All-trans Retinoic Acid Regulates the Balance of Treg-Th17 Cells through ERK and P38 Signaling Pathway

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ABSTRACT

Background: All-trans retinoic acid (ATRA) potentiates TGF-β-dependent regulatory T cells (Treg) induction, while it inhibits pro-inflammatory interleukin-17-producing T helper cells (Th17) differentiation. Combined application of ATRA and TGF-β may shift Treg-Th17 balance towards Treg. **Objective:** To investigates the effect of ATRA on the regulation of Th17-Treg balance through ERK and p38 signaling pathway. **Methods:** Mice naive CD4+T cells were isolated and co-cultured with 100 nmol/ml ATRA and 5 ng/ml TGF-β. The effect of ATRA on the phosphorylation of ERK and P38 was evaluated. The induction of Treg and Th17 was investigated before and after the application of the inhibitor of ERK and P38. **Results:** The expression of p-ERK1 and p-ERK2 increased significantly when the cells were incubated for 3 days with both TGF-β and ATRA. The upregulated expression of p38 was found after incubation for 1 day. The inhibition of ERK prohibited Treg induction and promoted Th17 development. However, the inhibition of p38 only had inhibitory effect on Treg induction. **Conclusions:** ERK and p38 pathways participated in ATRA-activated Treg-Th17 balance adjustment.

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Keywords: All-trans Retinoic Acid, Erk, P38, Regulatory T Cells, Th17 Cells

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INTRODUCTION

Regulatory T cells (Treg), also known as CD4+CD25+forkhead box P3(Foxp3)+ Tregs, play a crucial role in maintaining the allograft-tolerance after solid organ transplantation including kidney (1), heart (2,3), trachea (4), liver (5), and cornea (6,7). It has been considered as the biomarker to tailor immunosuppression and to predict graft outcomes (8,9). However, Tregs can be subverted by inflammatory conditions and converted into interleukin-17-producing T helper cells (Th17), which contribute to allograft failure (10). Regulating Treg-Th17 balance towards Treg has been proved to be a potential therapeutic target for allograft rejection.

The development pathways of Treg and Th17 are reciprocal although both of them develop from CD4+ T cells. The balance between Treg and Th17 can be influenced by many factors. Proinflammatory cytokines such as interleukin (IL)-6 and IL-23 (11), and metabolic inputs signaled by hypoxia inducible factor 1α (12) could skew the balance towards Th17 differentiation over Treg development. On the contrary, the polarization towards Treg can be induced with the presence of transforming growth factor-β (TGF-β), IL-2, and rapamycin (7).

All-trans retinoic acid (ATRA), the key metabolite of Vitamin A, plays a crucial role in the regulation of cellular differentiation, proliferation, and apoptosis, including tumor cells and lymphocytes (13,14). Disrupted ATRA metabolism or signaling causes altered homing or impaired functional differentiation of lymphocytes (15, 16). Nevertheless, ARTA potentiates TGF-β-dependent Treg induction, while it reciprocally inhibits pro-inflammatory Th17 differentiation (17,18). Our previous study confirms that combined application of ATRA and TGF-β may shift Th17-Treg balance towards Treg and facilitate the induction of immunological tolerance after allogenic corneal transplantation (19).

With the presence of TGF-β, ATRA is believed to promote the development of Tregs through Smad3 signaling pathway (20). In addition to Smad signaling, MAPKs including ERK, JNK and p38 constitute major non-Smad signaling pathways that play a supplementary role in mediating the intracellular responses to ATRA (21, 22). It has been recently reported that ATRA regulates the differentiation of acute promyelocytic leukemia cells (23) and the migration of lung cancer cells (24) through ERK signaling pathway. ERK signaling pathway has been revealed to have a close relationship with the development of Treg and inhibition of Th17 (25). P38 has also been shown to participate in the cell differentiation modulated by ATRA (26). Therefore, the current study investigates the effect of ATRA on the regulation of Treg-Th17 balance through ERK and p38 signaling pathway.

MATERIALS AND METHODS

Animals. Male C57BL/6 (H-2b) mice (weighing 20-24g, 6-8-weeks-old) were purchased from the Department of Laboratory Animal, Fudan University. All mice were housed in a specific pathogen-free (SPF) environment. All experimental manipulations were undertaken in accordance with the institutional guidelines for the care and use of laboratory animals and ARVO Statement for the use of animals in Ophthalmic and Vision Research Centers. The study was approved by the Animal Ethics Committee of Eye & Ear, Nose Throat Hospital of Fudan University.

Reagents and antibodies. RPMI 1640, HEPES, penicillin-streptomycin, ATRA, dimethyl sulfoxide, PMA, and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant IL-2 and TGF-β protein, as well as Dynabeads® Mouse T-Activator CD3/CD28, were purchased from Invitrogen (Carlsbad, CA, USA). Anti-mouse p44/42 MAPK (ERK1/2) (137F5), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E), p38 MAPK (D13E1), phospho-p38 MAPK (Thr180/Tyr182) (D3F9), SAPK/JNK (56G8) and phospho-SAPK/ JNK (Thr183/Tyr185) (81E11) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Small molecule inhibitors SB203580 and SCH772984 were purchased from Selleck (Houston, TX, USA). Fluorescein (FITC)-, allophycocyanin (APC) - or phycoerythrin (PE)-conjugated antibodies specific for mouse CD4 (RM4-5), CD25 (PC61.5), Foxp3 (FJK-16s), CD3e (145-2C11), CD8a (53-6.7), IL-17 (TC11-18H10.1), and their isotype controls were purchased from eBioscience, or BioLegend (San Diego, CA, USA). Monensin (GolgiStop) was purchased from BD Biosciences (Becton, NJ, USA). The stock solution of ATRA, with 10 mM ATRA dissolved in DMSO (vehicle), was prepared in a glove bag under an atmosphere of inert gas and stored in the dark at -80°C as aliquots before use.

Naive CD4+T cell purification and stimulation. Naive CD4+T cells (CD4+CD62L+) were isolated from spleen using CD4+CD62L+ T cell isolation kit from MACS Miltenyi Biotec, following manufacturer's instructions. The isolated naive CD4+ T cells were activated with dynabeads® Mouse T-Activator CD3/CD28 and IL-2 in 24-well plates. Then the cells were cultured for 1 day or 3 days with TGF-β (5 ng/ml), ATRA (100 nmol/ml) plus TGF-β (5 ng/ml), or DMSO respectively. For inhibition experiment, small molecule inhibitors, at the final concentration of 10 μM/ml, were added into the cultures one hour before the inclusion of TGF-β and ATRA, and repeated after 48-hour co-incubation. Each group contained at least six wells of cells, and the experiments were repeated for three times.

Western blot. Cell lysates were prepared in lysis buffer containing protease and phosphatase inhibitor cocktails for western blot. A total of 50 μg of protein was separated by SDS-PAGE and transferred to nitrocellulose filter paper, then incubated with anti-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK (ERK1/2), anti-p38 MAPK, anti-phospho-p38 MAPK, anti-SAPK/JNK and anti-phospho-SAPK/JNK antibodies. For the staining of phospho- and total proteins, we employed parallel Western blotting using equal amounts of loading protein to avoid any cross-reactivity. To confirm equal sample loading and transfer, membranes were stripped, re-blocked, and re-probed using an anti-mouse beta-actin Ab.

Flow cytometric analysis. Single-cell suspensions were prepared and cell concentration was adjusted to 1×10^7/ml. Then each sample was divided into two parts. One was incubated with anti-mouse FITC-CD4 and APC-CD25 mAb for at least 30 minutes at 4°C in the dark, and then washed, fixed, permeabilized, and stained with anti-mouse PE-Foxp3 mAb for another 30 min at 4°C in the dark. The other part was stimulated with 0.1 μg/mL PMA, 1 μg/mL ionomycin, and 1μl/ml monensin for 4 hours at 37°C. Following that, it was incubated with anti-mouse APC-CD3 and FITC-CD8 mAb for at least 30 minutes at 4°C in the dark, and then washed, fixed, permeabilized, and stained with anti-mouse PE-IL-17 mAb for another 30 min at 4°C in the dark. In addition, we used FITC-CD4/CD8-only, APC-CD3/CD25-only, PE-Foxp3/IL-17-only, and blank cells to observe the spectral overlaps and adjust for the compensation before applying the co-staining cell analyses. Finally, the cells were re-suspended with flow cytometry

staining buffer, and analyzed using a FACS Calibar flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest software. The gating strategy was shown in Supplementary Figure 1.

Statistical Analysis. Data are presented as the mean ± SD. Statistical significance was determined using one-way analysis of variance (ANOVA), Student's t-test or Kruskal-Wallis test. For each ANOVA, post-hoc Fisher PLSD (protected least significant difference) was performed to determine each individual group difference. All tests were considered statistically significant at $p<0.05$ (SPSS for Windows, version 13.0; SPSS, Inc., Chicago, IL).

RESULTS

ATRA cause upregulated phosphorylation of ERK and P38 in a time-dependent manner. Figure 1 shows that after incubation with TGF-β and 100 nmol/ml ATRA, the expression of p-ERK1 and p-ERK2 was significantly upregulated at the 3rd day compared to the 1st day (both $p<0.01$). However, such effect was not obvious in the expression of ERK. The upregulated expression of p-P38 was found after incubation with TGF-β and ATRA for 1 day. Nevertheless, the treatment of TGF-β alone did not affect the expression of p-P38.

Inhibition of ERK and P38 prohibit Treg induction. Figure 2 shows that TGF-β significantly promoted the induction of Treg. Such effect was further augmented when the cells were incubated with both ATRA and TGF-β. However, the inductive effect of ATRA and TGF-β was significantly prohibited when inhibitor of either ERK or P38 was added into the incubation system.

Inhibition of ERK rather than P38 promote Th17 development. The development of Th17 cells was significantly increased when incubated with TGF-β alone. However, the induction of Th17 cells was significantly inhibited with the combined use of ATRA and TGF-β. Nevertheless, this inhibitory effect was significantly abolished by the use of ERK inhibitor, just as shown in Figure 3. Further prohibition of Th17 cells development was found with the application of P38 inhibitor. However, the reduction is not statistically significant.

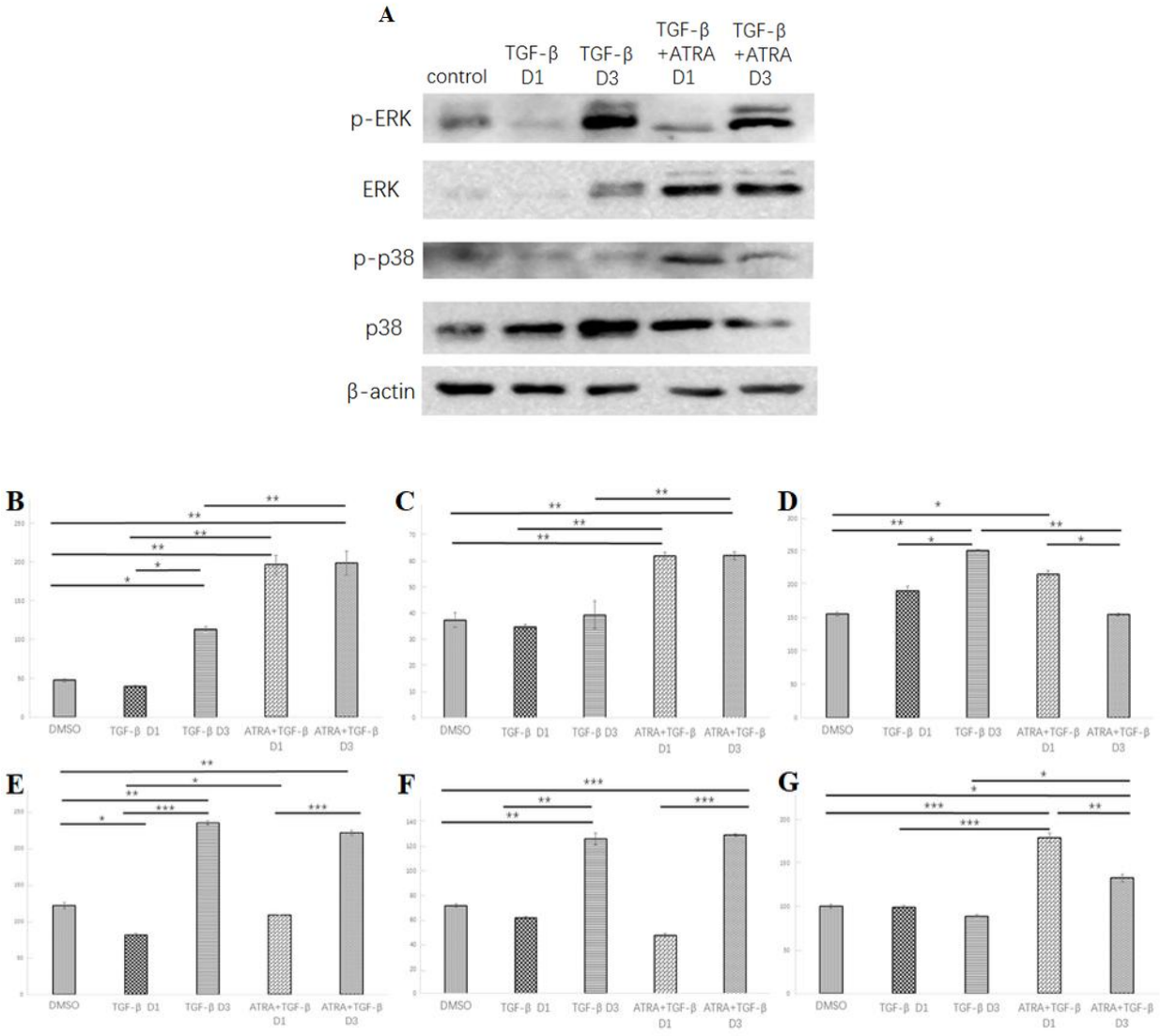

Figure 1. Western blot (A) and analysis of ERK1 (B), p-ERK1 (E), ERK2 (C), p-ERK2 (F), P38 (D) and p-P38 (G) after naïve T cells were incubated with 100 nmol/ml ATRA and TGF-β for 1 day and 3 days. Compared with the 1st day, the expression of p-ERK1 and p-ERK2 was significantly upregulated at the 3rd day when incubated with either TGF-β alone or TGF-β+ATRA. However, such effect was not obvious on the expression of ERK. The upregulated expression of p-P38 was found after incubation with TGF-β+ATRA for 1 day. Nevertheless, the treatment of TGF-β alone did not affect the expression of p-P38. Data are presented as the mean ± standard error and are representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

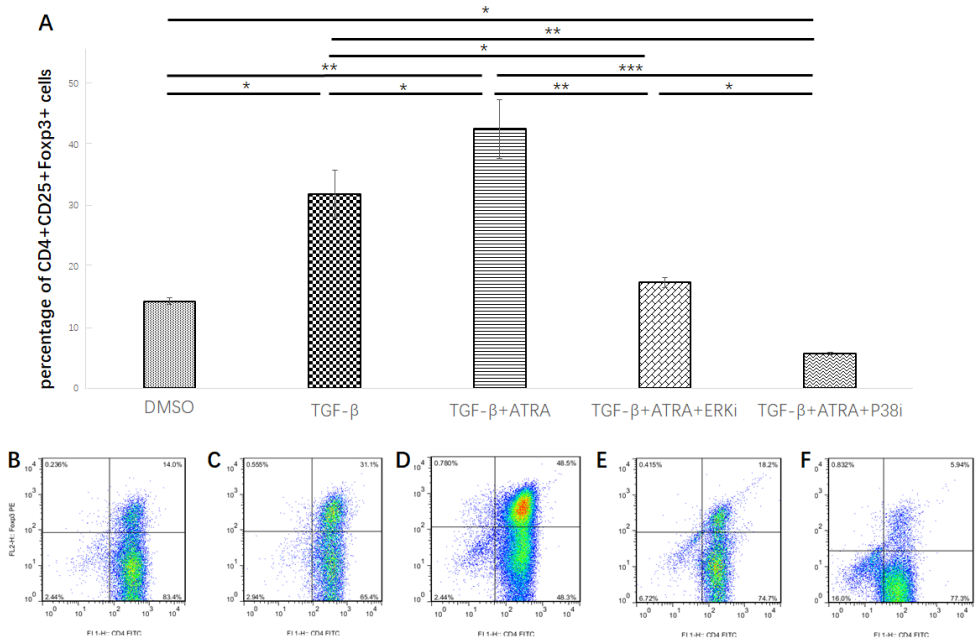

Figure 2. The induction of Tregs from naïve T cells. (A) show the native T cells after incubation with 100 nmol/ml ATRA and the inhibitor of ERK and P38. Compared with control group (B), TGF-β significantly promoted the induction of Tregs (C). Such effect was further augmented when the cells were incubated with both ATRA and TGF-β (D). However, the inductive effect of ATRA and TGF-β was significantly prohibited when inhibitor of either ERK (E) or P38 (F) was added into the incubation system. Data are presented as the mean ± standard error and are representative of at least three independent experiments. * p<0.05, ** p<0.01, ***p<0.001.

DISCUSSION

The present study demonstrates that two MAPK signaling pathways, p38MAPK and ERK, participated in the regulation of ATRA-induced Treg-Th17 balance, and these two pathways may play different roles in the process. These data met and extended previous findings on non-genomic pathways of retinoic acid (RA) and their contribution to cell differentiation. For example, RA has been shown to activate ERK pathway in embryonic stem cells (27) and neural cells (28), and ATRA-induced Foxp3 expression enhancement on naïveCD4+ T cells was found to be involved in increased ERK expression (29). In addition, in leukemia cells, ATRA was shown to trigger p38 pathway, which was involved in subsequent differentiation (26). Our study indicates that ERK pathway is required in not only the promotion of Treg differentiation but also in the inhibition of Th17 differentiation. P38 pathway is involved in helping Treg, but not suppressing Th17 differentiation.

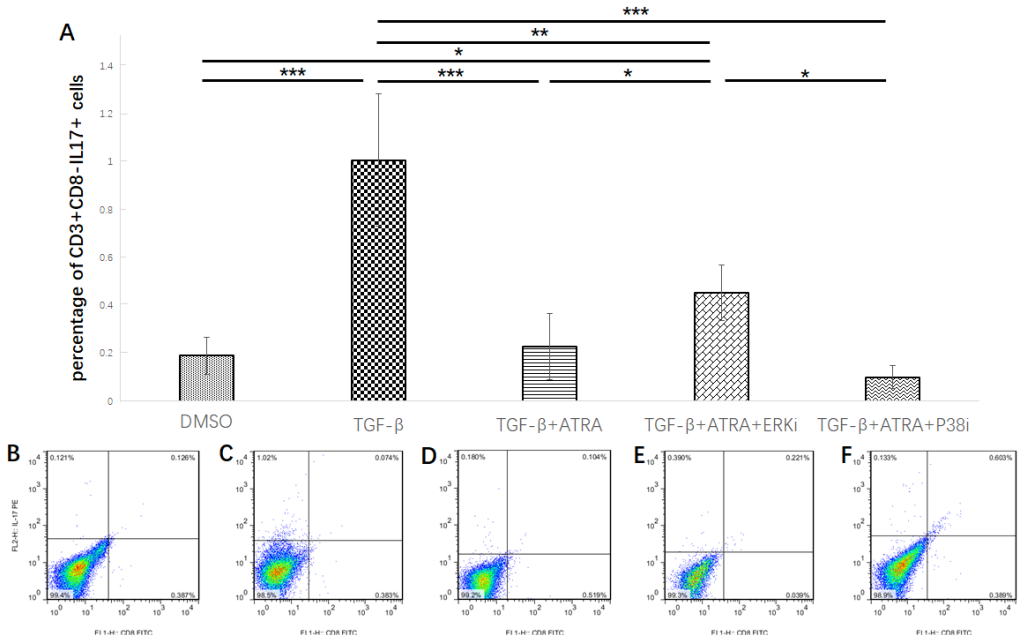

Figure 3. The induction of Th17 cells from naïve T cells after incubation with 100 nmol/ml ATRA and the inhibitor of ERK and P38 (A). Compared to control group (B), the development of Th17 cells was significantly increased with the incubation with TGF-β alone (C). However, the induction of Th17 cells was significantly inhibited with the combined use of ATRA and TGF-β (D). This inhibitory effect was significantly abolished by the use of ERK inhibitor (E). The application of P38 inhibitor caused decreased level of Th17 cells (F). However, the reduction was insignificant compared to cells cultured with ATRA and TGF-β. Data are presented as the mean ± standard error and are representative of at least three independent experiments.* p<0.05, ** p<0.01, ***p<0.001.

The MAPK superfamily including p38 MAPK, ERK, and JNK, play important roles in cell differentiation, proliferation, and apoptosis. Recent studies have also implicated MAPK in CD4 effector T cell differentiation, whose mechanism remains unclear. P38 signaling has been shown to be activated in TGF-β-induced Treg differentiation from CD4+CD25- T cells (30), and our data showed that p38 was also activated and even had a higher expression level of phosphorylation when the naive CD4+ T cells were treated with both ATRA and TGF-b, suggesting that the combined use of ATRA and TGF-b had a potential amplification effect on p38. Although ERK was required for Treg induction from naïve CD4+ T cells (28), it was reported that the activation of ERK in CD4+CD25-T cells was inhibited by TGF-β in 30 minutes (31). Our study has the similar finding that the activation of ERK in naïve CD4+ T cells was inhibited after TGF-β treatment at day 1. Nevertheless, significantly enhanced ERK activation was found at day 3. The difference between day 1 and day 3 might be attributed to the differential effect of TGF-β. It has been reported that in pancreas cells, pERK participated TGF-β-mediated cell cycle arrest in a differential way, initially facilitating it and later antagonizing it (32). The activation of ERK in naïve CD4+ T cells by TGF-β probably acts in a similar way. Co-treatment of TGF-β and ATRA activated ERK at day 1 and induced further activation within 3 days. ATRA has been shown to accelerate the

naïve CD4+ T cell cycle, inhibited cell apoptosis, and favored Treg production with the presence of TGF-β (5,14,16,33). It might be the possible explanation for the difference between single and combined treatment.

Our data also shows that ERK, not p38, is required for ATRA-induced Th17 production impairment, which is consistent to a previous finding on berberine-activated Th17 differentiation in type 1 diabetic mice indicating that ERK, not p38, plays a negatively regulating role (34). However, ERK and p38 were also found to be positively involved in Th17 development in lymph node cells induced by concanavalin A (35). The difference may be attributed to the cell culture conditions and inducing factors. Lymph node cells, containing many different subtypes, may secrete various cytokines that could result in complex effects on IL-17 production, whereas naïve CD4+ T cell culture is relatively more homogenous. In addition, it is possible that different pathways will be involved for Th17 differentiation triggered by different factors. This issue indicated that a more comprehensive understanding about MAPK pathways in Th17 development is required for further studies. Our data is, to some extent, consistent with Lu *et al*. finding (33). However, two major differences need to be of note. One is that the previous study focused on enhanced expression of Treg induced by ATRA, while our study emphasizes on Treg-Th17 balance. The other difference is that we found that ERK, but not p38, is involved in ATRA-induced Th17 inhibition, which is not totally opposite to Treg stimulation pathways.

Two limitations of the present study should be addressed. First, we chose the concentration of ATRA based on published literature and our previous study (7,36). The dose-dependent effect of ATRA on Treg-Th17 balance was still unclear. Second, in vivo immunosuppressive potential of Tregs induced by ATRA and TGF-β in vitro was unknown. It needs investigations and validations by further allogenic organ/tissue transplantation.

Taking together, this study serves as a preliminary step to understand the role of MAPK superfamily members, ERK and p38 pathways, in ATRA-activated Treg-Th17 balance adjustment. These data raised the questions of exact mechanisms of MAPK signaling pathways during the iTreg and Th17 differentiation including trigger factors and time sensitivity. Another issue is ERK-p38 interaction. Are they, by any chance, involved in one signal cascade, or still separately in different pathways but leading to a same target, in some particular cell differentiation progresses? Anyway, our findings suggest the complexity of MAPK pathways in T cell differentiation, and more investigations are needed to uncover this exact intracellular traffic mechanism.

In conclusion, combined use of ATRA and TGF-β regulated the balance of Treg-Th17 cells towards Treg. ERK and p38 pathways participated in ATRA-activated Treg-Th17 balance adjustment.

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