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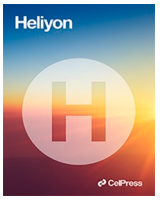

Research article

Characteristic features of *in vitro* differentiation of human naïve CD4$^+$ T cells to induced regulatory T cells (iTreg) and T helper (Th) 17 cells: Sharing of lineage-specific markers

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ABSTRACT

In vitro induced regulatory T cells (iTreg) and IL-17 producing T cells (Th17-like cells) can be generated in culture from native CD4$^+$ T cells in peripheral blood by different sets of cytokines. In the presence of transforming growth factor (TGF)-β plus interleukin (IL)-2, cells differentiate into Treg cells with increased expression of the forkhead box P3 (FOXP3). In the presence of TGF-β, IL-6, IL-1β and IL-23, cells differentiate into Th17 cells that produce IL-17A. However, protocols for the generation of human iTreg and Th17 are still controversial. In this study, we characterized the biological features of iTreg and Th17 cells differentiated from peripheral blood naïve CD4$^+$ T cells in vitro using the established protocols. We showed that cells obtained from Treg or Th17 culture conditions shared some phenotypic markers. Cells under Treg conditions had an up-regulated *FOXP3* gene and a down-regulated RAR-related orphan receptor C (*RORC*) gene. Cells derived from the Th17 condition exhibited a down-regulated *FOXP3* gene and had significantly higher *RORC* gene expression than Treg cells. Both resulting cells showed intracellular production of IL-17A and IL-10. Th17 condition-cultured cells exhibited more glycolytic activity and glucose uptake compared to the Treg cells. The findings suggest that cells obtained from established protocols for the differentiation of iTreg and Th17 cells in vitro are possibly in the intermediate stage of differentiation or may be two different types of cells that share a lineage-specific differentiation program.

1. Introduction

CD4$^+$ T cells are a key player in the adaptive immune system, since they can differentiate into various subsets of cells with specialized effector functions or suppressive functions [1]. Differentiation of different populations depends on specific cytokine signaling coupled with microenvironmental stimuli and activation of lineage-specific transcription factors. Despite their differences in functions, regulatory T cells (Treg) and T helper 17 cells (Th17) have a reciprocal developmental pathway and display a high grade of plasticity [2,3]. The balance between these two cell types is important to maintain physiological condition during an immune response. Specifically targeting molecules involved in the differentiation and function of these two cell types is significant for future therapeutic approach in diseases in which their dysregulations are involved, including autoimmune diseases, allergies, and cancer. To study the

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molecular mechanisms underlying the differentiation of these cells, a large number of cells is required. In humans, there is a limited number of naturally occurring Tregs and Th17 in the peripheral blood [4,5]. The generation of cell populations in vitro can be useful for functional assays and molecular studies, in addition to the evaluation of the effect of certain compounds on these cells.

In vitro-induced regulatory T cells (iTreg) and IL-17-producing T cells (Th17-like cells) can be generated in culture from naive human $CD4^+$ T cells in peripheral blood by different combinations of cytokines. Specifically, when cells are exposed to TGF-β and IL-2, they undergo differentiation into FOXP3-positive cells [6]. FOXP3 is a lineage specification factor for Tregs and relative to their suppressive functions [7]. Under the condition with TGF-β, IL-6, IL-1β and IL-23, naïve $CD4^+$ T cells differentiate to IL-17A-producing T cells with a positive retinoic acid-receptor (RAR)-related orphan receptor (ROR) gamma t (RORγt), a key molecule for Th17 differentiation [8,9]. Therefore, TGF-β seems to be essential for the differentiation of Treg and Th17 cells. TGF-β, in combination with IL-2, is necessary to induce the differentiation of $CD4^+$ T cells into Treg cells. Conversely, when pro-inflammatory cytokines, IL-6, IL-1β, and IL-23 are present, they promote the differentiation of Th17 cells by inducing the expression of RORγt [10,11]. Until now, there are various protocols for the generation of human iTreg and Th17 from peripheral $CD4^+$ T cells, which vary in terms of cytokine concentrations and the duration of cell culture, and are currently subject to debate. Given the substantial plasticity between the differentiation of Treg and Th17, together with the fact that $FOXP3^+$ Treg can produce IL-17A under certain conditions [12,13], and conversely, Th17 can express FOXP3 when exposed to specific stimuli 13, the generation of these cells of $CD4^+$ T cells in peripheral blood can produce indistinct types of cells that do not clearly align with the characteristics of Treg or Th17 cells.

This study was carried out to investigate the distinctive biological characteristics of iTreg and Th17 cells that are generated from peripheral blood naïve $CD4^+$ T cells by in vitro differentiation using established culture protocols. The principal objective was to validate that these established protocols produce the desired cell types suitable for subsequent applications.

2. Materials and methods

2.1. Human T cell isolations

Human peripheral blood mononuclear cells (PBMCs) were freshly isolated from a healthy donor buffy coat derived from the Siriraj Blood Donation Center. The PBMCs were separated by the density gradient centrifugation method using Histopaque-1077 (Sigma-Aldrich, MA, USA). PBMCs were stored overnight at 37 °C in RPMI medium (HyClone™, UT, USA) containing 10 % FBS (Hyclone™, Pasching, Austria). Naïve $CD4^+$ T cells were separated by immunomagnetic-negative selection using the human Naïve $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Gladbach, Germany) with cell separation column according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). The purity of more than 95 % of naïve $CD4^+$T cells was obtained, as confirmed by immunostaining with APC-Cy7-conjugated anti-CD45RA and APC-conjugated anti-CD4 (BD Biosciences, Heidelberg, Germany).

2.2. Cell culture and differentiation

After naïve T cell isolation, cells were rested overnight and then plated in a 96-well plate at a density of 2.5×10^5 cells/ml in 200 μl of TexMACS Medium (Miltenyi Biotec, Gladbach, Germany). Cells were cultured with the Treg differentiation condition or the Th17 differentiation condition. For the Treg condition, cells were cultured as previously described with some modification [14]. Cells were supplemented with soluble anti-CD3 (5 μg/ml), anti-CD28 (1 μg/ml), IL-2 (100 U/ml) and TGF- β1 (5 ng/ml) (all from Miltenyi Biotec, Gladbach, Germany). For the Th17 condition, cells were supplemented with soluble anti-CD3 (5 μg/ml), anti-CD28 (1 μg/ml), TGF-β1 (5 ng/ml), IL-6 (25 ng/ml), IL-1β (10 ng/ml) and IL-23 (25 ng/ml) (all from Miltenyi Biotec, Gladbach, Germany). Cells supplemented with 100 U/ml of IL-2 were used as control. Cells were incubated for 6 days with media replacement on day 4. After culture for 6 days; Th17 cells were restimulated with anti-CD3 (5 μg/ml) for 24 h, Treg cells were rested for 3 days in TexMACS medium supplemented with IL-2 (50 U/ml) before restimulation with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) for 4 days. The optimal dose for each cytokine and antibody was used on the basis of the dose titration in preliminary experiments.

2.3. Cell viability and proliferation

Cell viability was determined using trypan blue staining and counted with a hemocytometer under light microscope. Cell viability was also determined by flow cytometry using live/dead staining with BD Horizon™ Fixable Viability Stain 780 (BD Biosciences, Heidelberg, Germany).

2.4. Flow cytometric analysis

Cells were harvested after culture and restimulated with PMA (10 ng/ml) and ionomycin (375 ng/ml) in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich, MA, USA) for 4 h before flow cytometry. Cells were stained with APC-conjugated anti-CD4, FITC-conjugated anti-CD25, APC-Cy7-conjugated anti-CD45RA, PE-cy7-conjugated anti-CD127, PE-conjugated anti-IL-17A, PE-Cy7-conjugated anti-IL10, PerCP-cy5.5-conjugated anti-FOXP3 and FITC-conjugated anti-RORγt (all from BD Biosciences, Heidelberg, Germany). Fluorescence Minus One (FMO) controls were performed to establish the appropriate gates for multicolor staining. The FMO controls were performed by staining cells of interest with all fluorochromes in a panel minus the one measured in the flow cytometry experiment. All staining performed included a Live/Dead marker (BD Biosciences, Heidelberg, Germany) to exclude false positive staining. Flow cytometric analysis was performed with the FACSLyric instrument (BD Biosciences, Heidelberg, Germany)

instrument. FACS data was analyzed using FlowJo version 10.0 (BD Biosciences, Heidelberg, Germany).

2.5. Glucose uptake assay

Cells from each condition were washed with PBS before seeding in a 96-well plate at a density of 5×10^4 cells in 100 μl of media and starved in glucose-free medium overnight. The next day, the cell culture supernatants were assayed for glucose uptake using the

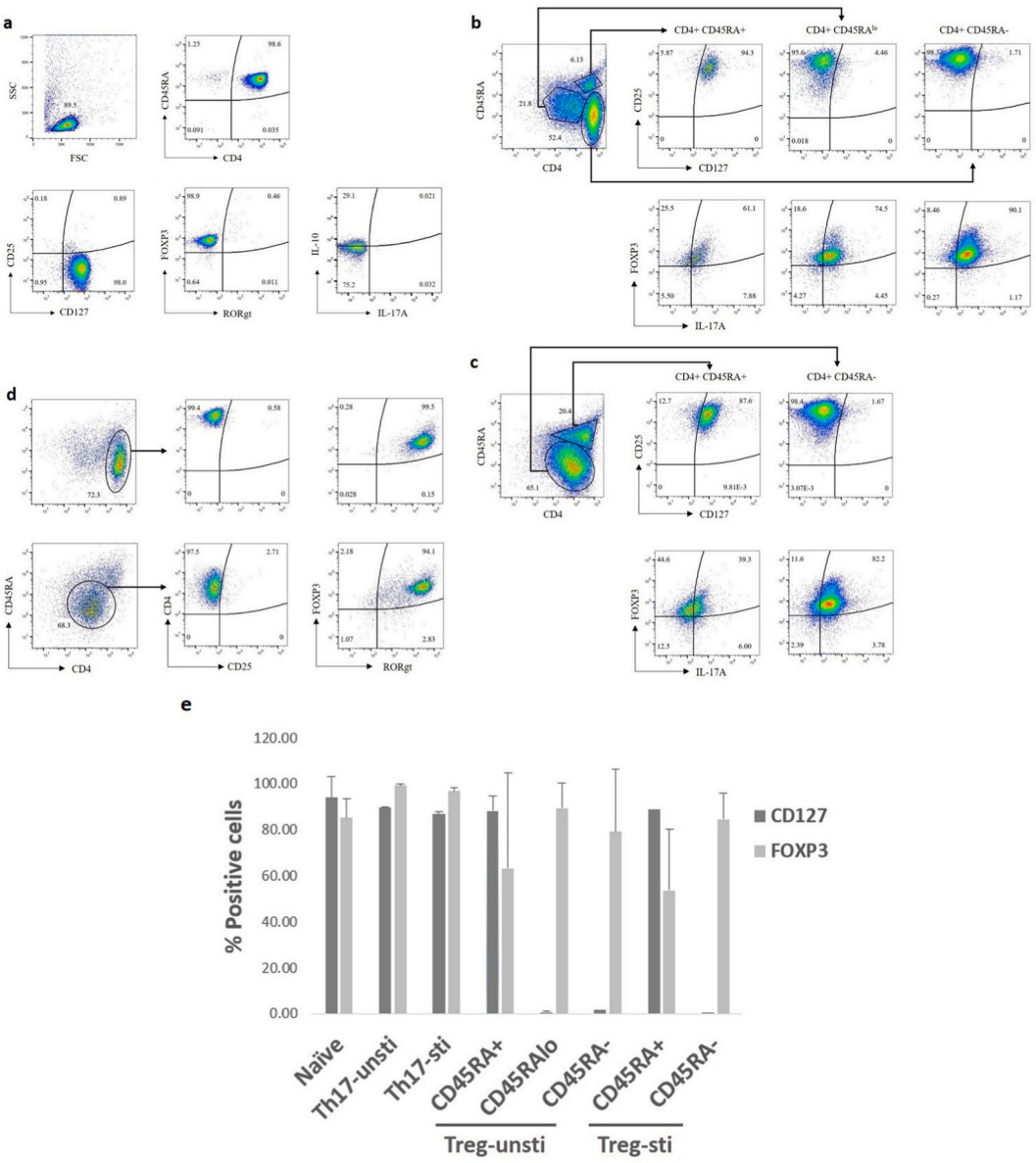

Fig. 1. Surface and intracellular marker expression of naïve T cells (a), Treg cells (b–c), and Th17 cells (d) by flow cytometry. Human naïve $CD4^+$ T cells were separated from PBMCs by negative selection and stained for specified markers. **a)** The lymphocyte population was identified using FSC vs. SSC plots. Naïve $CD4^+$ T cells were identified as $CD4^+CD45RA^+$ cells. Gated in $CD4^+CD45RA^+$ cells, flow cytometric expressions of CD25, CD127, FOXP3, RORγt, intracellular IL-17A, and IL-10 were illustrated. **b-c)** Naïve $CD4^+$ T cells were cultured in the presence of TGF-β and IL-2 with anti-CD3/-CD28 antibodies for 6 days in serum-free medium and further incubated with anti-CD3/-CD28 antibodies for 4 days (unstimulated). Then cells were stimulated with PMA/ionomycin for 4 h (stimulated). Unstimulated cells (d) were gated according to CD45RA expression in $CD45RA^+$, $CD45RA^{lo}$ and $CD45RA^-$ cells (left to right panel). Stimulated cells (e) were gated according to CD45RA expression in CD45RA and $CD45RA^-$ cells (left to right panel). **d)** Naïve $CD4^+$ T cells were cultured in the presence of TGF-β, IL-1β, IL-6 and IL-23 with anti-CD3/-CD28 antibodies for 6 days in serum-free medium and further incubated with anti-CD3 antibodies for 24 h (upper panel). Then cells were stimulated with PMA/ionomycin for 4 h (lower panel). **e)** Compilation of the percentage of CD127 and FOXP3 positive cells from the cultures described in **a-d**. The results demonstrated mean ± SD of three biological replicates.

glucose uptake colorimetric assay kit (Abcam, Cambridge, United Kingdom) according to the manufacturers instructions. Briefly, cells were washed with PBS before preincubation with KRPH/2 % BSA buffer (20 mM HEPES, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, pH 7.4). A 10-mM 2-Deoxyglucose (2-DG) was added to activate cells from each condition and control cells were left untreated to show background. Cells were lysed and proceeded to a freeze/thaw cycle to promote endogenous degradation of NAD(P). The reaction mix was then added and the output was read at OD 412 nm using a microplate reader (EPOCH, BioTek, CA, USA) in kinetic mode according to the standard curve.

2.6. Glycolysis and oxidative phosphorylation assay

The cells were seeded in a 96-well plate at a density of 1×10^4 cells in 100 μl of medium and cultured overnight at 37 °C. The next day, the culture medium was removed and cells were transferred to the glycolysis/OxPHOS test kit (DOJINDO Laboratories, Kumamoto, Japan) according to the manufacturers instructions. In brief, 1.25 μmol/l of oligomycin solution or 22.5 μmol/l of 2-DG were added to the indicated wells. Untreated cells were used as a control. After 5 h of incubation, the supernatant of each condition was

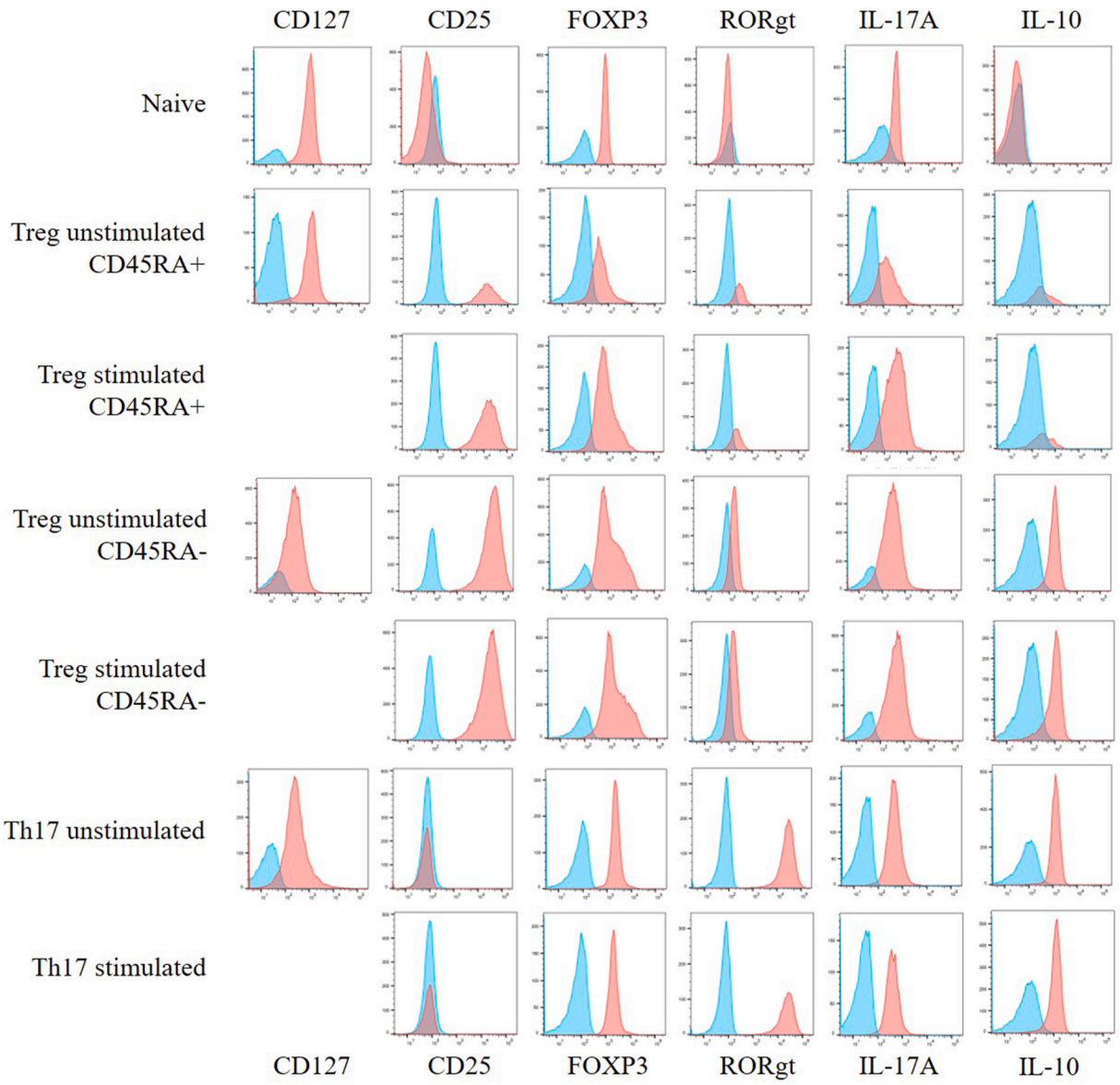

Fig. 2. Histograms of CD127, CD25, FOXP3, RORγt, IL-17A and IL-10 expression on naïve $CD4^+$ T cells, $CD4^+CD45RA^+$ and $CD4^+CD45RA^-$ populations of unstimulated and stimulated Treg, as well as unstimulated and stimulated Th17. The blue peaks represent fluorescence minus one (FMO) unstained control, and the red peaks represent antibody staining of specific markers. The results were representative of at least three biological replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

harvested to measure the amount of lactate released by the absorbance method using water-soluble tetrazolium formazan and the concentration of intracellular ATP by the luciferase luminescence method. The absorbance at 450 nm and the luminescence were measured using a microplate reader (EPOCH, BioTek, CA, USA).

2.7. RNA isolation and real-time quantitative reverse transcription PCR (qRT-PCR)

Cultured T cells from each condition were recovered, lysed, and RNA was extracted with TRIzol (Invitrogen, MA, USA). For each sample, a total of 500 ng of RNA was used for the RT-PCR assay using the SuperScript™ III Platinum™ One-Step qRT-PCR kit (Invitrogen, MA, USA). The amounts of *FOXP3* and *RORC* were detected using real-time PCR (LightCycler® 480 Instrument II, Roche, Basel, Switzerland). The primers and TaqMan probe were supplied by Applied Biosystems: *RORC* (Hs01076112_m1), *FOXP3* (Hs01085834_m1) and a housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (HS99999905_m1) as internal control.

2.8. Statistical analysis

Data are represented as mean ± standard error of the mean (SEM). Comparisons between experimental groups were analyzed using the Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Surface and intracellular marker expression in naïve cells

Naïve $CD4^+$ T lymphocytes were isolated from PBMCs by negative selection. This yielded naïve T cells at a purity of more than 95 %, as identified by positive expression of CD4 and CD45RA in the cell population from the FSC vs. SSC plot (Fig. 1a). Naïve T cells were positive for CD127, FOXP3, and IL-10, and negative for IL-17A and RORγt (Fig. 1a).

3.2. Phenotypes of cells in Treg condition

After culture with the Treg differentiation protocol, cells were separated into 3 populations according to CD45RA expression (Fig. 1b). Cells with negative (52.4 %) and low (21.8 %) CD45RA showed high expression of CD25 and negative for CD127, compared to cells with positive CD45RA (6.13 %), which demonstrated positive expression of CD127. Then, $CD4^+CD45RA^-CD25^+CD127^-$ cells were considered to be the iTreg population. When examining the expression of FOXP3 and RORγt, the iTreg population exhibited dual positive expression of FOXP3 and RORγt. After stimulation with PMA plus ionomycin, this population expanded and had higher expression of FOXP3 (Figs. 1c and 2). Surprisingly, after evaluating intracellular expression of IL-17A and IL-10, the iTreg population demonstrated positivity for both IL-17A and IL-10 (Fig. 2). In contrast, the $CD4^+CD45RA^+$ population exhibited lower IL-10 expression, indicating a compromised capacity for IL-10 production.

3.3. Phenotypes of cells under Th17 condition

Cells cultured under Th17 conditions were positive for CD45RA (Fig. 1d). We found double positive expression of FOXP3 and RORγt in unstimulated cells (Fig. 1d upper panel). These cells had negative expression for CD25 molecules (Fig. 2). After stimulation

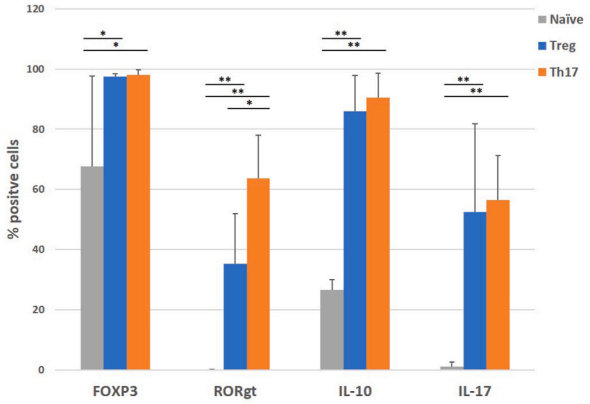

Fig. 3. Compilation of the percentage of positive cells from cultures described in Fig. 2. The percentages of positive cells for FOXP3, RORγt, IL-10 and IL-17A were compared in naïve, unstimulated Treg and unstimulated Th17 cells. The result demonstrated mean ± SD of at least 3 biological replicates. *P*-values were analyzed using Student's *t*-test. * indicates $p < 0.05$; ** indicates $p < 0.01$.

with PMA plus ionomycin, cells expressed a similar level of expression of RORγt and FOXP3 (Fig. 1d lower panel and Fig. 2). The resulting Th17 cells expressed significantly higher RORγt than iTreg cells (Fig. 3). When comparing IL-17A and IL-10 expression, Th17 cells demonstrated expression levels of both IL-17A and IL-10 comparable to those of iTreg cells (Figs. 2 and 3).

3.4. Glucose uptake and intracellular metabolism

Glucose uptake of cells was measured by a glucose uptake assay. The results showed that Th17 and Treg cells treated with 2-DG had elevated glucose uptake compared with naïve cells that had an uptake capacity equivalent to baseline (Fig. 4a). At the endpoint, Th17 cells showed a trend of higher glucose uptake levels compared to Treg cells, although this difference was not statistically significant. Intracellular metabolisms of cells were evaluated by assessing glycolytic capacity and oxidative phosphorylation (OXPHOS) employing the glycolysis/OXPHOS test kit. This assay measured the concentration of intracellular ATP production and lactate release from cells, enabling the evaluation of whether cells depend on the glycolytic pathway or OXPHOS for energy production. The result demonstrated mean of 3 independent experiments for naïve cells. For Th17 and Treg condition, the results showed value from 1 experiment. In the presence of 2-DG or oligomycin, ATP production in Th17 and Treg decreased compared to untreated cells (Fig. 4b), suggesting that the metabolisms of Th17 and Treg cells depend on both the glycolytic pathway and OXPHOS. Regarding lactate production, the absorbance of the lactate amount that we could measure was overall low in all cell types (Fig. 4c). The results showed that in the presence of oligomycin, lactate production in Th17 cells increased compared to untreated cells. This suggests that Th17 cells have more glycolytic reserve than Treg.

3.5. RNA expression

Quantitative real-time RT-PCR was performed to detect the expression of *FOXP3* and *RORC* genes in cells from each condition. We found that naïve T cells seemed to express both genes at a low level. Using naïve cells as control, Treg exhibited the upregulation of

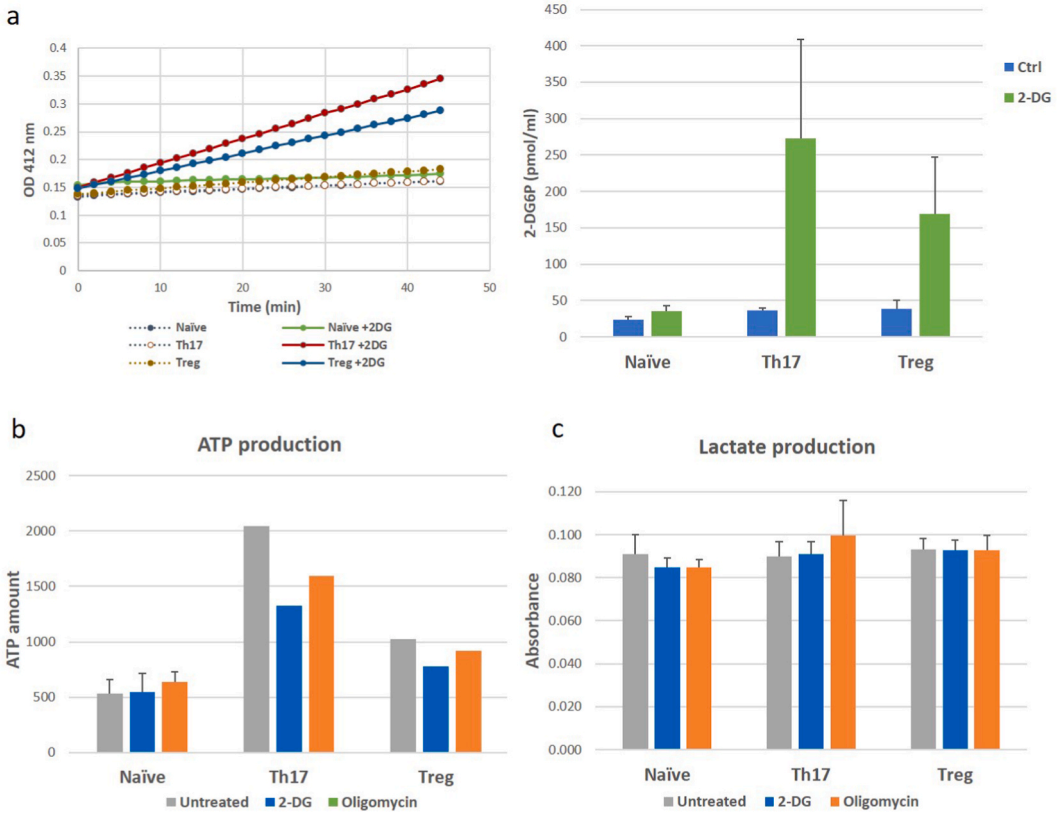

Fig. 4. Metabolisms of Treg and Th17 cells compared with naïve T cells. **a)** The glucose uptake of cells was measured by a glucose uptake calorimetric assay. The kinetic and end-point levels of glucose uptake at the absorbance OD 412 nm were illustrated. Ctrl = cells left untreated to show background. **b)** Intracellular energy production was evaluated by measuring the amount of ATP produced by cells in the presence of 2-DG or oligomycin. Untreated cells were used as control. For naïve cells, the result demonstrated mean ±SEM of three biological replicates. For Th17 and Treg condition, the results showed value of n = 1. **c)** Glycolysis activity was evaluated by measuring the amount of lactate produced by cells in the presence of 2-DG or oligomycin. The result demonstrated mean ± SEM of three biological replicates. *P*-values were analyzed using Student's *t*-test. 2-DG = 2-deoxyglucose.

FOXP3 and downregulation of *RORC* (Fig. 5). On the contrary, *FOXP3* was down-regulated in Th17 cells. *RORC* expression in Th17 cells was also down-regulated, but the level was significantly higher than that in Treg cells.

4. Discussion

In this study we demonstrated that naïve $CD4^+$ T cells cultured under Treg condition (TGF-β plus IL-2 with anti-CD3 and anti-CD28 stimulation) showed Treg phenotypic markers in peripheral blood, which is the combination of $CD25^+CD127^{lo}FOXP3^+$ [,15,16]. Furthermore, naïve $CD4^+$ T cells cultured under Th17 condition (TGF-β plus IL-1β, IL-6 and IL-23 with anti-CD3 and anti-CD28 stimulation) showed phenotypic markers of Th17, which include IL-17A$^+$ and RORγt$^+$ [9,17]. From this perspective, it is plausible to assume that we are obtaining the correct cell populations from the culture. In our experiments, we observed that when we simultaneously examined cells for markers associated with Treg and Th17, cells cultured under Treg or Th17 conditions displayed coexpressions of FOXP3 and IL-17A.

Of note, naïve $CD4^+$ T cells isolated from PBMCs also showed positive FOXP3 expression, leading to an elevated baseline level of FOXP3 expression in all populations originating from these naïve cells. However, the percentage of naive cells positive for FOXP3 was significantly lower than that of Treg and Th17 cells. It should be noted that these naïve cells were not natural naïve Treg cells because they expressed a high level of CD127 (the IL-7 receptor α-chain) and did not express CD25 [18]. Compared between the two resulting cells, we found that Th17 exhibited a comparable expression of FOXP3 with Treg, but significantly higher RORγt expression than Treg cells. This is consistent with the expression of *RORC* genes by qRT-PCR, showing that Th17 cells expressed significantly higher expression of the *RORC* gene than Treg cells. While Treg cells appeared to up-regulated *FOXP3* expression and down-regulated *RORC* expression, Th17 cells expressed both *FOXP3* and *RORC* genes comparable to naïve cells.

Since human Th17 cells are characterized by their intracellular expression of IL-17A and RORγt, whereas human Treg cells are marked by $CD25^+CD127^{lo}FOXP3^+$ expression, it is plausible to suggest that cell populations obtained during the duration of culture in our study may represent an intermediate stage of differentiation between regulatory T cells and Th17 cells [19]. However, even though cells under the Th17 condition showed coexpression of FOXP3, IL-10, and IL-17A, they did not exhibit CD25 positivity and displayed elevated levels of RORγt, distinguishing them from typical Treg cells. It should be noted that CD25 negative $CD4^+$ T cells can undergo differentiation into Treg cells when activated, and these cells are identified as precursors of Treg cells [20,21,]. Therefore, it is reasonable to propose that the Th17 condition, which produces a cell population with a $CD25^-FOXP3^+$ phenotype, may generate Treg precursors that can be converted into functional Treg cells upon further activation.

In cell metabolic assays we demonstrated that the energy production of Treg and Th17 relies on the glycolytic pathway and OXPHOS. Th17 appeared to be more dependent on glycolysis than Treg, since inhibition of OXPHOS by oligomycin enhanced lactate production in Th17. Additionally, Th17 had a higher glucose uptake capacity than Treg in the glucose uptake assay. It has been reported that resting naive T cells exert low metabolic activity and produce energy via the tricarboxylic acid (TCA) cycle and the fatty acid oxidation with low levels of glycolysis [22]. Treg cells rely on OXPHOS for energy production and are less dependent on glycolysis compared to Th1, Th2 and Th17 cells [,23,24]. These evidences support our findings that T cells cultured under Th17 conditions demonstrated trends of elevated glycolytic activity and glycolytic reserve compared to those under Treg conditions. However, because of their plasticity and an ambiguous stage of differentiation, the difference did not reach statistical significance. In summary, based on the established protocols that we used for Treg differentiation [,25,26] or Th17 differentiation [27], we observed that the resulting cells exhibited phenotypic markers and transcriptional regulators that direct the differentiation program of Treg and Th17 cells. These two populations demonstrated disparate metabolic activities, suggesting that they might represent either an intermediate stage of cells or two distinct cell types.

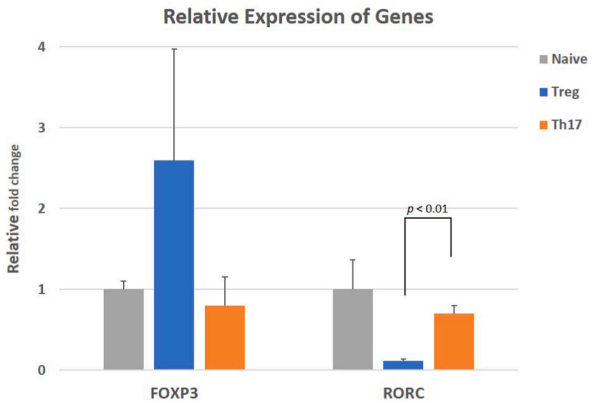

Fig. 5. Relative expression of the *FOXP3* and *RORC* genes. Quantitative real-time reverse transcription PCR (qRT-PCR) was performed in cells obtained from each condition and in naïve T cells as control. Cycle thresholds were compared using *GAPDH* as a reference gene for normalization. The relative fold change was calculated by 2ˆ(–delta delta CT) of gene expression in Treg or Th17 in comparison with naïve T cells. The result demonstrated means of three biological replicates. *P*-values were analyzed using Student's *t*-test.

5. Conclusions

In this study, our findings postulate that with established protocols for iTreg and Th17 cell differentiation in vitro, the obtained cells are possibly at the intermediate stage of differentiation. Otherwise, it is likely that the culture systems give rise to two distinct types of cells at suboptimal conditions that share a lineage-specific differentiation program. Further studies at the transcriptional or epigenetic level may further elucidate these cell types. We suggest the confirmation of cell phenotypes when using these in vitro culture systems to develop the inducing regulatory T cells and Th17 cells before employing the cells in a further research experiment.

Ethical approval

This study was reviewed and approved by the Siriraj Institutional Review Board with the approval number: Si683/2022, dated September 23, 2023. All participants (or their proxies/legal guardians) provided written informed consent to participate in the study and for their data to be published.

Data availability

No secondary data is associated with this study. Primary data will be made available upon request.

CRediT authorship contribution statement

Jarupa Soongsathitanon: Writing – original draft, Validation, Formal analysis, Conceptualization. **Ticha Homjan:** Methodology, Investigation. **Suthatip Pongcharoen:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT 3.5 to check and correct grammatical errors during the manuscript writing process. After using this tool/service, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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