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Fatty Acids Effect on T Helper Differentiation in Vitro

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Abstract: Autoimmunity shows a concerning growth recently. It seems that role of diet is important for development of autoimmunity. Nutritional elements can affect immune system functions. Oversupply or deficiency of specific metabolites may change performance of immune cells, especially that of T helper lymphocytes. For example, glucose, omega 3 and poly unsaturated fatty acids can induce specific Th subsets which play a critical role in autoimmune diseases. T lymphocytes, mostly T $CD4^+$ (Th cells) not only play a critical role in orchestrating immune responses, but also they have major role in pathogenesis of some autoimmune disease. Hence, we designed a research to find out effects of oleic and palmitic acids on differentiation of Th cells. We added oleic acid, palmitic acids and combination of them on peripheral blood mononuclear cells (PBMC) culture and cells were harvested for RT-PCR and flow cytometry analysis after seven days. Our results showed that palmitic and oleic acids induce Th1 and Th17 subsets (p-valu<0.05).

Keywords: T Helper Cells, Differentiation, Metabolism, Fatty Acids, Mtor

1. Background

"During last years, there is a special focus on diet's role in possible pathogenesis of autoimmunity [1-3]". "It has been showed that specific nutritional elements can affect performance of immune system [4-6]". "It is interesting that some metabolites can shift immune response by their effects on differentiation of Th cells [7-14]". "It is evident that immune signals regulate Th cells differentiation and consequent fate of immune response [15-18]". T helper lymphocytes are one of the most important cells in orchestrating responses of immune system. "Th cells can secrete wide range of cytokines and thereby direct B lymphocytes and cytotoxic T cells functions [19, 20]". Th cells are classified into effector (Th1, Th2 and Th17) and regulatory subsets. Here, a question may be raised whether could metabolic signals affect Th differentiation? In recent years, there is a body of evidence supporting this theory. Immunometabolism has been emerged as a new field of immunology. "Effects of metabolites on Th differentiation are focus of many studies [21-23]". Fundamental idea of these researches came from a basic fact: after activation of Th cells they change their metabolic programs to get ready for clonal expansion.

"Naïve T cells use autophagy and lipid oxidation to supply their energetic demands. Interestingly, these cells show Warburg Effect after the activation [14-16, 24]", which is, switching metabolic program from autophagy and lipid oxidation to glycolysis. Because of this shift from catabolism to anabolism it could be accepted that metabolic condition, depletion or addition of specific metabolites, can change Th differentiation. Differentiated Th subsets consist of T effector

(Th1, Th2 and Th17) and T regulator (Treg). Teff response against diverse pathogens and at the final phase of immune responses, Treg suppresses function of effector T cells. Teff subsets and Treg have different metabolism. "Studies show that Teff subsets primarily are glycolytic but Treg cells rely on lipid β-oxidation [14, 15, 25]". "Although some studies have investigated fatty acid effects on Th differentiation but the results are somewhat vague [25, 26]". Hence, we designed a study to find out the effect of palmitic and oleic acids on Th cells differentiation. Palmitic and oleic acids are abundant in plasma phosphatidyl choline. We evaluated potential of oleic and palmitic acids to induce Th subsets differentiation. In fact, we investigated effects of same energy sources on Th subsets differentiation. In this study, we analyzed T-bet, ROR-γt, GATA3 and FoxP3 relative gene expression to investigate effects of these fatty acids on Th1, Th2, Th17 and Treg differentiation respectively. Moreover mTOR and TGFβ genes expression were evaluated by RT-PCR. Also three color flow cytometry was used to determine $Treg^{CD4+\ CD25+\ FOXp3+}$ differentiation.

2. Material and Methods

2.1. Pbmc Isolation

Venous blood from 6 healthy adult nonsmokers, nonalcoholic men without any symptom of immunologic disorders, was collected in heparin tubes. PBMCs were separated by Ficoll gradient centrifugation All study participants provided informed written consent prior to study enrolment.

2.2. Cell Culture

The PBMC cells suspension were added to 1μg/mL anti CD3 (human CD3 MAb elabscience) coated plate and then 1μg/L anti CD28 (human CD28 Mab elabscience) was added to X-vivo15 (X-Vivo 15 Lonza) containing wells, and cells were incubated at 37°C, 5%CO_2 for 3 days. Then, 100 unit rIL-2 (human rIL-2 ebioscice,) was added per well, cells were incubated at 37°C, 5%CO_2 for 2 days. For the next step, in three separate groups, 1mM oleic acid (Merck), 1mM palmitic acid (Sigma-Aldrich) and 1mM oleic acid in combination with 1mMpalmitic acid were added (total well volume was 1ml) and cells were incubated at 37°C, 5%CO_2. 2 days after adding fatty acid (totally 7 days), cells were harvested for a differentiation analysis by RT-PCR and flow cytometry. All assays performed in triplicate.

2.3. Rna Extraction and Cdna Synthesis

Total RNA was extracted by using RNX plus (Sina colon), according to the manufacturer's instructions. RNA quality was assessed using a Nano Drop A&E lab instruments (guangzhou) spectrophotometer. Then 1μg RNA according to cDNA synthesis kit (Viva 2steos RT-PCR Kit with M-MuLV, Sina colon) was used for cDNA synthesis.

2.4. Real Time Pcr

cDNA, Forward and Reverse primers, ROX and syber-green master (TaKaRa, SYBR Premix Et Taq^{TM}II (Ti RNaseH Plus) used for each reaction.

2.5. Flow Cytometry

Cells were prepared for 3 Color flow cytometry. Appropriate amount of conjugated CD4 (PerCP-Cy TM 5.5 mouse anti-human CD4, BD Pharmingen) and CD25 (FITC mouse anti-human CD25, BD Pharmingen) were added. 1x working solution Human FoxP3 Buffer C and conjugated FoxP3 antibody (alexa flour 640 anti-human FOXp3, BD Pharming) were added at appropriate concentration. Cells were suspended in fix buffer and analyzed immediately. To do proper staining, BD Pharmigen protocol was fallowed precisely.

2.6. Statistical Analysis

All the data were analyzed using IBM SPSS version 21 and GraphPad prism6 software. Normality tests and homogeneity of variance were done by Shapiro-Wilk and Leven test respectively, for each gene expression. All comparisons were performed by non-parametric tests including Friedman test for real time PCR results and Kruskal-Wallis test for flow cytometry results were used. P-value < 0.05 was considered to be statistical significant and power in all of the analysis was more than 0.80. Data for each of gene expression were displayed as the mean ± standard deviation.

3. Results

Our RT-PCR data analysis showed Foxp3 and GATA3 genes expression were significantly down regulated in all groups p-value<0.05.T-bet, mTOR and TGF-β were up-regulated in all three separate groups significantly p-value<0.05. But ROR-γt only was up-regulated in Combination of oleic and palmitic acids (figure1 and 4). Flow cytometry data analysis shows no significant change in Foxp3 gene expression. CD25 expression was increased because of IL-2 presence in cell culture (figure 2 and 3). All data indicate presence of fatty acids suppress Treg and Th2 differentiation and induce Th1 and Th17 differentiation.

In three separated group, 1mM palmitic acid, 1mM oleic acid and their combination (1mM palmitic acid + 1mM oleic acid) were added. FOXp3 dramatically suppressed. TGF-β, T-bet and mTOR are induced in all groups and ROR-γt only induced in combination group. Differences among these fatty acids also were evaluated by pair comparison of groups. There is no significant differences between saturated, unsaturated or combination of them in our results. Each bar represents the average of three independent experiments.

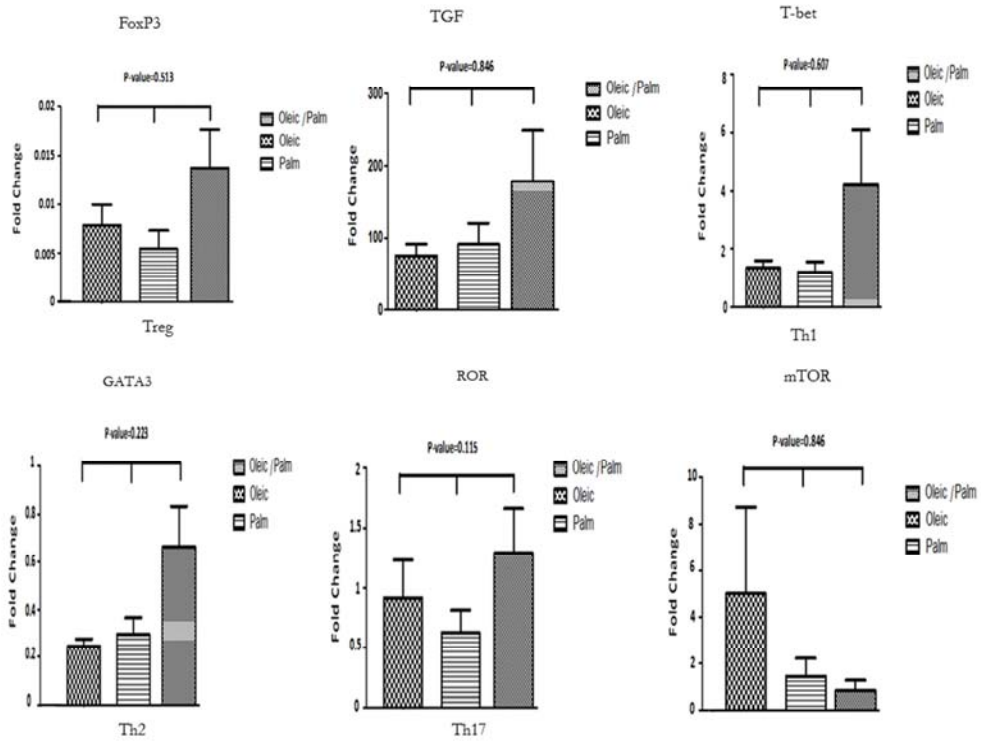

Figure 1. *FOXp3, ROR-γt, mTOR, TGF-β, T-bet and GATA-3 relative gene expression.*

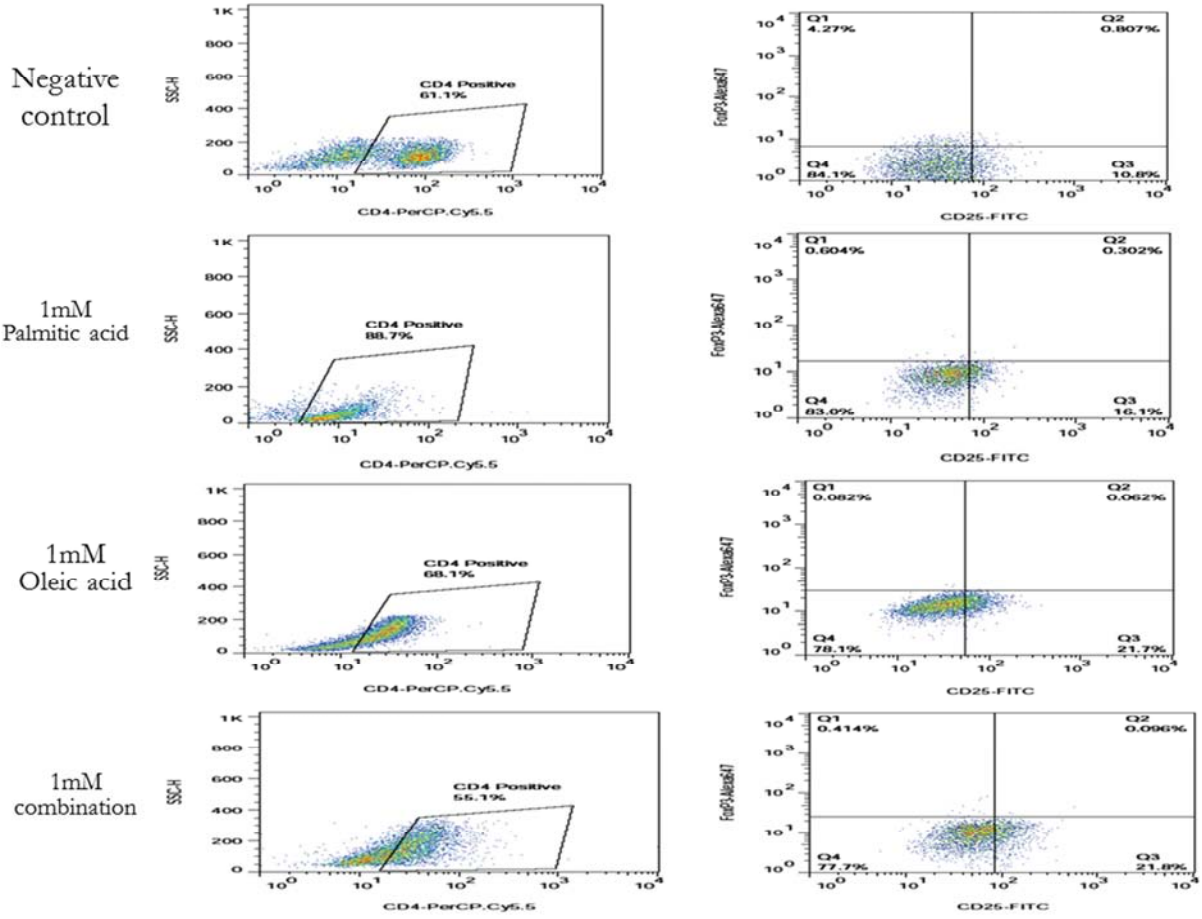

Figure 2. *Three color flow cytometry results of treated and untreated PBMCs.*

PBMCs were cultured in four groups then after seven days were harvested for flow cytometry analysis. Treated groups were compared negative control for CD4, CD25 and FOXp3 expression.

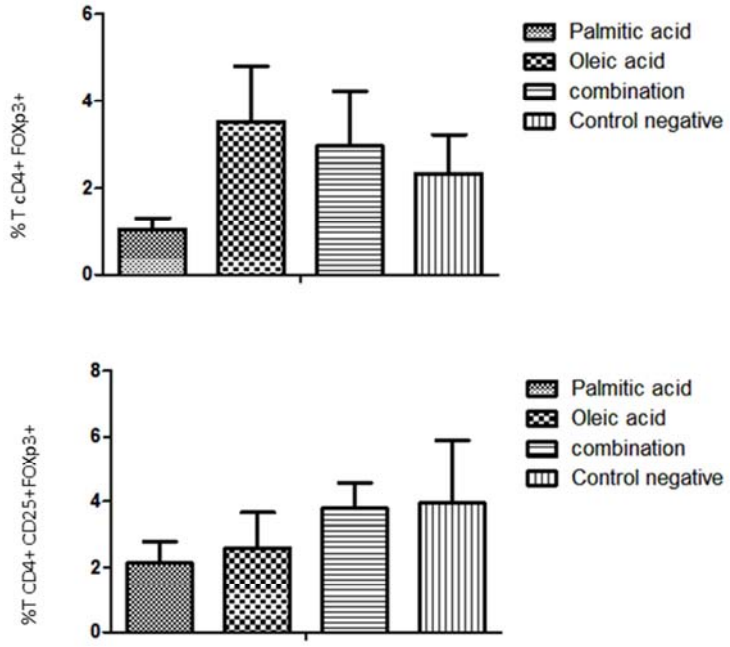

Figure 3. *Statistical analysis of flow cytometry staining.*

In normal human 5-10% T $CD4^{+}FOXp3^{+}$ cells exist. Our negative control result also shows a normal person T cells population. There are no significant changes in treated groups in compare to negative control. It can be concluded that palmitic, oleic acids and their combinations are not able to induce Treg differentiation.

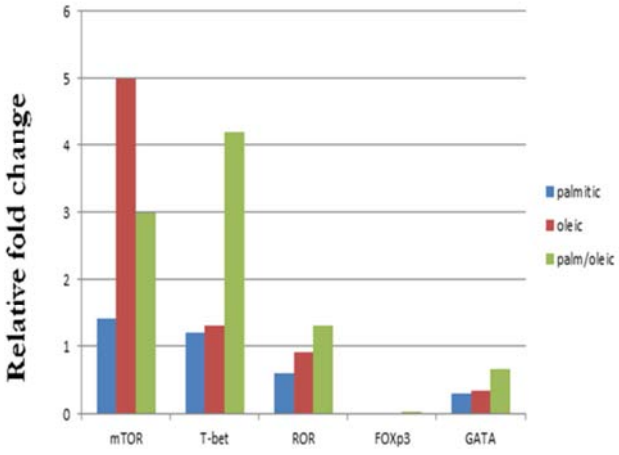

Figure 4. *Palmitic and oleic acid effect on Th signature genes and mTOR expression.*

All genes expression except TGF-β is shown here. As illustrated above, significant FOXp3 suppression and significant mTOR and T-bet induction in all three treated groups are seen. But ROR-γt just induce in oleic and palmitic acid combination.

4. Discussion

Immunometabolism is an interesting field which is investigated links between immune system function and metabolic status of human body. Th cells role in immune system is very crucial. In HIV^{+} patients, T $CD4^{+}$ important role is clear. T $CD4^{+}$ different subsets including Th1, Th2, Th17 and Treg show different functions in immune system. They also recruit different metabolic machinery after activation. Teff and Treg cells use glucose and fatty acids to supply their metabolic demands (respectively). Some studies have been examined the metabolism manipulation to induce specific Th subsets. Hence there is a possibility that available fatty acids can induce Th differentiation. Because saturated and unsaturated fatty acids play distinct role on immune system, it can be assumed that they may induce different Th cells subsets.

As our results indicated, mTOR gene expression was up-regulated. These data are according with Th1 and Th17 differentiation. Because of Th1 and Th17 induction it can be concluded that mTORC1 was up-regulated. In michalech et al study, Th subsets skewing condition was used. For example, IL-12 and anti-IL-4 were added to cell cultures to induce Th1 differentiation, and then fatty acids were added [25]. But we did not use any skewing condition for specific Th subsets. It seems that adding alternative energy sources is not sufficient for Treg induction. FOXp3 suppression

supports this theory. It could be explained that until glucose exists in cell culture, Th cells do not use any existing alternative energy source [24, 25]. In fact, Th cells prefer to use glucose to obtain their energetic demands. Because Treg subset was not induced, mTOR gene expression also was not down-regulated. All data showed that adding only1mM oleic and palmitic acids change Th cells metabolism and induce Th1 and Th17 induction.

"Palmitic acid effects on the immune system have been studied. It has pro-inflammatory effects, including activation of NF-kB and induction of IL-6, TNF-α secretion [27, 28]". Our results also are accord to these pro-inflammatory effects. Th1 and Th17 subsets are pro-inflammatory lymphocytes. "Unlike palmitic acid, oleic acid effects remain controversial [29, 30]". Although some studies showed anti-inflammatory role of oleic acid on the immune system, but results of some other studies are against them. They showed that oleic acid containing diet is not immunosuppressive as omega3 fatty acid diet. "On the other hand oleic acid containing diet does not suppress anti-microbial response [30]". Our results also showed that oleic acid may induce Th1 and Th17 cells differentiation.

Because FOXp3 gene expression strongly suppressed in this study, only possible source of TGF-β production, will be macrophage. Classical macrophage activation results in M1 macrophage polarization and alternative macrophage activation results in M2 macrophage polarization. "M1 cells are pro-inflammatory agents and M2 cells act as an anti-inflammatory cells and they secret TGF—β and IL-10 to mediate their function [31, 32]". Also M1 and M2 cells are different metabolically. M1 cells are glycolytic and M2 rely on oxidative phosphorylation for supply their energetic demands. "Some studies showed that fatty acids can polarize M2 cells and M2 cells increase their fatty acid uptake and oxidation [32]". Hence it seems that oleic and palmitic acid can affect macrophage polarization and TGF- β production by M2 consequently.

5. Conclusion

Metabolism can influence our immune system functions. T lymphocytes metabolic status change during their activation, and they show glycolytic activity after differentiation. This metabolic reprogramming is needed for their clonal expansion. Among Th1, Th2, Th17 and Treg subset, just Treg relies on lipid oxidation. Our data showed that adding fatty acids to cell culture is not sufficient for Treg induction. It is necessary to reduce glucose level and enhances fatty acid.

For future studies, it recommended that, fatty acids added to cell culture after depletion of glucose. However, different fatty acids concentration may be useful for Treg induction. Raising the number of samples from both sexes can be influenced results.

This study focused on T lymphocytes as important cells in immune system. They play central and critical role to orchestrating immune response. Finding metabolism role on Th cells differentiation may help us to find out how diet can affect immune system and our health status. But because of limited financial source several weaknesses have been faced including; we couldn't assay other important genes like AMPK that may involve in Th cell differentiation or other related cytokines like IL-4, IL-5, Il-17 and IFN-γ. Moreover we were not able to use more concentration of anti-CD3 and anti-CD28 to stimulate Th cells. It is possible that more potent Th cells stimulation results in different results.

Nomenclature

FOXp3; Forkhead box P3, T-bet; T-box transcription factor, GATA-3; Trans-acting T-cell-specific transcription factor, and ROR-γt; RAR-related orphan receptor gamma are transcription factors of T regulatory, Th1, Th2 and Th17 (respectively).

PBMC; Peripheral Blood Mononuclear Cells contain T and B lymphocytes, NK cells and monocyte.

T CD4+; also are named T helper cells (Th cells) can be divided to Teff; T effector cells and Treg; T regulatory cells.

HIV; Human Immunodeficiency Virus is virus that causes HIV infection. HIV+ patients have decreased number of Th cells and because of immune system failure they suffer opportunistic infection and cancers.

NF-κB; Nuclear Factor Kappa-light-chain-enhancer of activated B cells is a transcription factor involved in inflammatory signaling pathway.

TGF-β; Transforming Growth Factor beta, IL-6; Interleukin 6, IL-10; Interleukin 10 and TNF-α; Tumor Necrosis Factor alpha are anti-inflammatory and pre-inflammatory cytokines which a range of immune cells are source of them.

mTOR; The mechanistic Target of Rapamycin and mTORC; The mechanistic Target of Rapamycin Complex were described before.

RT-PCR; Real Time-PCR is a technique that measure amount of target DNA in real time. It helps us to evaluate gene expression level.

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