Naturally Occurring Regulatory T Cells Regulate the Differentiation of Pro-inflammatory Th17 Cells through PI3K-dependemt Mechanisms

Introduction

Helper T cells (Th cells) are playing central roles in every immune reaction; they connect innate immunity (*e.g.* phagocytosis by macrophages) to adaptive immunity (*e.g.* antibody production from activated B cells). Their depletion in HIV infection results in a severe immunodeficiency. There are three famous lineages of Th cells. All of them derive from naive CD4-positive T cells through antigen presentation from dendritic cells in lymph nodes, and their lineages are determined by the combination of cytokines released from dendritic cells during antigen presentation (Figure 1). Th1 cells differentiate in the presence of IL-12 and produce interferon-γ (IFN-γ) to activate macrophages. Th2 cells are induced by IL-4, activating B cells through CD40/CD40L interaction to produce antibodies. Th17 cells were identified in 2003 as a new lineage producing IL-17 and activated by IL-23 (Aggarwal *et al*., 2003; Murphy *et al*., 2003), and they are now changing our understandings of adaptive immunity, which has long been investigated based on the Th1/Th2 theory published in 1986 (Mosmann *et al*., 1986). Th17 cells are now said to be important in the induction of inflammation. Besides these Th cells, induced regulatory T cells (iTregs) arise from naive CD4-positive T cells when only TGF-β is released from surrounding mesenchymes

(Chen *et al*., 2003).

Th17 cells are now particularly spotlighted not only because they are newly found, but also because they are highly pathogenic. Th17 cells differentiate from naive CD4-positive T cells in the presence of IL-6 and TGF-β (Bettelli *et al*., 2006). During differentiation they secret IL-21 in an autocrine fashion to proliferate (Nurieva *et al*., 2007), and IL-23 secreted from mesenchymal cells contributes to their further maturation. Th17 cells are important in the induction of inflammatory reactions, and their long-lasting activation results in a variety of chronic inflammatory disorders such as Crohn's disease and rheumatoid arthritis (Langrish *et al*., 2005). Correspondingly, large amount of Th17 cell infiltration has been detected in the tissues of those who suffer from chronic inflammatory disorders (Pène *et al*., 2008), and experimentally, oral dose of anti-IL-23 Ab could ameliorate the severity of experimental autoimmune encephalomyelitis in mice (Chen *et al*., 2006).

Interestingly, the differentiation of proinflammatory Th17 cells and anti-inflammatory iTregs from naive CD4-positive T cells share a lot of mechanisms (Figure 2). Both need TGF-β from mesenchyme, which activate the phosphorylation of Smad2 and Smad3 in these cells, followed by the induction of both Foxp3 and RORγt. In this condition containing only TGF-β, Foxp3 directly binds to RORγt and suppresses its transcriptional effect, and iTregs mainly differentiate from naive CD4-positive T cells (Zhou *et al*., 2008; Ichiyama *et al*., 2008; Yang *et al*., 2008). Therefore, Foxp3

knockout mice cannot produce regulatory T cells (Hori *et al*., 2003). When IL-6 is added to this condition, JAK2 associating with IL-6 receptors begins phosphorylation of STAT3, and phosphorylated STAT3 dimerize in cytosol and translocate to nucleus, where they further activate the transcription of RORγt. Therefore, in the Th-17 condition (IL-6 + TGF-β), RORγt/Foxp3 balance shift toward a RORγt-dominant state, polarizing naive CD4-positive T cells into Th17 cells. Actually, the knockout of RORγt and mutations in STAT3 depleted the differentiation of Th17 cells (Ivanov *et al*., 2006; Milner *et al*., 2008). There is also a report that RORα is also important in the differentiation of Th17 cells, but its role and the problem whether they interact with Foxp3 like seen in RORγt are still largely unknown (Yang *et al*., 2008).

During antigen presentation, signals from T cell receptors (TCRs) and from co-stimulatory molecules CD28 are also essential for further maturation of naive CD4-positive T cells. In this process, phosphoinositide 3-kinase (PI3K) becomes activated in the downstream of these molecules. PI3K is a molecule that has a variety of important roles in many kinds of cell types in our bodies. PI3K consists of catalytic domain and regulatory domain, and the combination of catalytic domain p110δ and regulatory domain p85α is mainly expressed in immune cells (Koyasu, 2003); for instance, PI3K is important in the determination of IL-12/IL-10 balance secreted from dendritic cells (Ohtani *et al*., 2008; Weichhart *et al*., 2008; Fukao *et al*., 2002). The activation of PI3K in the downstream of TCR and CD28 results in the up-regulation of

many signal cascades. In many cells, PI3K phosphorylates Akt, which then results in following phosphorylation of mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3 (GSK3). These mTOR and GSK3 have been reported to phosphorylate STAT3 (Kusaba et al., 2004; Baurel and Jope, 2008), which is one of the key molecules in the induction of proinflammatory Th17 cells as described above. Given all these facts, I used p85α knockout mouse to investigate the roles of PI3K in the differentiation of Th17 cells *in vitro*.

Materials and Methods

Mice

Wild-type BALB/c mice (WT), Balb/c p85$\alpha^{+/-}$ (KO) and p85$\alpha^{-/-}$ (HT) mice were used in accordance with the guidelines of the Institutional Animal Care and Use Committee of Keio University School of Medicine.

Isolation of naive CD4-positive T cells

For negative sorting, splenocytes was incubated with biotinylated anti-CD8α Ab, anti-B220 Ab, anti-DX5 Ab, anti-MAC-1 Ab, anti-CD11c Ab and anti-TER119 Ab. The cells were then incubated with magnetic beads coupled with streptavidin and negatively sorted on autoMACS (miltenyibiotec). The negative fraction was treated with biotinylated anti-CD62L Ab and then streptavidin-magnetic beads again, followed by positive sorting by autoMACS. The purity of $CD44^{-}CD62L^{+}CD4^{+}$ T cells (naive

CD4-positive T cells) was >90%. When $CD4^+CD62L^+CD25^-$ T cells were especially required, we further negatively sorted $CD4^+CD25^+$ T cells using anti-CD25 Ab, magnetic beads coupled with streptavidin, and autoMACS.

Differentiation into Th1 and Th17 cells

1x106 cells were cultured for three to four days in RPMI1640 containing 10% FCS with plate-bound anti-CD3ε Ab (10μg/ml) and soluble anti-CD28 Ab (1μg/ml). For Th1 or Th17 cell differentiation, IL-12 (10ng/ml), or IL-6 (30ng/ml), TGF-β (3ng/ml), IL-21 (100ng/ml) and IL-23 (10ng/ml) were added, respectively.

Intracellular cytokine staining

Cultured cells were stimulated by PMA (50ng/ml) and ionomycin (1μg/ml) for two hours followed by brefeldin A (5μg/ml) treatment for another two hours. Cytokine staining was performed using Intra Prep (Beckman Coulter) following manufacture's instruction. For Foxp3 staining, Foxp3 Staining Buffer Set (eBioscience) was used. Cells were stained by FITC-labeled anti-IFN-γ Ab (2.5μg/ml) and PE-labeled anti-IL-17A Ab (1μg/ml) and analyzed by FACS. In another experiment, cells were stained by FITC-labeled anti-IL-17A Ab (1μg/ml), Alexa-Fluor-labeled anti-IL-17F Ab (1μg/ml), and PE-labeled anti-Foxp3 Ab (1μg/ml).

ELISA

Supernatant of cultured medium was harvested and the concentration of each cytokine was analyzed by Quantikine Mouse IFN-γ and Mouse IL-17 (R&D systems).

Western blotting

Cell lysates at 0, 30, and 90 minutes after stimulation for Th17 cell differentiation were separated by SDS-PAGE and transferred to PVDF membrane. After the treatment by TBS-T containing 5% skimmed milk, membrane was treated with primary Abs: rabbit anti-pAkt Ab, rabbit anti-Akt Ab, rabbit anti-pTyr^{705} STAT3 Ab, rabbit anti-pSer^{727} STAT3 Ab, rabbit anti-STAT3 Ab, mouse anti-pERK2 Ab, rabbit anti-ERK2 Ab, and rabbit anti-c-Fos Ab. After washed by TBS-T containing 5% skimmed milk, membrane was further treated by secondary Ab (HRP-conjugated anti-rabbit-IgG Ab or HRP-conjugated anti-mouse-IgG Ab). Each band was detected by LAS3000 (Fuji) with an ECL detection kit. For the detection of pAkt, Akt, and c-Fos, ECL advance (GE Healthscience) was adopted.

RT-PCR

Total RNA at 0, 24, 36, and 48 hours after stimulation for Th17 cell differentiation was extracted, and cDNA was synthesized. For PCR reaction, the following probes were used: RORγt, 5'-ACC TCC ACT GCC AGC TGT GTG CTG TC-3' and 5'-TCA TTT CTG CAC TTC TGC ATG TAG ACT GTC CC-3'; Foxp3, 5'-CAC CCA GGA AAG ACA GCA ACC-3' and 5'-GCA AGA GCT CTT GTC CAT TGA-3': IL-17F, 5'-CAG CAG CGA TCA TCC CTC AAA G-3' and 5'-CAG GAC CAG GAT CTC TTC CTG-3'.

Results

$CD4^+CD62L^+$ T cells from KO mice showed impaired ability for Th17 differentiation.

In order to compare the abilities of naive CD4-positive T cells from KO and HT mice to differentiate into Th17 cells, I first sorted $CD4^+CD62L^+$ T cells from spleens of these mice, and the cells were cultured in both Th1- and Th17-cell conditions. After three days, cells were harvested, stained for IFN-γ and IL-17, and analysed using FACS. In the FACS analysis, $CD4^+CD62L^+$ T cells from KO mouse showed a decreased rate of Th17 cell differentiation, which was <50% of the one marked by the $CD4^+CD62L^+$ T cells from HT mouse (Figure 3A).

Next I examined the concentration of secreted IFN-γ and IL-17 in the culture medium on Day 3 after stimulation using ELISA. In Th1-cell condition, the concentrations of IL-17 in the culture medium were strikingly low both in the cells from KO and HT mice, while high amount of IFN-γ secretion was marked in both, however, little difference in the concentration of secreted IFN-γ was detected between the cells from KO and HT mice (Figure 4A). In the Th17-cell condition, low IFN-γ concentration and high IL-17 concentration in both culture medium was detected as expected, and IL-17 concentration in the medium containing cells from KO mouse was nearly only the one-third of that from HT mouse (Figure 4B), which corresponds to the result that Th17 cell differentiation is suppressed in $CD4^+CD62L^+$ T cells sorted from KO mice.

No difference was detected in the phosphorylation level of STAT3 between $CD4^+CD62L^+$ T cells from KO mice and HT mice.

Given the result that p85α depletion impaired Th17 cell differentiation, I next investigated the phosphorylation level of STAT3 at Tyr705 and Ser727 because previous studies had shown that the activation of mTOR and GSK3 in the downstream of PI3K and Akt had increased the phosphorylation level of STAT3 (Kusaba et al., 2004; Baurel and Jope, 2008). I sorted $CD4^+CD62L^+$ T cells from spleens of both KO and HT mice, and then stimulation for Th17 cell differentiation was added to both. At 0, 30, and 90 minutes after stimulation, cell lysates were harvested and examined the amount of proteins listed in Figure 5 by western blotting.

As a result, I ascertained the phosphorylation of Akt was strongly inhibited in $CD4^+CD62L^+$ T cells from KO mouse; however, there were no differences between $CD4^+CD62L^+$ T cells from KO and HT mice in the phosphorylation level of STAT3 at both Tyr705 and Ser727. Therefore, impaired differentiation of Th17 cells in $CD4^+CD62L^+$ T cells from KO mice was not due to a decreased phosphorylation level of STAT3 in cytosol.

On the other hand, I could detect that the phosphorylation level of Erk was slightly suppressed in $CD4^+CD62L^+$ T cells from KO mouse, in which the expression level of c-Fos was also largely decreased.

$CD4^+CD62L^+$ T cells from KO mouse rather showed RORγt-dominant mRNA expression pattern.

Given the results of recent studies that Foxp3 directly inhibits RORγt and that the balance between RORγt and Foxp3 is important in the differentiation of both Th17 and iTreg cells (Zhou *et al*., 2008; Ichiyama *et al*., 2008; Yang *et al*., 2008), I next performed RT-PCR analysis for RORγt, Foxp3, IL-17A, and IL-17F mRNA expressions in $CD4^+CD62L^+$ T cells from KO and HT mice. $CD4^+CD62L^+$ T cells were sorted and whole mRNA was harvested at the point of 0, 24, 48, and 72 hours after the stimulation for Th17 cell polarization. Surprisingly, $CD4^+CD62L^+$ T cells from KO mouse revealed lower Foxp3 mRNA level and elevated RORγt mRNA level compared to the counterparts from HT mouse (Figure 6). Therefore, from the present our understandings of Th17 cell differentiation, $CD4^+CD62L^+$ T cells from KO mouse themselves have rather a tendency to differentiate into Th17 cells, with their RORγt/Foxp3 balance shifted strongly toward a RORγt-dominant state.

On the other hand, the amount of IL-17A and IL-17F mRNA expression were both lower in $CD4^+CD62L^+$ T cells from KO mouse than these cells from HT mouse; therefore, these results strongly implies the presence of other mechanisms controlling the differentiation of Th17 cells besides RORγt/Foxp3 balance.

Little difference in the ability for Th17 differentiation was detected between

$CD4^+CD62L^+CD25^-$ T cells from KO mice and HT mice.

There is 10 to 20% of $CD4^+CD25^+$ naturally occurring regulatory T cells (nTregs) in the population of $CD4^+CD62L^+$ T cells, hence I negatively sorted the $CD4^+CD25^+$ T cell population from $CD4^+CD62L^+$ T cells both from KO and HT mice and differentiated these newly obtained $CD4^+CD62L^+CD25^-$ T cells into Th17 cells. Three days later, cultured cells were stained for IL-17A, IL-17F, and Foxp3 and compared by FACS analysis (Figure 3B).

Because nTregs were depleted from the cultured population, twofold increase in the amount of IL-17A expressing cells was detected in the cultured cell population from HT mouse as expected, and surprisingly, the difference in the rate of Th17 cell differentiation between KO and HT seen in $CD4^+CD62L^+$ cells vanished. Since our laboratory previously observed that there was little difference in the amount of $CD4^+CD25^+$ T cell population between KO and HT mice (data not shown), it can be assumed that the ability of nTregs in KO mice to suppress Th17 cell differentiation would have been elevated due to the p85α depletion.

Discussion

In this experiment, impaired STAT3 phosphorylation during Th17 cell differentiation due to p85α depletion could not be detected, although there are many molecular biological studies concluding that mTOR and GSK3 in the downstream of PI3K and Akt

could increase the phosphorylation level of STAT3 (Kusaba et al., 2004; Baurel and Jope, 2008). Hence, the impaired Th17 cell differentiation in KO mouse couldn't be explained by an alteration in phosphorylation level of STAT3. Instead, I could detect a slight decrease in pErk level and a large drop in c-Fos expression level; hence, now it can be assumed that the amount of transcriptional factor AP-1, which has also important roles in immune system, would be lower in KO mice during Th17 cell differentiation. New phenotype in Th17 cell differentiation and in chronic inflammation could be observed with a c-Fos-depleted mouse.

In another study, it was reported that weaker TCR signaling and inhibition of PI3K/Akt pathway resulted in elevated Foxp3 mRNA expression and increased differentiation of iTregs (Sauer *et al*., 2008). Actually, I could get the same result in iTreg differentiation; however, when it comes to Th17 cell differentiation, depletion of p85α contrary decreased the level of Foxp3 mRNA expression and elevated the level of RORγt mRNA expression. Hence, the mechanism of impaired Th17 cell differentiation after p85α depletion also can not be explained by impaired expression of RORγt.

For an explanation of this impairment, the existence of nTregs in the cultured population and their interaction with naive CD4-positive T cells now seem to be important. In this study, the effect of p85α depletion on Th17 cell differentiation vanished when $CD4^+CD25^+$ T cells (nTregs) were removed from the cultured population, implying the importance of PI3K signaling in the function of nTregs. The

roles of PI3K in the differentiation and function of both Th17 cells and nTregs have not been investigated well, although it is a well-known fact that PI3K has a lot of roles in immune cells. The suppressive function of nTregs on effective Th cells is mainly mediated by the expression of CTLA4 and the secretion of IL-10 (Jonuleit *et al*., 2001; Wing *et al*., 2008), therefore the ability of KO mice to express CTLA4 and to produce IL-10 should be examined as a next step, although there is an ambivalent report that p110$\delta^{-/-}$ mice revealed an impaired ability in IL-10 secretion during IL-2 stimulation (Patton *et al*., 2006). There is also another possibility that naive CD4-positive T cells from KO mice are more susceptive to the suppressive function of nTregs. Hence, the susceptivity of naive CD4-positive T cells from KO and HT mice to CTLA4 and IL-10 expressed by nTregs should also be compared. What is more, our laboratory's data suggest that depletion of p85α shows little difference in Th1 and Th2 differentiation; hence, the distinctive role of nTregs on the differentiation of Th17 cells must also investigated.

This nTreg's particular suppression of Th17 cell differentiation and proliferation seems to have a clinical significance. Now, the treatment of chronic inflammatory disorders is highly dependent on the use of immunosuppressants such as corticosteroids and methotrexate. These regimens frequently result in compromised immunities. Recently, antibody drugs such as anti-TNFα antibody (infliximab) have been developed as more potent and side-effect-free regimens, however, they are still very expensive.

Now that it is widely accepted that extreme activation of Th17 cells is important in the pathogenesis of these chronic inflammatory disorders and nTregs are necessary to alleviate these long-lasting immune reactions, targeting PI3K/Akt pathway to modulate the balance between nTreg function and Th17 cell differentiation could serve as a strong or an alternative regimen in the future.

Conclusion

Depletion of p85α resulted in an impaired ability for Th17 cell differentiation from naive CD4-positive T cells, and this was observed only when nTregs were co-cultured with naive CD4-positive T cells. From the results of previous studies and this experiment, it could be assumed that this impairment in Th17 cell differentiation would be achieved by either or both of following two mechanisms: an elevated function of nTregs to suppress the differentiation and proliferation of Th17 cells, and an elevated susceptivity of Th17 cells to these nTreg functions.

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Figures

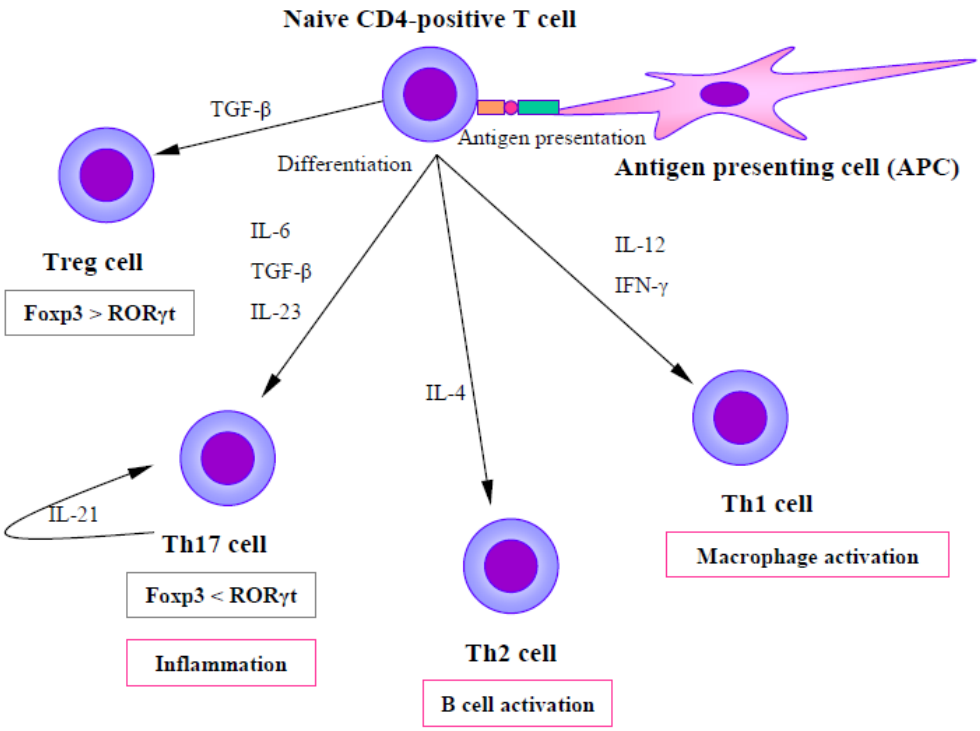

Figure 1 | Antigen presentation and differentiation of Th cells.

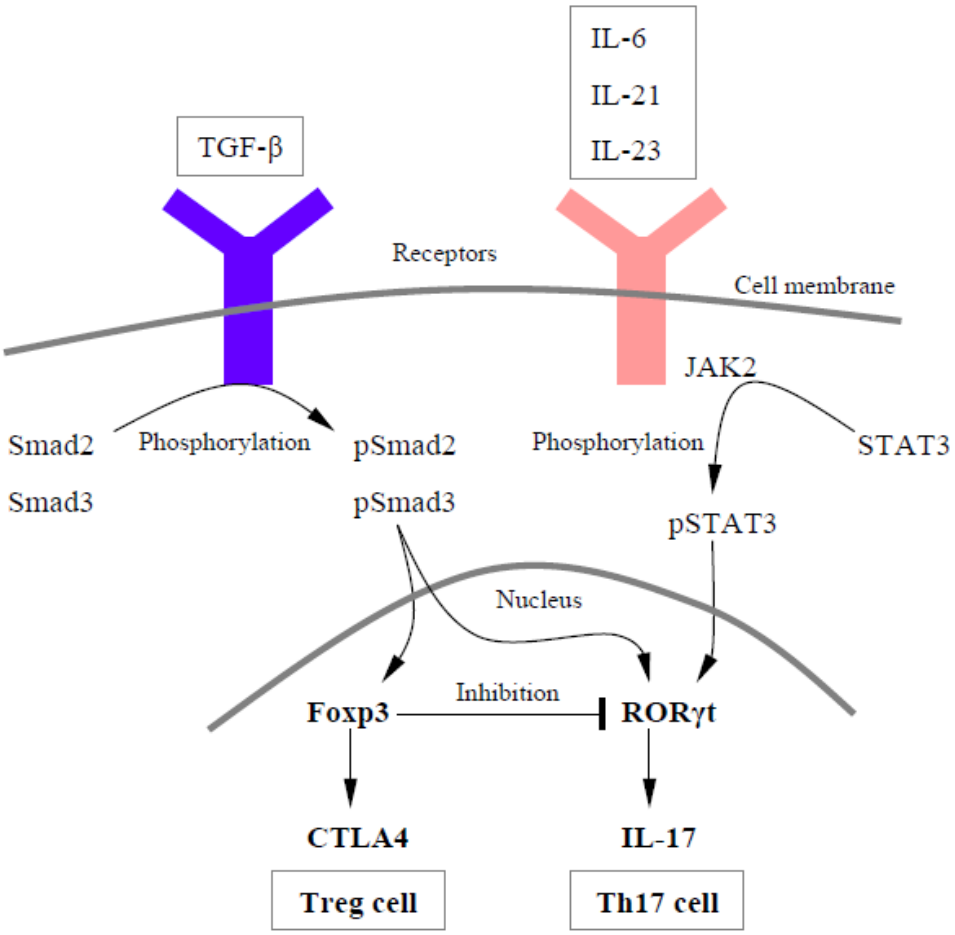

Figure 2 | Intracellular signaling cascades in the down stream of cytokine receptors.

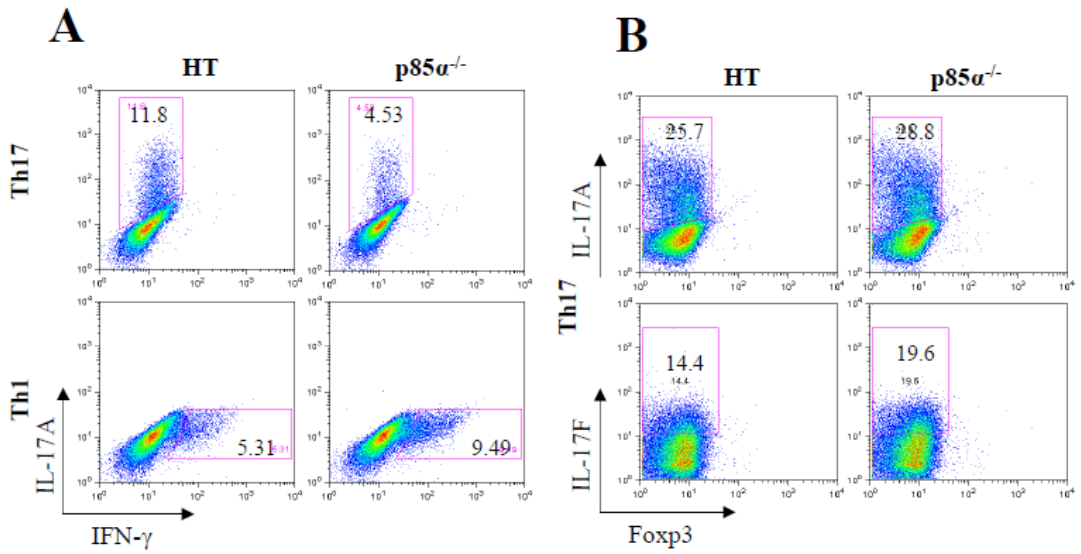

Figure 3 | Results of FACS analysis. (A) Impaired Th17 cell differentiation in $CD4^+CD62L^+$ T cells of KO mouse. $CD4^+CD62L^+$ T cells from KO and HT mice were sorted and differentiated into Th1 and Th17 cells. After three days, cells were stained for IL-17A and IFN-γ. (B) No impairment in Th17 cell differentiation was detected when $CD4^+CD62L^+CD25^-$ T cells from KO and HT mice were compared. $CD4^+CD62L^+CD25^-$ T cells from KO and HT mice were sorted and differentiated into Th17 cells. After three days of culture, they were stained for Foxp3 and IL-17A or IL-17F.

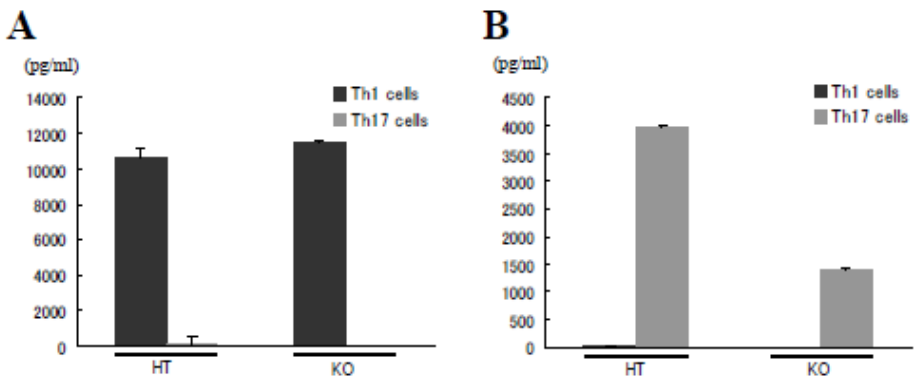

Figure 4 | Results of ELISA. (A) Little difference in IFN-γ secretion from Th1 cells differentiated from $CD4^{+}CD62L^{+}$ T cells of KO and HT mice. (B) Impairment in IL-17 secretion from Th17 cells differentiated from $CD4^{+}CD62L^{+}$ T cells of KO mouse.

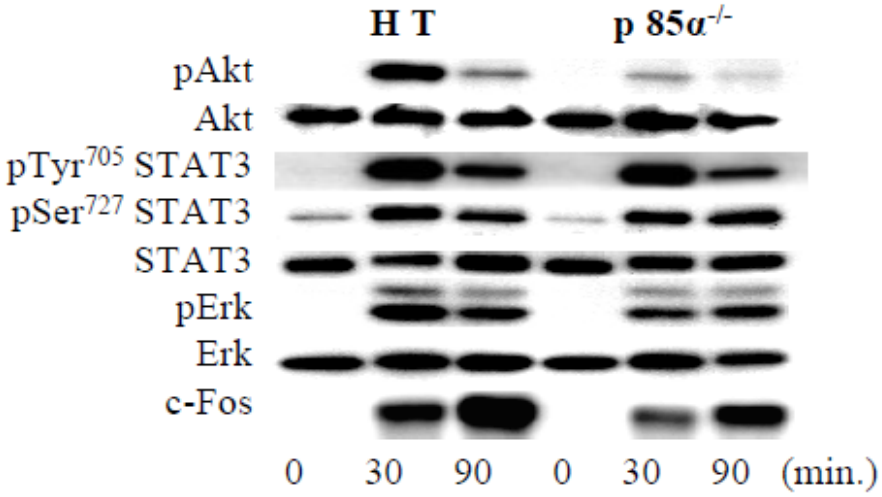

Figure 5 | Results of western blotting. The phosphorylation level of Akt was strongly suppressed in $CD4^{+}CD62L^{+}$ T cells from KO; however, there ware no differences in the phosphorylation level of STAT3 at both Tyr705 and Ser727 between these cells from KO and HT.

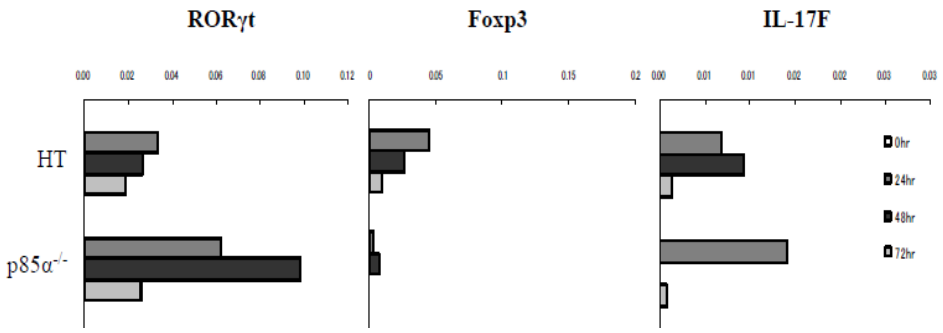

Figure 6 | Results of RT-PCR. The Foxp3/RORγt balance in the $CD4^{+}CD62L^{+}$ T cells from KO was rather RORγt-dominant.