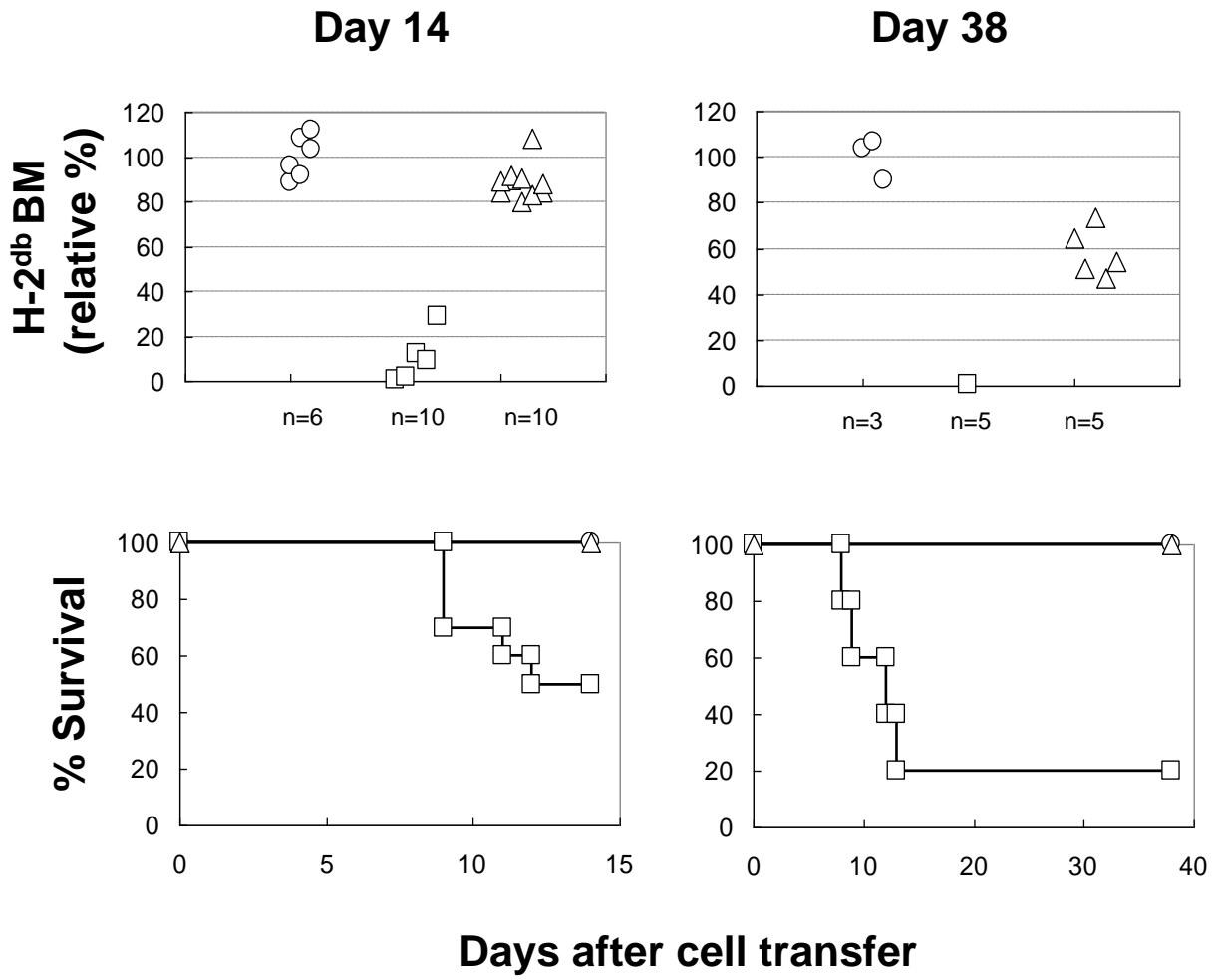


Fig. 6

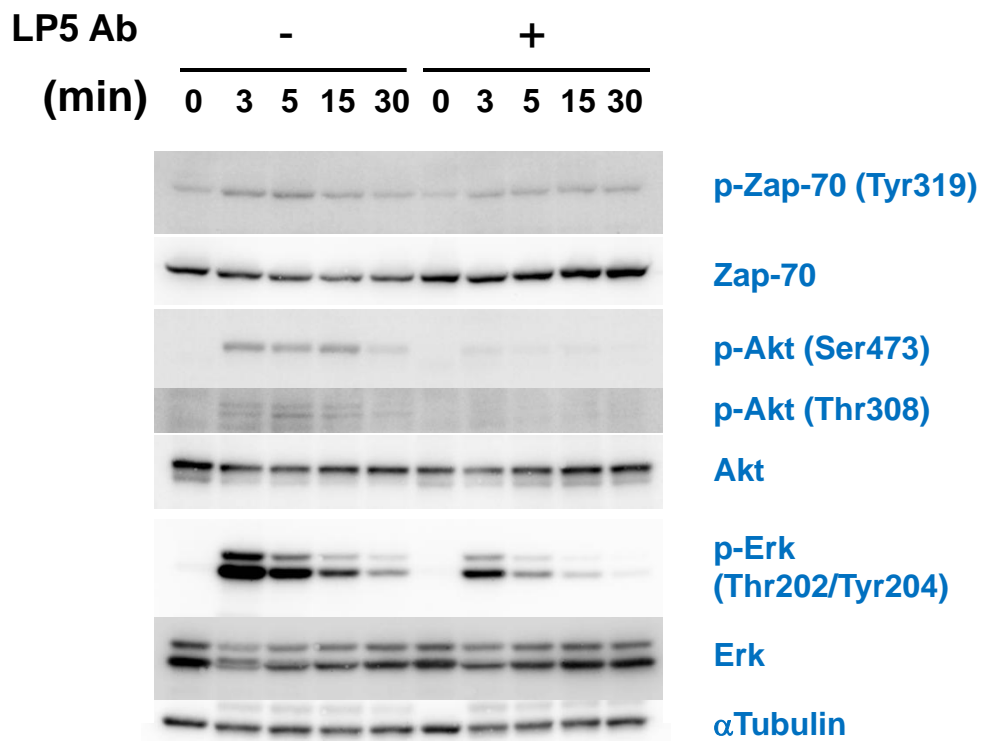


Figure S1

Effect of LP5 on signaling molecules associated with T cell activation.

CD4 T cells purified by negative selection were treated with or without LP5 (50% SN) for 90 min at 37°C, and then anti-CD3 Ab (2C11, hamster IgG), anti-CD28 Ab (clone 37.51, hamster IgG), and finally anti-hamster IgG Ab was added. After the indicated period, cell lysate was prepared, subjected to SDS-PAGE under reducing conditions, transferred onto a membrane, blotted with the indicated Ab, and visualized. Data are representative of three independent experiments.

We examined signaling molecules implicated in TCR-driven T cell activation, and whether they are influenced by LP5 (Fig. S1). Zap-70 is activated following TCR stimulation. In accordance with this, the activation of Zap-70, that is, an increase in phosphorylated Zap-70 (Tyr319), was evident by 5 min post-stimulation with the anti-CD3 and anti-CD28-Abs. However, by pre-treating cells with LP5 for 90 min, phosphorylation of Zap-70 (Tyr319) was inhibited; no increase in p-Zap-70 (Tyr319) at any time-point examined. Furthermore, phosphorylation of Akt and Erk, both required for the activation of T cells, was also significantly inhibited by the Ab. Taken together, these results demonstrate that LP5 inhibits normal signal transduction leading to T cell-activation mediated by the TCR/CD3 complex, resulting in the inhibition of T cell-activation.

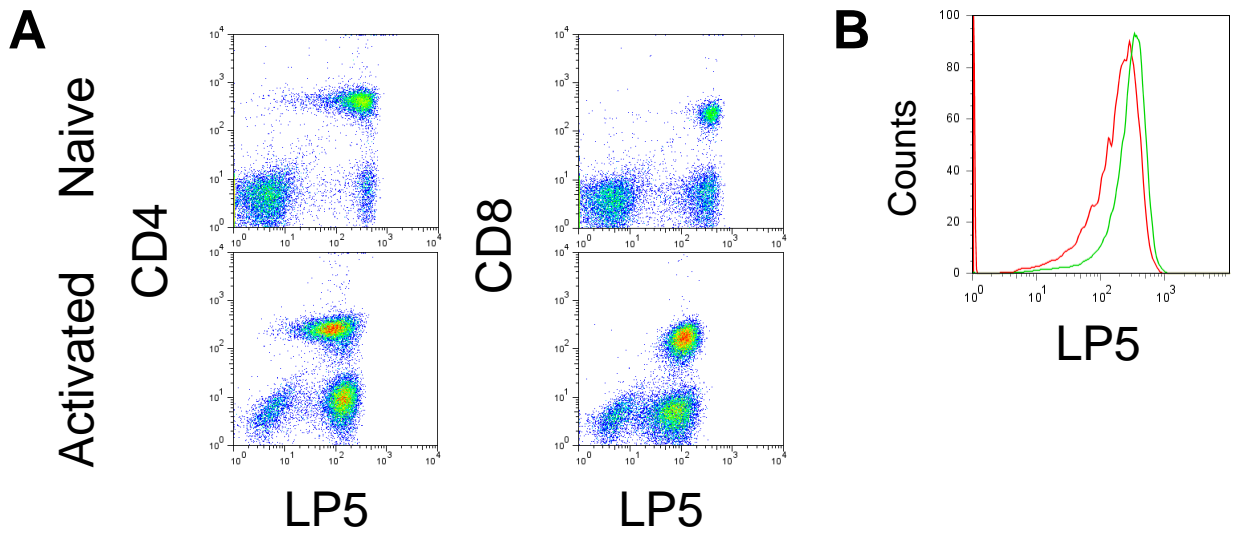


Figure S2

Antigen recognized by LP5 is expressed on almost all T cells.

(A) BALB/c spleen cells freshly prepared (naïve) or activated with anti-CD3 Ab for two days (activated) were stained with LP5 and anti-CD4 or anti-CD8. (B) Naïve spleen cells were stained with LP5, anti-CD4, and anti-Foxp3. The expression of LP5-target molecules on CD4⁺Foxp3⁻ (green) or CD4⁺Foxp3⁺ (red) cells is indicated. Data are representative of more than five (A, B) independent experiments.

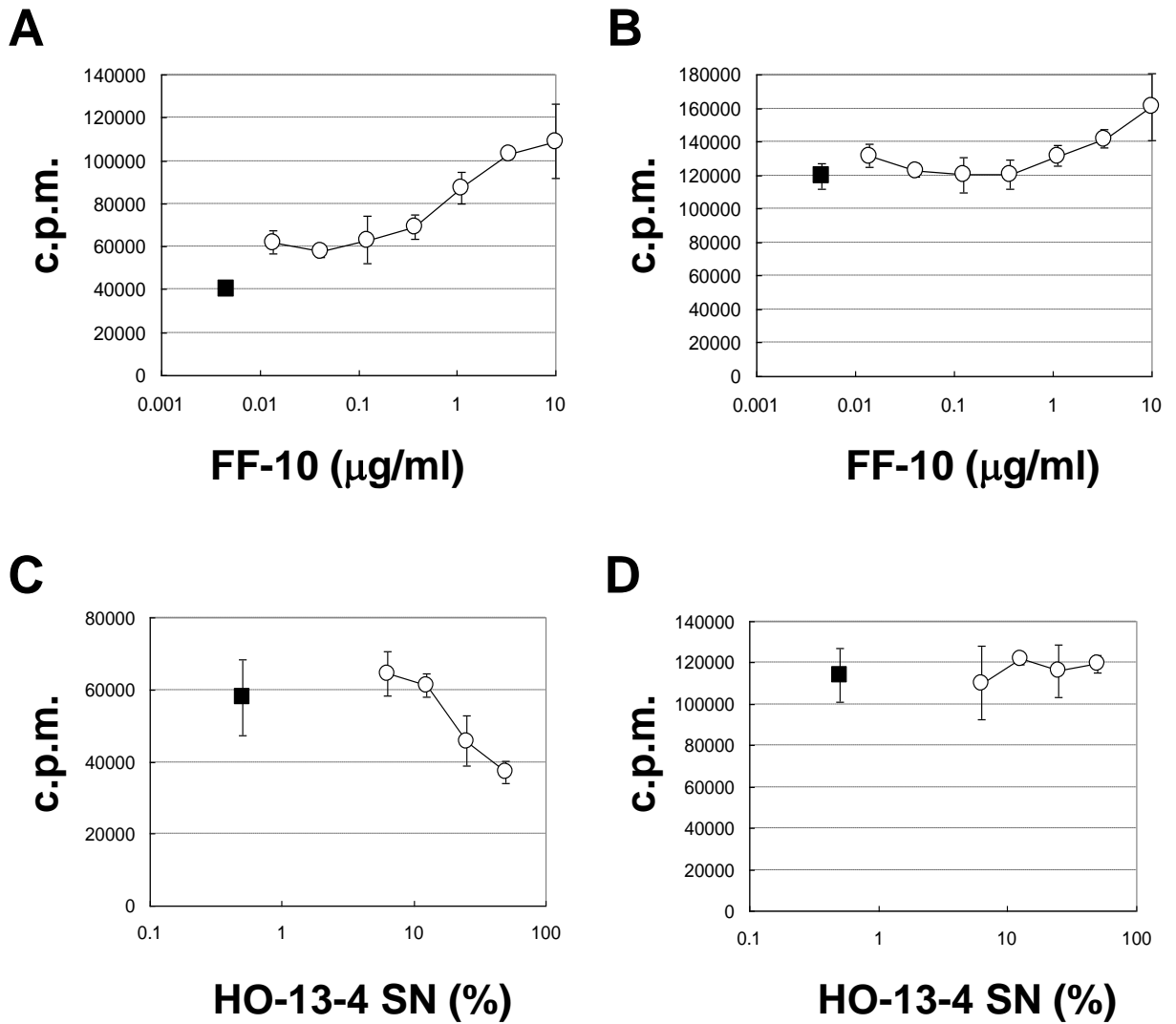


Figure S3

Effect of anti-CD90 Abs, FF-10 and HO-13-4, on the activation of CD4 T cells.

BALB/c CD4⁺CD25⁻ T cells were cultured with anti-CD3/anti-CD28 Ab-coated beads (A and C) or anti-CD3 Ab (5% SN) along with APCs (B and D) in the presence (open symbols) of titrated amount of FF-10 Ab (upper panels) or HO-13-4 Ab (lower panels). As a control, cells were cultured in the absence of anti-CD90 Abs (closed). Three days later, cell proliferation was measured.

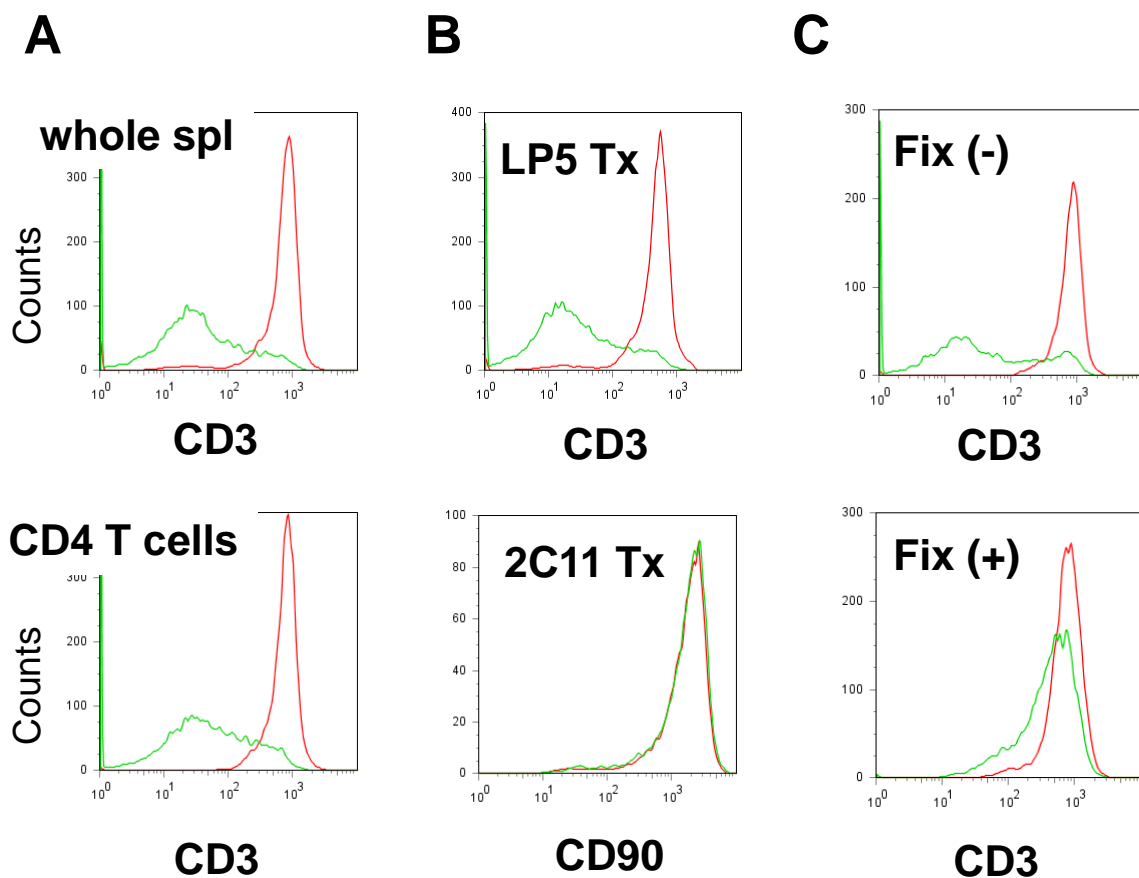


Figure S4

Characterization of LP5-induced decrease in CD3.

(A) BALB/c whole spleen cells or CD4 T cells purified by negative selection were treated with LP5 (green) or medium (red) for 60 min, washed, and stained with anti-CD4 and anti-CD3 Abs. The expression of CD3 in CD4 T cells is shown as a histogram. (B) Purified splenic CD4 T cells were cultured, as in (A), in the presence of LP5 (top panel, green) or anti-CD3 Ab (bottom, green), then stained with anti-CD3 or anti-CD90 Ab, respectively. As a control, cells were cultured in the absence of Abs (red). (C) Purified CD4 T cells were treated with or without 1% paraformaldehyde (Fix), washed, treated with LP5 (green) or medium (red), and stained with anti-CD3 Ab. We confirmed that the fixed cells could be stained with LP5 without any decrease in staining intensity (data not shown). Data are representative of more than three independent experiments.

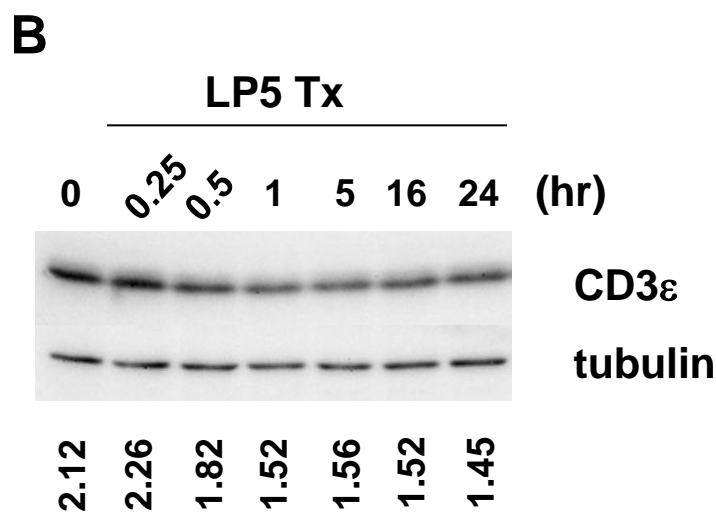
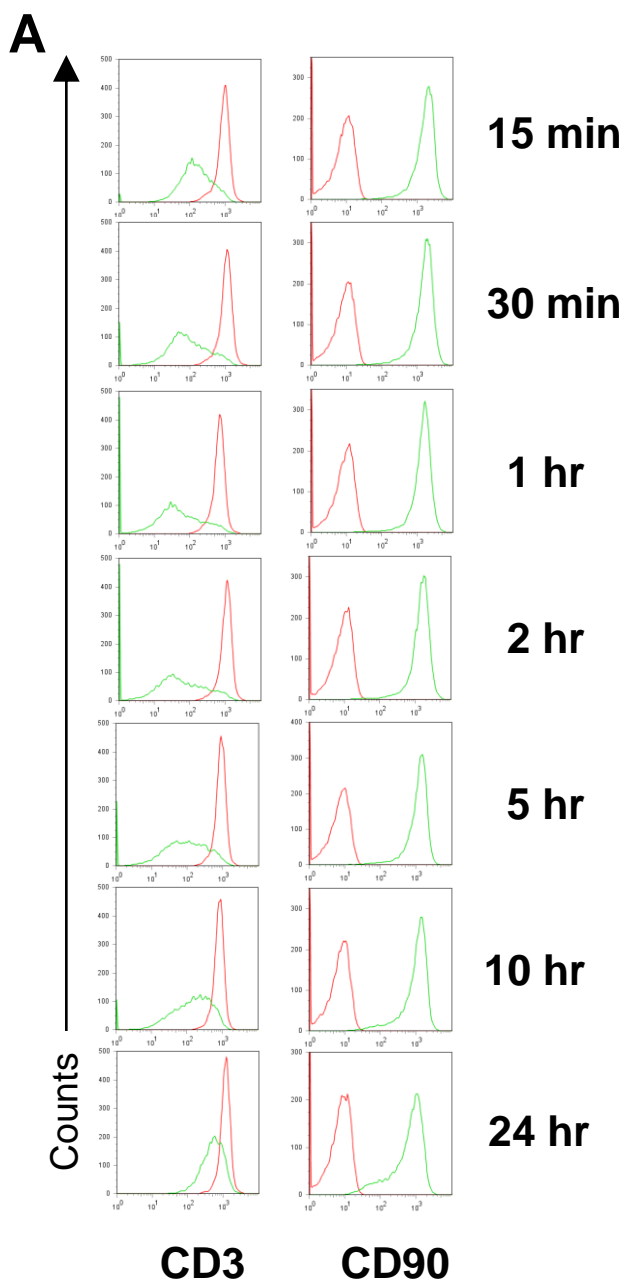


Figure S5

LP5 induces a temporary decrease in CD3.

(A) BALB/c spleen cells were cultured for the indicated period with (green) or without (red) LP5, and then stained with anti-CD4 and anti-CD3 Ab or anti-rat Ig Ab. All CD4⁺ T cells were analyzed for CD3 (left panels) or CD90 (right panels) staining. (B) Purified CD4 T cells from BALB/c mice were treated with LP5 (50% SN) at 37°C. After the indicated period (hours), cell lysate was prepared, and examined for the existence of CD3ε, as in Fig. S1. As a control, the amount of tubulin was also examined. Optical density ratios (indicated in the bottom) were calculated by comparing the density of individual CD3ε bands with the corresponding tubulin band.

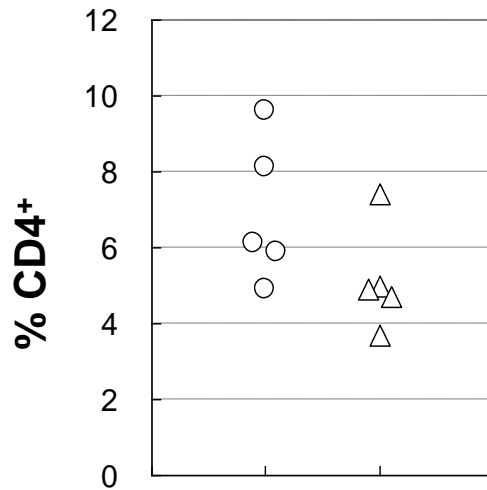


Figure S6

The pre-treatment of BALB/c CD4 T cells with LP5 has no cytotoxic effect in vivo.

CD4 T cells prepared from BALB/c mice were pre-treated with LP5, washed three times, and transferred intravenously into CB17.scid mice (9×10^5 /mouse). Fourteen days later, splenic cells were stained with anti-CD4 Ab, and examined for the remaining CD4 T cells (triangle). As a control, untreated CD4 T cells were transferred (circle). Each symbol represents an individual recipient. Statistical significance of the difference between untreated (6.9 ± 1.9 %) and LP5-treated (5.1 ± 1.4 %): $p=0.127$.