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CD4 T cell-intrinsic IL-2 signaling differentially affects Th1 and Th17 development

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ABSTRACT

IL-2 signaling is involved in clonal expansion of antigen-specific CD4 T cells. IL-2 is also reported to promote Th1 but inhibit Th17 differentiation, although in vivo relevance remains unclear. In addition, IL-2-dependent Foxp3+ CD4 Tregs suppress T cell proliferation, complicating the in vivo role of IL-2 in the development of Th cell responses. To elucidate the roles of cell-intrinsic IL-2 signaling in CD4 T cells, we cotransferred TCR-Tg CD4 T cells from IL-2Rα (CD25)-deficient and WT mice and analyzed development of antigen-specific Th1 and Th17 responses. It was revealed that Th17 development of antigen-specific CD4 T cells was largely unaffected, whereas Th1 development was impaired by the lack of IL-2 signaling. Similar data were obtained from mixed BM chimera experiments using BM cells from CD25-deficient and WT mice. In addition, although in vitro blockade of IL-2 during Th17 development greatly increased the percentages of Th17 cells, it did not affect their numbers, indicating that in vitro Th17 development is also IL-2-independent. Th1 development was dependent on IL-2 in vitro as well. Thus, our data suggest that cell-intrinsic IL-2 signaling is critical for Th1 development but plays a limited role in Th17 development in vitro as well as in vivo.
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Introduction

IL-2 is a T cell growth factor involved in the clonal expansion of antigen-specific T cells, a hallmark of adaptive immune responses. IL-2 is transiently produced by activated T cells and binds to the high-affinity IL-2R, which is also transiently induced on the activated T cells. The high-affinity IL-2R is a trimolecular complex composed of IL-2Rα (CD25), IL-2Rβ (CD122), and the γc (CD132), among which, IL-2Rβ and γc are involved in signal transduction [1]. IL-2 signaling includes STAT5 and Shc pathways, which induce cell proliferation and survival [1, 2]. Reagents inhibiting IL-2 production or IL-2 signaling have been used clinically as immunosuppressants for the prevention of graft rejection and for the treatment of autoimmune diseases [3]. However, it is also known that T cells can proliferate in the absence of IL-2 [4–6] and that IL-2 even promotes activation-induced cell death [7, 8], complicating the role of IL-2-signaling in T cell responses. Furthermore, IL-2 plays indispensable roles in the development, maintenance, and functions of Foxp3+ CD4 Tregs, which are known to inhibit T cell proliferation in vivo as well as in vitro [9, 10]. Consequently, mice deficient in IL-2 or CD25 spontaneously develop autoimmunity with polyclonal expansion of T cells [11, 12]. This also indicates the IL-2-independent expansion of pathogenic Teffs and the importance of paracrine function of IL-2 in the regulation of T cell responses. In fact, it was shown that adoptively transferred, IL-2-deficient TCR Tg CD4 T cells expand comparably with or even more than WT CD4 T cells after immunization with the antigen [4, 5]. On the other hand, CD25-deficient TCR Tg CD4 T cells proliferated less than WT cells [13]. Thus, the actual role of cell-intrinsic IL-2 signaling is, although not absolutely required, to promote antigen-specific CD4 T cell expansion in vivo. This emphasizes the importance of using CD25-deficient cells in determining the functions of cell-intrinsic IL-2 signaling.

Along with clonal expansion, antigen-primed, naïve $CD4^+$ T cells differentiate into distinct Th cell subsets, depending on the cytokine milieu. IL-12 and IFN-γ induce differentiation of Th1 cells that produce IFN-γ and mediate delayed-type hypersensitivity responses. IL-2 has often been categorized as a Th1 cytokine and promotes Th1 differentiation [14, 15]. TCR stimulation of naive CD4 T cells in the presence of TGF-β and IL-6 induces differentiation of Th17 cells, another Th cell subset

Abbreviations: γc=common γ-chain, BM=bone marrow, CD62L=CD62 ligand, Foxp3=forkhead box P3, KO=knockout, OT-II=OVA-specific CD4 TCR transgenic mice, RORγτ=retinoic acid-related orphan receptor γτ, T-bet=T-box transcription factor, Teff=T effector cell, Tg=transgenic, Treg=regulatory T cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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that produces IL-17A (IL-17) and IL-17F and is also involved in many inflammatory disorders [16]. Interestingly, it was reported that STAT5-mediated IL-2 signaling inhibited the differentiation of Th17 cells [17]. An addition of exogenous IL-2 in Th17-inducing culture decreased the frequency of Th17 cells, whereas neutralizing IL-2 exhibited opposite effect. IL-2-deficient CD4 T cells showed an enhanced Th17 development in vivo. Several molecular mechanism have been proposed, which include competitive inhibition of STAT3 by STAT5 [18] and/or down-regulation of IL-6Rα [15]. However, IL-2-induced suppression of the Th17 response seems to conflict with the clinical effect of IL-2-targeting therapy. IL-2-induced clonal expansion of antigen-specific T cells might critically affect the number of differentiated Th cells. Furthermore, the precise role of CD4 T cell-intrinsic IL-2 signaling in Th cell development remains to be determined.

In the present study, we used CD25-deficient mice to investigate the role of IL-2 signaling in the development of antigen-specific Th cell responses. It was revealed that cell-intrinsic IL-2 signaling did not significantly affect Th17 development, although it was critically involved in Th1 development.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Charles River Breeding Laboratories (Yokohama, Japan). CD25 KO mice and OT-II mice (Tg mice expressing TCR specific for the $OVA_{323–339}$ peptide on $I\text{-}A^b$) and Ly5.1-congenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). CD25 KO mice were crossed with OT-II mice to generate CD25-deficient OT-II cells. Mice were maintained in specific pathogen-free conditions in our institute, and sex- and age-matched mice were used for the experiment at 4–8 weeks of age. This study design was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine (Kyushu University, Fukuoka, Japan). Experiments were carried out under the control of the Guidelines for Animal Experiment.

Flow cytometric analysis

Fluorochrome-conjugated mAb and reagents used for flow cytometric analysis were as follows: Alexa Fluor 488 anti-IFN-γ (XMF1.2), Alexa Fluor 647-conjugated anti-IL-17A (TC11-18H10), anti-IFN-γ (XMF1.2), antiphospho-STAT5 (pY694; 47), PE-conjugated anti-IL-17A (TC11-18H10), anti-IFN-γ (XMF1.2), and allophycocyanin-conjugated anti-CD25 (PC61) mAb (BD Biosciences, San Jose, CA, USA); and PE-conjugated anti-CD154 (MR1), anti-T-bet (eBio4B10), anti-RORγt (AFKJS-9) mAb, and allophycocyanin-conjugated anti-IL-2 (JES6-5H4) mAb (eBioscience, San Diego, CA, USA). To exclude dead cells from the analysis, we added PI (1 μg/mL) to the cell suspension, just before run on a flow cytometer. To detect cytokine production, cells were cultured for 5 h in the presence of PMA (25 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) or $OVA_{323–339}$ peptide (10 μg/ml). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 4 h of incubation. Intracellular staining was performed using the BD Cytofix/
Cytoperm kit (BD Biosciences). To detect phospho-STAT5, draining LN cells were washed and cultured with serum-free medium for 2 h. Subsequently, cells were treated with human rIL-2 (10 IU/ml) for 30 min and stained for phospho-STAT5, according to the manufacturer's instructions. Stained cells were run on a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using BD CellQuest software (BD Biosciences).

Naive CD4 T cell purification

CD4 T cells were negatively selected by staining with anti-CD8 (2.43), anti-I-A/E (M5/114.15.2), and anti-CD25 (PC61) mAb (BD Biosciences) for 20 min at 4°C, followed by incubation with Dynabeads sheep anti-rat IgG (Invitrogen, Carlsbad, CA, USA) at 4°C. Naive CD4 T cells were purified by using anti-CD62L MicroBeads and an autoMACS cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD44^{lo}CD62L^{hi}CD25^{-}CD4^{+}$ T cells was >95%.

Adoptive transfer experiments

Naïve CD4 T cells from WT (Ly5.1/5.1) or CD25 KO (Ly5.2/5.2) OT-II mice were mixed (5×10^4 cells/each) and injected i.v. into WT (Ly5.1/5.2) recipient mice. The recipient mice were injected s.c. into the base of the tail with a emulsion containing 100 μg OVA and CFA (Difco, Detroit, MI, USA) on the next day. For the CFSE-labeling experiment, mixed, naïve CD4 T cells (1×10^5 cells/each) were labeled with 1 μM CFSE (Invitrogen) in PBS for 15 min at 37°C. After stopping the reaction by adding an equal volume of FCS, the cells were washed twice with PBS before injection.

Mixed BM chimera mice

BM cells from WT (Ly5.1/5.1) and CD25 KO (Ly5.2/5.2) TCR non-Tg mice were depleted of T cells by using anti-CD3 mAb (17A2; eBioscience) and Dynabeads sheep anti-rat IgG (Invitrogen). As we had observed in the preliminary experiments that KO BM cells repopulated less efficiently than WT cells, WT and KO BM cells were mixed at a ratio of 1:2 and injected i.v. into lethally irradiated (10 Gy) WT-recipient mice (Ly5.1/5.2). After confirming reconstitution 8 weeks after BM transfer, recipients were immunized s.c. with 100 μg $OVA_{323–339}$ peptide emulsified in CFA.

Induction of Th1 and Th17 cells in vitro

Naive CD4 T cells (1×10^5) were cultured in 96-well plates, precoated with 5 μg/ml anti-CD3 mAb (145-2C11), in the presence of 1 μg/ml soluble anti-CD28 mAb (37.51). Th1 cells were induced by adding 1 ng/ml mouse rIL-12 (PeproTech, Rocky Hill, NJ, USA) and 10 μg/ml anti-IL-4 mAb (11B11) to the culture. Th17 cells were induced by adding a combination of 20 ng/ml mouse rIL-6 (Miltenyi Biotec) and 5 ng/ml human TGF-β1 (R&D Systems, Minneapolis, MN, USA) with 10 μg/ml anti-IFN-γ (XMG1.2) and anti-IL-4 mAb to the culture. Where indicated, 10 μg/ml anti-mouse IL-2 (JES-1A12; BD PharMingen, San Diego, CA, USA) was also added to the culture. The amount of cytokines in the culture supernatant was measured by using ELISA kits (R&D Systems). Cells were stimulated with PMA and ionomycin before intracellular staining of IFN-]γ and IL-17.

Statistics

Statistical significance was calculated by the two-tailed Mann-Whitney U-test using Prism software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Lack of IL-2 signaling differentially affected Th1 and Th17 development in vivo

To investigate the roles of cell-intrinsic IL-2 signaling during in vivo development of Th1 and Th17 cells, naive CD4 T cells from CD25 KO and WT OT-II mice with different CD45 allotypes were mixed at a ratio of 1:1 and transferred into recipient mice, which were subsequently immunized with OVA-emulsified in CFA. Clonal expansion of KO and WT OT-II cells was

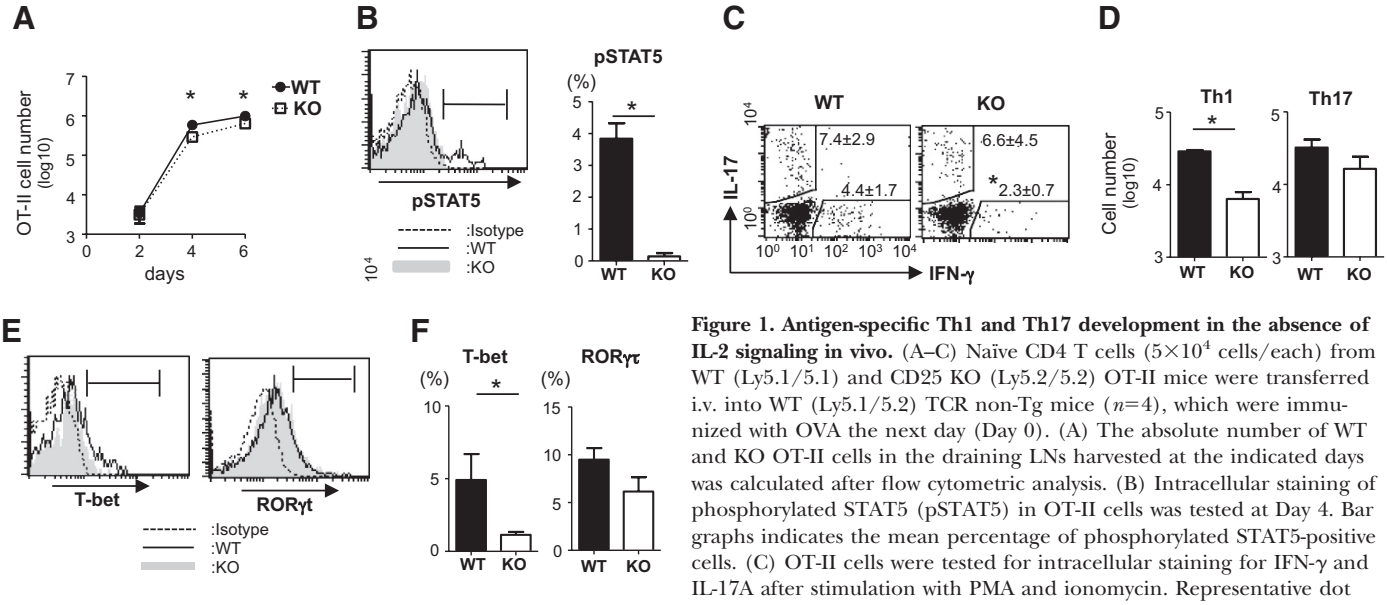

Figure 1. Antigen-specific Th1 and Th17 development in the absence of IL-2 signaling in vivo. (A–C) Naïve CD4 T cells (5×10^4 cells/each) from WT (Ly5.1/5.1) and CD25 KO (Ly5.2/5.2) OT-II mice were transferred i.v. into WT (Ly5.1/5.2) TCR non-Tg mice (n=4), which were immunized with OVA the next day (Day 0). (A) The absolute number of WT and KO OT-II cells in the draining LNs harvested at the indicated days was calculated after flow cytometric analysis. (B) Intracellular staining of phosphorylated STAT5 (pSTAT5) in OT-II cells was tested at Day 4. Bar graphs indicates the mean percentage of phosphorylated STAT5-positive cells. (C) OT-II cells were tested for intracellular staining for IFN-γ and IL-17A after stimulation with PMA and ionomycin. Representative dot plots at Day 4 are shown. Upper numbers in left and right panels are the mean ± SD percentages of Th17 and Th1 cells, respectively. (D) The absolute number of Th1 and Th17 cells at Day 4 was calculated. (E) Expression of T-bet and RORγτ in OT-II cells was examined by intracellular staining at Day 4. (F) The mean frequency of T-bet (left)- or RORγτ (right)- expressing cells is shown. Error bars represent mean ± SEM. *$P<0.05$ Data are representative of three separate experiments.

clearly seen after immunization, and there was only a slight reduction in the total number of KO OT-II cells (**Fig. 1A**). This is not likely a result of incomplete inhibition of IL-2 signaling by only CD25 deficiency, as we did not detect phosphorylated STAT5 in KO cells (Fig. 1B). Therefore, in vivo clonal expansion of antigen-specific T cells is largely IL-2 signaling-independent.

However, the frequency of Th1 cells was reduced significantly in KO OT-II cells, whereas the frequency of Th17 cells was not significantly different between KO and WT OT-II cells (Fig. 1C). By calculating the absolute number, it was revealed that the number of Th17 cells was decreased slightly by the lack of IL-2 signaling, whereas the number of Th1 cells was decreased severely (Fig. 1D). We obtained similar results by transferring fewer numbers of OT-II cells (1×10^4/mouse; data not shown). There was no difference in the expression levels of IL-6R, TGF-βR, or IL-12R between WT and KO OT-II cells (Supplemental Fig. 1). We also examined the development of IL-17F-positive cells, but there was also no difference (data not shown). Foxp3+ cells scarcely developed from WT or KO OT-II cells (data not shown). As it remains possible that IL-2 signaling affects cytokine production but not differentiation of Th cells, we examined the expression of T-bet and RORγt, key transcription factors of Th1 and Th17 cells, respectively. Whereas the frequency of T-bet-expressing cells decreased in CD25 KO OT-II cells, that of RORγt-expressing cells was comparable between WT and KO cells (Fig. 1E and F). Thus, the lack of IL-2 signaling did not enhance in vivo development of antigen-specific Th17 cells, whereas it reduced Th1 development.

To elucidate the roles of IL-2 signaling in the physiological number of antigen-specific CD4 T cells, we investigated Th cell development in mixed BM chimera mice reconstituted with WT and KO TCR non-Tg BM cells. This system also has an advantage to exclude the possible functional difference at the level of naive CD4 T cells developed in KO and WT environment. The chimera mice were immunized with OVA peptide, and antigen-specific T cells were detected by intracellular staining of CD154 [19] after ex vivo stimulation with OVA peptide. The frequency of Th1 and Th17 cells in the antigen-specific T cells was examined by additional staining of IFN-γ and IL-17. As shown in **Fig. 2**, the number of antigen-specific CD4 T cells (CD154+) was comparable between WT and KO cells, but Th1 cells among them were decreased significantly in KO rather than WT cells. Th17 cells also tended to decrease in KO CD4 T cells, but there was not a significant difference from WT cells. Thus, IL-2 signaling did not suppress Th17 development, even in a condition with a physiological number of antigen-specific T cells.

IL-2-independent differentiation and expansion of Th17 cells in vivo

A simple explanation for the above observation is that in vivo Th17 differentiation is not susceptible to IL-2-induced suppression. However, it remains possible that IL-2 indeed suppresses differentiation of Th17 cells, but IL-2-induced clonal expansion overcomes the effect. To test these possibilities, we examined cell division of differentiating Th1 and Th17 cells by labeling OT-II cells with CFSE before transfer. There was not a large difference in the extent of cell division of whole OT-II cells between WT and KO during the observation period (**Fig. 3A**), which is consistent with the small difference in the total number of OT-II cells (Fig. 1A). IFN-γ-positive cells appeared from Day 3 in the divided cell population in WT and KO cells (Fig. 3B and D). Fewer IFN-γ-positive cells developed in KO cells, although no difference was found in the extent of cell division

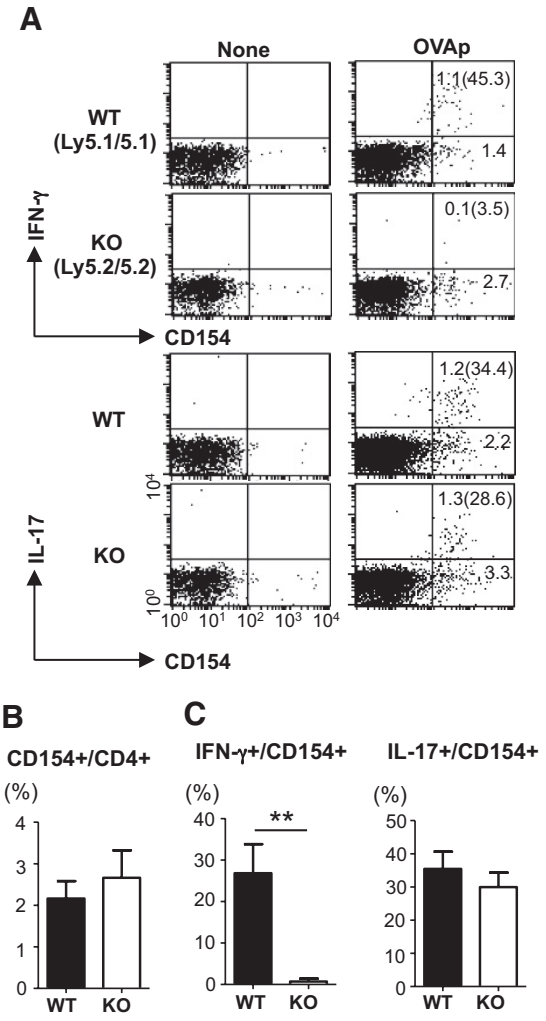

Figure 2. Antigen-specific Th1 and Th17 development in mixed BM chimera mice. Mixed BM chimera mice reconstituted with WT (Ly5.1/5.1) and KO (Ly5.2/5.2) TCR non-Tg mice were immunized with OVA peptide (OVAp; $n=4$). After 14 days, antigen-specific Th1 and Th17 cells were detected by intracellular staining of CD154, IFN-γ, and IL-17 after ex vivo stimulation, with or without the OVA peptide. (A) Representative dot-plot analysis of the spleen cells is shown. Numbers indicate the percentage of cells in each quadrant, whereas the numbers in the parentheses indicate the percentage of IFN-γ- or IL-17-positive cells in CD154-positive cells. Similar data were obtained from the analysis of draining LN cells. (B and C) The frequency of OVA peptide-specific T cells (CD154+) in CD4 T cells (B) and IFN-γ- or IL-17-producing cells within CD154+ CD4 T cells (C) was shown as bar graphs ($n=4$). Data are representative of three separate experiments. Error bars represent mean ± SEM. $^{**}P < 0.01$.

between WT and KO IFN-γ-positive cells. This suggests that the differentiation rather than proliferation of Th1 was impaired in the absence of IL-2 signaling. This is consistent with the recent publication showing the importance of IL-2 signaling in Th1 differentiation [15]. IL-17-positive cells also appeared from Day 3 in the divided cell population, although there was no difference in the number of IL-17-positive cells between WT and KO cells (Fig. 3C and D). Importantly, there was also no difference in the extent of cell division of IL-17-positive cells between WT and KO, suggesting similar efficacy of Th17 differentiation.

IL-2R expression during in vivo development of Th cell subsets

These results indicated that the in vivo development of Th17 is relatively independent of or insensitive to IL-2 signaling compared with that of Th1. Therefore, we examined the expression of CD25 on developing Th1 and Th17 cells from WT OT-II cells (**Fig. 4A**). CD25 was expressed on a large part of OT-II cells at early stages after OVA immunization, when few cells differentiate into Th1 or Th17 cells. Thereafter, IFN-γ- or IL-17-producing cells appeared, but most of them were CD25-negative. Thus, we could not detect differences in the expression levels of CD25 between Th1 and Th17 cells. There was also no difference in the percentage of IL-2-positive cells in Th1 and Th17 cells (Fig. 4B).

IL-2 blockade did not affect the number of Th17 cells developed in vitro

Our data seem to conflict with the results of previous studies showing a suppressive effect of IL-2 on Th17 development [17]. This might be because CD25-deficient CD4 T cells behaved differently from IL-2-deficient CD4 T cells or CD4 T cells in the culture with neutralizing anti-IL-2 mAb, but it is also possible that we evaluated in vivo development of Th17 cells by calculating cell numbers. So, we investigated the effect of in vitro IL-2 neutralization during Th17 development by focusing on cell numbers. Naive CD4 T cells were stimulated with anti-CD3 and anti-CD28 mAb in a Th1- or Th17-inducing condition, in the presence or absence of anti-IL-2 mAb. Consistent with the previous studies [17], neutralizing IL-2 during in vitro Th17 development greatly increased the frequency of Th17 cells (**Fig. 5A** and **B**). However, whereas the number as well as the frequency of Th1 cells were reduced by neutralizing IL-2, it did not affect the absolute number of Th17 cells throughout the culture period (Fig. 5C). We also measured the cytokines in the culture supernatants and found that there was no significant difference in the amount of IL-17, whereas IFN-γ was greatly decreased by neutralizing IL-2 (Fig. 5D). Thus, there was not a large difference in the roles of IL-2 signaling between in vivo and in vitro development of Th cell subsets as estimated by the cell number.

Differences and similarities in the effect of IL-2 signaling during in vivo and in vitro development of Th17 cells

In contrast to the in vivo situations, where the total number of antigen-specific T cells was only slightly decreased in the absence of IL-2 signaling, the total cell number, especially in Th17-inducing culture, was greatly reduced by neutralizing IL-2 (Fig. 5C). Th17-inducing culture generated a large number of IL-17-nonproducing cells, which failed to expand by neutralizing IL-2 (Fig. 5C). Thus, the increased percentage of Th17 cells by neutralizing IL-2 was mainly attributed to the decrease of these IL-2-sensitive cell populations. In this regard,

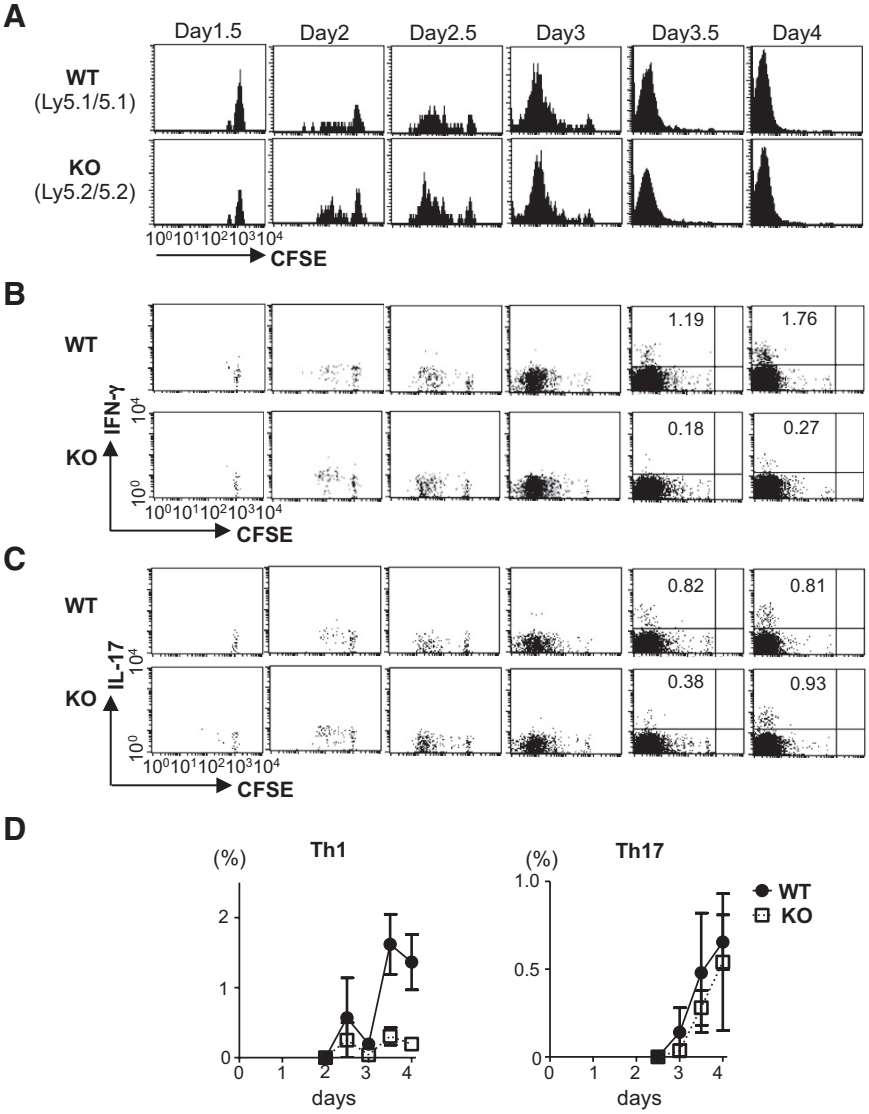

Figure 3. Lack of IL-2 signaling did not affect cell division and Th17 differentiation in vivo. Naïve CD4 T cells (1×10^5 cells/each) from WT (Ly5.1/5.1) and KO (Ly5.2/5.2) OT-II mice were mixed, labeled with CFSE, and transferred i.v. into WT mice (Ly5.1/5.2). The recipient mice were immunized s.c. with OVA on the next day (Day 0), and the cells were harvested from the draining LNs at the indicated time-points. (A) Histograms show CFSE staining of WT (upper panels) and KO (lower panels) OT-II cells. Cells were stimulated with PMA and ionomycin before flow cytometric analysis for IFN-γ (B) and IL-17 (C) staining. Numbers indicated are the percentages of cytokine-positive cells. Data are representative of three separate experiments. (D) Kinetics of the percentage of Th1 (left) and Th17 (right) cells are shown as line graphs.

there were several points worth noting. First of all, CD25 was continually expressed on in vitro-activated CD4 T cells, even after differentiation into Th1 or Th17 cells (**Fig. 6A**), which might explain the drastic effect of in vitro IL-2 neutralization. The expression level of CD25 on T cells in a Th17-inducing condition was slightly lower than those in a Th1-inducing condition. Interestingly, the level of IL-2 in the supernatants of Th17-inducing culture was much lower than that of a Th1-inducing culture (Fig. 6B), which contributed to the different level of CD25 expression, as neutralizing IL-2 equalized the expression levels of CD25 on cells in Th1 and Th17 conditions (Fig. 6A). Nevertheless, the clear effect of IL-2 neutralization in a Th17-inducing culture indicated that the low level of IL-2 was essential for the expansion of IL-17-nonproducing T cells, which likely further consume IL-2 in the culture. As these complicate the in vitro role of IL-2 signaling during Th cell development, we examined in vitro development of Th1 and Th17 cells in a coculture system using WT and CD25 KO cells to confirm the cell-intrinsic role of IL-2 signaling. We found that although Th1 cells were decreased greatly in KO rather than WT cells, the number of Th17 cells was not significantly different between KO and WT cells (Fig. 6C). The number of IL-17-negative cells in Th17-inducing culture decreased severely in the absence of IL-2 signaling. Thus, cell-intrinsic IL-2 signaling preferentially affected Th1 but not Th17 development in vitro as well.

DISCUSSION

Although IL-2 is known as an autocrine growth factor of activated T cells, IL-2 so produced is also used by Foxp3+ CD25+ CD4 Tregs that conversely suppress T cell proliferation, complicating the in vivo role of IL-2 in a Teff response [20]. To circumvent this problem, we set up experiments to compare CD25 KO with WT CD4 T cells in the same mice and found that cell-intrinsic IL-2 signaling only marginally promoted antigen-specific CD4 T cell expansion in vivo. Similar levels of re-

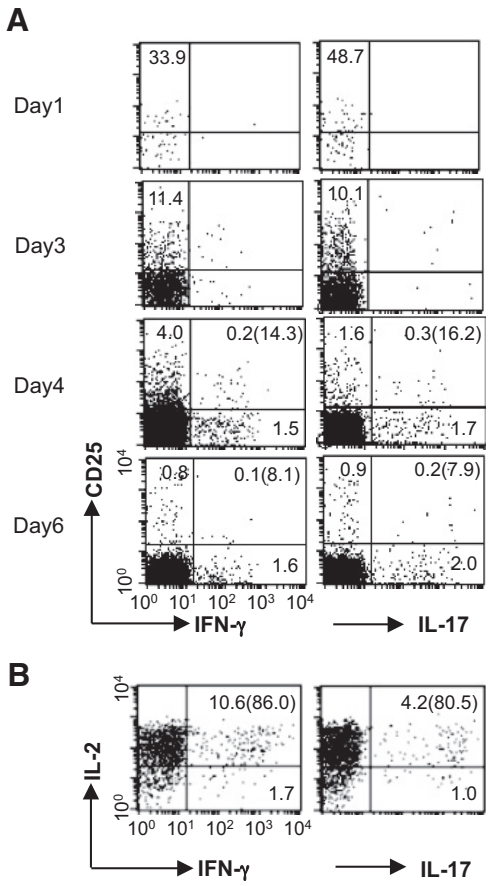

Figure 4. Lack of IL-2R expression on differentiating Th cells in vivo. Expression of CD25 (A) or IL-2 (B) in WT OT-II cells that have been transferred and primed in vivo as Fig. 3 was examined. Cells were harvested from the draining LNs at the indicated days (Day 4 in B) and stimulated with PMA and ionomycin to detect IFN-γ and IL-17 production. Numbers on each quadrant indicate the percentage of CD25- or IL-2-positive cells in OT-II cells, whereas the numbers in the parenthesis indicate the percentage of CD25- or IL-2-positive cells in cytokine-positive cells. Data are representative of three separate experiments.

duction in clonal expansion of CD25-deficient T cells were reported by Dooms et al. [13]. These suggest that in vivo proliferation of CD4 T cell is largely IL-2-independent. In addition, Williams et al. [6] reported that IL-2 signaling was dispensable for the primary CD8 T cell response. One may argue that reagents targeting IL-2 have been used as immunosuppressants that efficiently inhibit antigen-specific immune responses. We assume this is related to the severely impaired Th1 response in CD25-deficient T cells. In line with our results, it was reported that IL-2 played a crucial role for Th1 development, which included the expression of IL-12Rβ2 and T-bet [15]. Therefore, in the clinical settings where Th1 responses play critical roles, such as allograft rejection, suppression of Th1 development might be equivalent to the suppression of T cell clonal expansion.

It was shown that IL-2 signaling mediated by STAT5 suppresses Th17 development [17]. We also observed a striking increase of the frequency of Th17 cells by neutralizing IL-2 in a Th17-inducing culture. However, the results of our in vivo experiments, which showed comparable frequencies of WT and KO Th17 cells, suggested that in vivo development of antigen-specific Th17 cells was independent of IL-2 signaling. This might be related to the transient CD25 expression on Th17 cells in vivo. However, Th1 cells, which were reduced significantly by the lack of CD25, also down-regulated CD25 before differentiation. The absence of phosphorylated STAT5 in CD25 KO OT-II cells suggests that the lack of CD25 is sufficient for inhibiting IL-2 signaling in vivo. We further confirmed, by using in vitro-activated T cells—most of which from WT mice express CD25—that CD25-deficient T cells failed to induce phosphorylation of not only STAT5 but also S6, a key molecule downstream of the Shc pathway of IL-2 signaling [1, 21, 22] (Supplemental Fig. 2A). We showed comparable IL-2 production by in vivo-developed Th1 and Th17 cells, but interpretation of the results needs some caution. As the cells needed to be stimulated ex vivo to induce IFN-γ and IL-17 production for the detection of Th1 and Th17 cells, respectively, that might not reflect the in vivo level of IL-2 production but only showed the capability to produce IL-2. It was actually reported that in vivo IL-2 production by antigen-specific CD4 T cells was limited during the first 48 h after immunization [5], further indicating the transient nature of IL-2 signaling during in vivo Th cell differentiation.

We were surprised to find that the absolute number of Th17 cells was not increased by blocking IL-2 signaling in vitro, although the frequency was increased greatly as mentioned above. Therefore, we conclude that the development of Th17 cells is independent of IL-2 signaling in vitro as well as in vivo. This also means that the in vitro culture system recapitulates the in vivo Th17 development with regard to the effect of IL-2 signaling by measuring absolute cell numbers. The in vitro observation indicates that the lack of IL-2 signaling mainly affects the IL-17-nonproducing CD4 T cells developed in the Th17-inducing culture condition. These T cells were highly sensitive to IL-2, even more than Th1 cells, as estimated by the extent of reduction by neutralizing IL-2. The kinetics of cell number suggest that an impaired expansion is the main mechanism of the reduction. Although we observed that most of the IL-17-negative T cells in the Th17-inducing culture was Foxp3-negative (data not shown), they could be a sort of incompletely differentiated Tregs induced by TGF-β signaling. Further investigation is needed to test this hypothesis. Another question is whether these IL-2-sensitive T cells are generated during in vivo Th17 induction. However, we did not detect such a striking decrease of T cells producing neither IFN-γ nor IL-17 by the lack of IL-2 signaling in vivo. In addition, we observed these T cells capable of producing TNF-α (data not shown).

Our results conflict with the prevailing notion that IL-2 signaling suppresses Th17 development. The major reason for this discrepancy might be that most of the studies evaluated Th17 development by their frequency, whereas our conclusion was drawn by calculating their absolute number. Nevertheless, one may claim that there ha been molecular evidence for IL-2-induced suppression of Th17 development. These include competitive binding of STAT5 and STAT3 on an *IL-17α* locus [18] and down-regulation of IL-6R [15]. However, there have

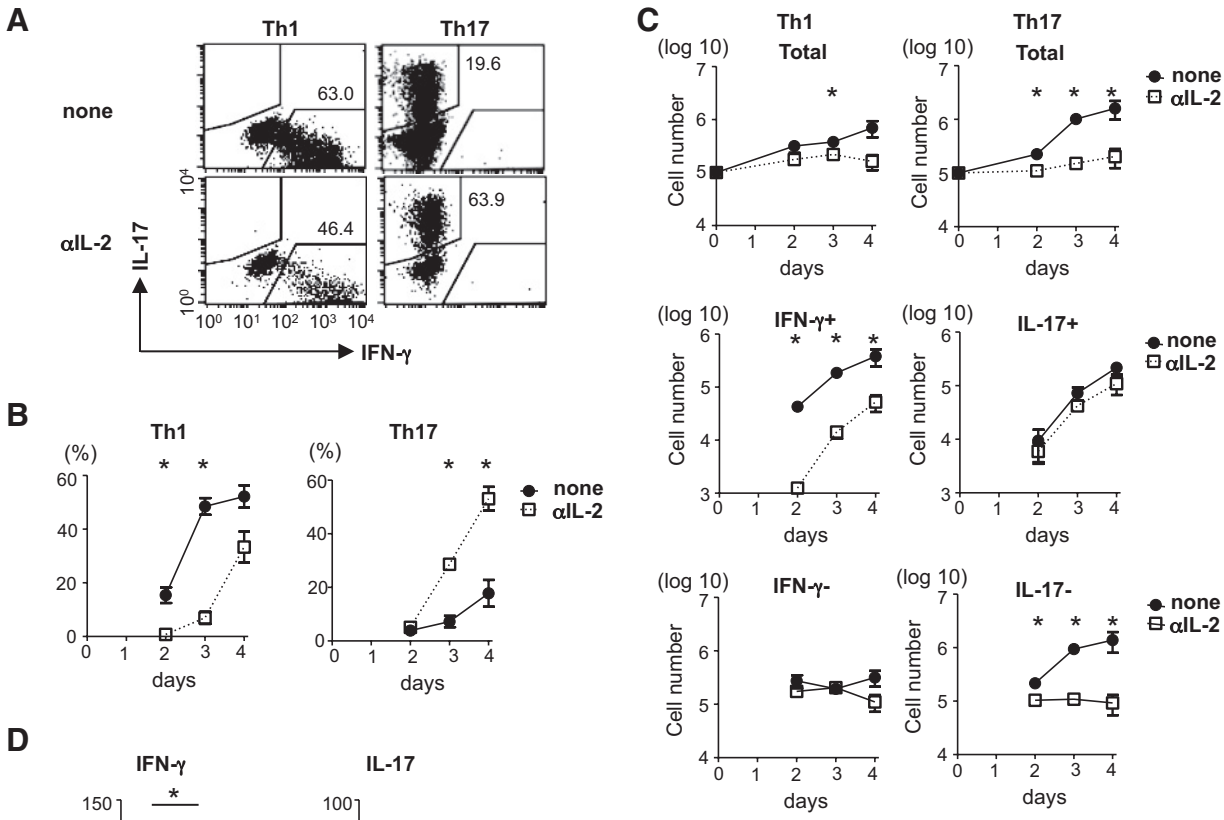

Figure 5. Effect of neutralizing IL-2 on the number of Th1 and Th17 cells developed in vitro. Naïve CD4 T cells from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAb under Th1- or Th17-inducing condition, with or without anti-IL-2 (αIL-2) mAb for 4 days. (A) IFN-γ and IL-17 production was determined after stimulation with PMA and ionomycin. Representative dot plots of 4-day-cultured cells are shown. The numbers indicated are the percentages of Th1 (left) and Th17 (right) cells. (B) Kinetics of the percentage of Th1 or Th17 cells are shown as line graphs. (C) Kinetics of the absolute numbers of total (top), IFN-γ- or IL-17-positive (middle), and IFN-γ- or IL-17-negative (bottom) cells in a Th1 (left column)- or Th17 (right column)-inducing condition are shown. (D) Concentration of IFN-γ and IL-17 in the culture supernatants of Th1- and Th17-inducing conditions, respectively, was measured by ELISA at Day 4. (B–D) Data are the pools of four independent experiments. Error bars in the figures represent mean ± SEM. $*P < 0.05$.

been conflicting data. For instance, transduction of IL-6R augmented Th17 development, even in the presence of IL-2 [15]. The expression of RORγt, a master regulator of Th17 development, was shown to be suppressed [17] or unaffected [18] by IL-2 signaling. It was shown that IL-2 signaling suppresses IL-17A production from Th17 cells but not Th17 development [18], whereas another report showed that IL-2 was affected at early time-points of Th17 development [23]. In addition, as the IL-2 blockade must have changed the proportion of Th17 cells within the cell samples used for the molecular analysis, the data could be the results but not the causes of the increased Th17 frequency. In vitro differentiation into Th17 cells, as estimated by mRNA expression of IL-17A, is achieved as early as 40 h after the beginning of the culture [24], whereas most of molecular analysis was performed at later time-points. So, we think this issue remains an open question, and these might be a general concern in examining molecular events during cell differentiation into different populations.

Quintana et al. [24] reported recently that IL-2 production was suppressed in Th17 cells, which was mediated by a member of the Ikaros family transcription factors, Aiolos. This is in line with our data that the amount of IL-2 in Th17-inducing culture was extremely low compared with Th1-inducing condition. Although Aiolos was shown to be induced by STAT3 and the Aryl hydrocarbon receptor [24], another study indicated an involvement of TGF-β signaling in the suppression of IL-2 production [25]. The reduced IL-2 production by Th17 cells might explain the IL-2 independence of Th17 cells. However, even such low levels of IL-2 were biologically significant, as its neutralization greatly reduced the IL-17-nonproducing cells. In addition, supplementation of IL-2 by the surrounding non-Th17 cells is possible in an in vivo situation. Interestingly, a careful look of the published data revealed that IL-6 signaling reduced STAT5 phosphorylation induced by IL-2 [18], suggesting a cell-intrinsic mechanism of IL-2 hyporesponsiveness. Needless to say, it is also possible that Th17 development is

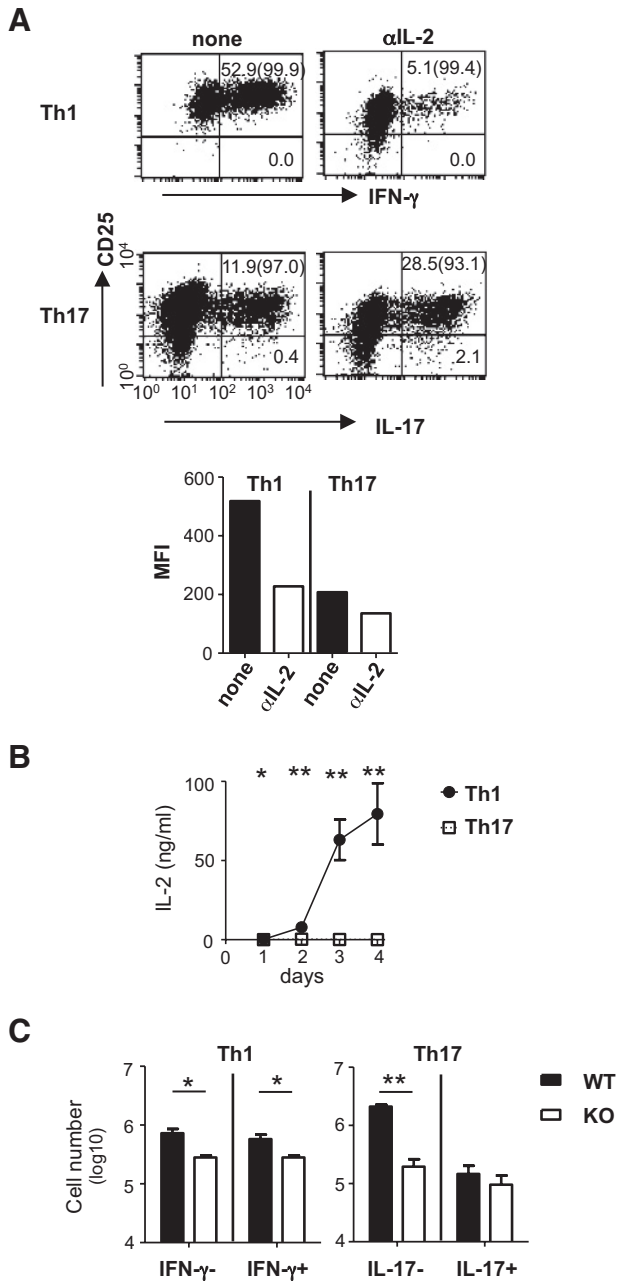

Figure 6. IL-2 signaling in the development of Th1 and Th17 cells in vitro. (A) CD25 expression on CD4 T cells from C57BL/6 mice cultured for 3 days in Th1- or Th17-inducing condition in the absence (left) or presence (right) of anti-IL-2 mAb was examined. The number indicates the percentage of cells in each quadrant, whereas the numbers in the parenthesis indicate the percentage of CD25+ cells within IFN-γ+ or IL-17+ cells. The graph shows mean fluorescence intensity (MFI) of CD25 expression on cytokine-positive cells. Representative data of three separate experiments are shown. (B) IL-2 concentration in the culture supernatants of Th1- or Th17-inducing condition (n=6 each) at the indicated day was measured by ELISA. (C) Naïve WT and CD25 KO OT-II cells (5×10^4 cells/each) were mixed and cultured in the Th1- or Th17-inducing condition for 4 days. The absolute number of cytokine (IFN-γ- or IL-17-positive or -negative cells) in Th1 (left)- and Th17 (right)-inducing conditions is shown. Data are pooled from eight independent experiments. Error bars in the figures represent mean ± SEM. *$P<$ 0.05; **$P<$ 0.01.

essentially IL-2-independent, which is the simplest explanation. The high amount of IL-2 in the Th1 culture raises a possibility that anti-IL-2 mAb rather enhances IL-2 signaling [26]; however, it is unlikely, as an addition of exogenous IL-2 increased Th1 cells (data not shown). Moreover, STAT5 phosphorylation was inhibited in the presence of anti-IL-2 mAb (Supplemental Fig. 2B).

In summary, our data demonstrated that IL-2 was involved in the induction of Th1 development but played a minor role in clonal expansion or Th17 development, supporting the assumption that the main role of IL-2 in immune responses in vivo is the regulation of Foxp3+ CD4 Tregs. Although it remains to be evaluated whether mouse and human CD4 Teff responses are similarly controlled by IL-2, our results would be important information for the clinical application of IL-2 targeting therapy in inflammatory disorders and graft rejection.

AUTHORSHIP

K.F. and H.Y. designed research. K.F., A.O., Y.I., and Y.Y. performed experiments and analyzed data. K.F., Y.Y., and H.Y. wrote the manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

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