ORIGINAL ARTICLE

Antagonistic Effect of Biosynthesized AgNPs from Garcinia Atroviridis Extract on Anti-inflammatory Properties of $CD4^+ILR^{high}$ Cells from Non Obese Resistance (NOR) Mouse Model

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ABSTRACT

Introduction: $CD4^+CD25^+$ $Foxp3^+$ T regulatory cell (Tregs) represents approximately 8-10% of the total CD4+ T cell population and are important for immune homeostasis and preventing autoimmune development. Thus, harnessing their functions as immune modulator may be coupled with the rapid advancement of nanotechnology development. Plant-mediated biosynthesis of silver nanoparticle (AgNP) is noteworthy due to simplicity, rapid rate and potentially render more biocompatibility with biomolecules. This study identified the effect of biosynthesized-AgNPs from Garcinia atroviridis (GA) in modulating inflammatory properties of Treg cells in Non-Obese Resistant (NOR). GA extract was used to biosynthesized AgNPs and was tested on the effect of inducing inflammatory properties in $CD4^+IL17R^{high}$ cells following 72hr *in vitro* treatment. **Methods:** Conventional $CD4^+CD25^-Foxp3^-$ cells from female NOR mice were sorted using magnetic separation and cultured in RPMI in the presence of anti-CD3/CD28 antibodies, TGF-β and IL-2 cytokines. Cells were then treated with or without GA-AgNPs for 48hr of iTreg cell induction and then re-cultured with new media treated with respective treatments received. After 72hr in vitro culture, cells were stained with fluorochrome-conjugated antibodies for flow cytometry. **Results:** Current result showed that AgNPs suppress CD4 expression in $CD4^+IL17R^{high}$ population. MAPK pathway proteins remain unchanged in both control and AgNP-treated groups. **Conclusions:** The preliminary findings may suggest the properties of GA-AgNPs in modulating $CD4^+$ T cell population in normal condition. Further studies are necessary to elucidate the molecular mechanisms involve in such interaction. Current findings serve as basis in further identifying the immunomodulatory profile of nanoparticle for potential therapeutic use.

Keywords: Pro-inflammatory, Immunomodulator, Nanoparticle, T-regulatory cells, *Gardinia atriviridis*

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INTRODUCTION

Understanding the immunobiology of $CD4^+$ T cell subset serves as the crucial step in the effort to manipulate our immune system as means of therapy. One of the most common approach is by modulating the immune induction and regulation components. In fact, immunomodulation has been extensively studied in research laboratories in cancer- and autoimmune-related diseases (1-5). One of the important component in immune induction is $CD4^+$Th17 cell subset which is produced from naive $CD4^+$ T cell in the presence of IL-21, IL-6 and TGF-β. This will lead to phosphorylation of STAT3 (6-9) and eventually upregulate transcription factor Retinoic acid-related Orphan Receptor C (RORC2) in naive $CD4^+$ T cells. As a results, these cells express IL17 receptors and release pro-inflammatory cytokines IL17 and IL-23 (8, 10-12). These cells are highly pro-inflammatory and functioned as assassins to tumour antigens and bacteria in which dysregulation will lead to self-destruction autoimmune disease (7, 8, 10, 11, 13).

Thus, immunoregulatory component is necessary to maintain immune homeostasis. One of the key player in immune homeostasis is $CD4^+CD25^+Foxp3^+$ T Regulatory (Treg) cells. It is one of the $CD4^+$ subset

and can be divided into two main subsets i.e natural T regulatory (nTreg) and induce T regulatory cell (iTreg) ((7, 14, 15). Natural nTreg cells are derived from the thymus while iTreg are produced from conventional T cell ($CD4^+$ $CD25^-$ $Foxp3^-$) in the peripheral site (7, 14, 15). Production of iTreg cells can increase when there is decreased or defective nTreg (16). This can also be induced in vitro upon TCR stimulation and cytokines IL-2 and TGF-β (16, 17).

Nanomaterial as a mean of cancer therapy has been widely studied in translational researches. In fact, its application as immune modulator is now a focal point in immunomodulation approach in cancer-related studies. Advanced method of nanomaterial biosynthesis such as plant-mediated silver nanoparticles (AgNPs) is highly preferred due to less harmful, cost effective, higher biocompatible with biomolecules as well as environmental-friendly waste management compared to chemical and physical synthesis of AgNP (18-20). Due to its differential effect on both innate and adaptive immune components (21-23), fundamental analysis is necessary to provide immunomodulatory profile of nanomaterials for therapeutic purposes.

Garcinia atroviridis (GA) is a tropical plant commonly consumed by locals for its edible fruits. In traditional medicine, GA has been used to treat abdominal pain, infections, gastric and pains associated with pregnancy (24). Tan et al. (2018) recently reported essential oils isolated from GA leaves having anti-proliferative property showing significant inhibitory effect against MCF-7 human breast cancer cells (25). Besides, GA has also been studied to provide potent anti-inflammatory, antioxidant, anti-microbial and anti-cholinesterase properties (26, 27). In fact, volatile constituents of GA fruit extract possess selective inhibitory activity against COX-2 compared to COX-1 thus suggest anti-inflammatory properties (26).

The aforementioned studies have inspired us to explore potential modulatory effect that may induce by GA on inflammatory components in $CD4^+CD25^-IL\text{-}17R^{high}$ cell fraction of healthy control. In the present study, GA leaf was used as bioreductant to biosynthesise silver nanoparticles (GA-AgNPs), knowing this is the first study to investigate the potential bioactivity of GA-AgNPs on anti-inflammatory activity. It is hoped to serve as basis immunomodulation properties of nanomaterial for future therapeutic applications.

MATERIALS AND METHODS

Biosynthesis of *Garcinia atroviridis* silver nanoparticles (GA-AgNPs)

Briefly, *G. atroviridis* leaves were washed thoroughly with distilled water and dried for 3 days in the hot air oven at 38-40 °C. The extract solution was prepared by boiling 10 g of ground leaves with 100 ml of deionised water for 10 min at 60 °. Freshly prepared aqueous extract was used for synthesis of silver nanoparticles. For synthesis of silver nanoparticles (GA-AgNPs), a known concentration of leaf extract was interacted with 0.1 M silver nitrate (AgNO3) solution at 1:4 mixing ratio to make up 100 ml final volume in conical flask. The flask was heated at 32 °C with continuous stirring for 72 h.

Mice

Eight-week old female Non-Obese Resistance (NOR) mice were purchased from the Jackson Laboratory, Maine USA. The permission from Veterinary Department, Ministry of Agriculture, Malaysia at Seberang Jaya District, Penang was obtained to import the animals. These animals were acclimatised at the Animal Research Centre, USM, prior to commencement of the experiment. All animals were housed in the cage with dimension of 29.5 cm x 12.5 cm x 15.5 cm and bedded with sterile dry sawdust. The mice were maintained in the animal facilities under specific pathogen-free in accordance with guidelines and regulations of the Universiti Sains Malaysia and were used at 12 week of age. All experiment protocols were approved from USM Animal Ethics Committee [USM/animal ethics approval/2015/97/704]. All protocols including the euthanasia and samples collection were carried out according to the recommendations of the Animal Ethics Committee.

Antibodies, reagents and flow cytometry analysis

Spleen of NOR mice were collected after mice were sacrificed by cervical dislocation. Spleen from all mice were pooled in one falcon tube containing total complete media before excised into small fragments. Mouse nTreg cells were isolated from spleen tissues by magnetic separation using negative and positive isolation kit (Miltenyi Biotech GmBh, German). Negatively isolated $CD4^+CD25^-$ T cells from NOR mice spleens were then stained with FITC-anti- mouse CD4, VioBlue-anti ICOS, VioBlue-anti mouse IL17R and PE-anti mouse TIGIT to determine the marker profile of isolated $CD4^+CD25^-$ cells (Miltenyi BioTech GmBh, German). Cell were cultured in RPMI 1640 supplemented with 10% FBS (Thermo Fischer Scientific, USA), 10 mM HEPES, 500 µL antibiotic stock solution containing 100 U/mL penicillin, 100 µg/mL streptomycin and 10 µM β-mercaptoethanol (Gibco BRL, New York) with or without growth factor 20 ng/mL IL-2 (BD Pharmingen, USA) and 5 ng/mL TGF-β (BD Pharmingen, USA) with or without 10 mg/mL GA-AgNP. Plate wells were coated with 10 µg/mL of anti CD3 prior to culture and anti-CD28 were added to respective groups before in vitro culture. Following in vitro treatment, cells were stained with similar markers in addition of fluorochrome tagged-antibodies against phosphorylated transcription factor protein i.e alexa fluor-anti STAT5, alexa fluor-anti p38 and alexa fluor-anti ERK1/2. Intracellular staining was performed using fixation and permeabilization technique and reagents were prepared from Transcription factor buffer kit (BD

Pharmingen). Mouse PE-and APC-conjugated IgG1 and FITC-conjugated IgG2a were used as isotype controls. Samples were analysed using flow cytometer BD FACSCanto II (BD Pharmingen, USA).

Statistical Analysis

Data were statistically analysed using Statistical Package for the Social Sciences (SPSS) 12.0.1 software. Kruskal-Wallis test was used to compare between the cellular and molecular profiling markers of treated groups with control groups. Data was presented as mean ± Standard Error of Mean (SEM). A significant difference of $p \leq 0.01$ was denoted as **.

RESULTS

Expression of cellular and molecular markers of $CD4^+CD25^-$ isolated cells from NOR mice before treatment

The markers expressed by $CD4^+CD25^-$ cells determined the functional analysis of $CD4^+$ cell subset. Thus, profiling the expressed markers of isolated $CD4^+$ $CD25^-$ T cells was performed by labelling the cells with surface and cytoplasmic markers. The surface markers identified were CD4, ICOS, TIGIT and intracellular Foxp3. Figure 1 and 2 showed the cellular profiling of isolated $CD4^+CD25^-$ T cells before treatment. Isolated cells from NOR mice spleen were predominantly expressed high levels of $CD4^+$ (93.0 ± 0.4%) and IL17 receptors or IL17R (72.5 ± 0.0 %) on the cell surfaces. However, these cells expressed lower number of inhibitory molecule TIGIT (3.6 ± 7.2%) and stimulatory molecule ICOS (2.4 ± 1.9%) as well as transcription factor Foxp3 (3.2 ± 1.5%). Figure 1 represented the dot plot showing the population of cells isolated from NOR spleens prior to treatment. $CD4^+CD25^-$ cells codominantly expressed IL17R while lower expression levels of TIGIT, ICOS and Foxp3 proteins.

Expression of cellular and molecular markers of $CD4^+CD25^-$ isolated cells from NOR mice after treatment

Figure 3 and 4 showed the cellular profiling after 120hr in vitro culture of isolated $CD4^+CD25^-IL17R^{high}$ from NOR mice. Treatment groups were divided into four groups which included $CD4^+CD25^-IL17R^{high}$ as control group for all AgNP treated and untreated groups supplemented with or without TGF-β. Group 2 comprised of $CD4^+CD25^-IL17R^{high}$ cells treated with TGF-β for induction of Foxp3-expressing $CD4^+$ cells i.e iTreg cells. Group 3 comprised of $CD4^+CD25^-IL17R^{high}$ cells treated with TGF-β and GA-AgNP to test the induction of GA-AgNP in inducing iTreg cells. Group 4 comprised of $CD4^+CD25^-IL17R^{high}$ cells treated with GA-AgNP only to test its effect on $CD4^+CD25^-IL17R^{high}$ cell subset.

The percentage of CD4 marker in stimulated cells expressing high IL17 receptor was not significantly

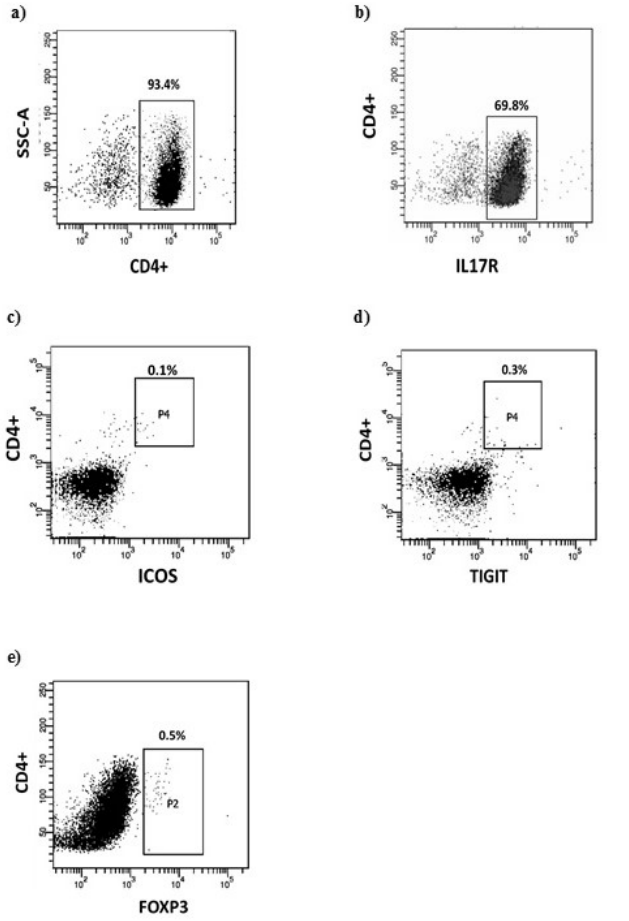

Figure 1: The expression of stimulatory ICOS and IL17R proteins, inhibitory TIGIT protein and transcription factor Foxp3. Dot plot shows the levels of protein expressed in isolated $CD4^+$ T cells before treatment. (n=5 mice/experiment).

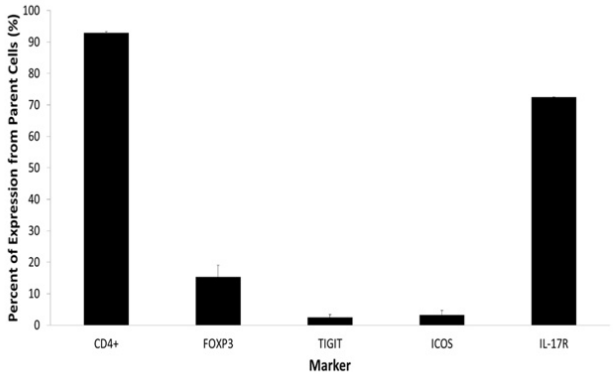

Figure 2: Mean of surface and intracellular protein profiling of isolated $CD4^+CD25^-$ cells before treatment. Isolated cells were codominantly expressed IL17R and lower expression levels of ICOS, TIGIT and Foxp3. Data shown are expressed as the percentage of cells expressing the markers from single population $CD4^+$ $CD25^-$ cells. This experiment was repeated three times and the graph was plotted based on the mean value. Error bar represents Standard Error Mean (SEM). n=5.

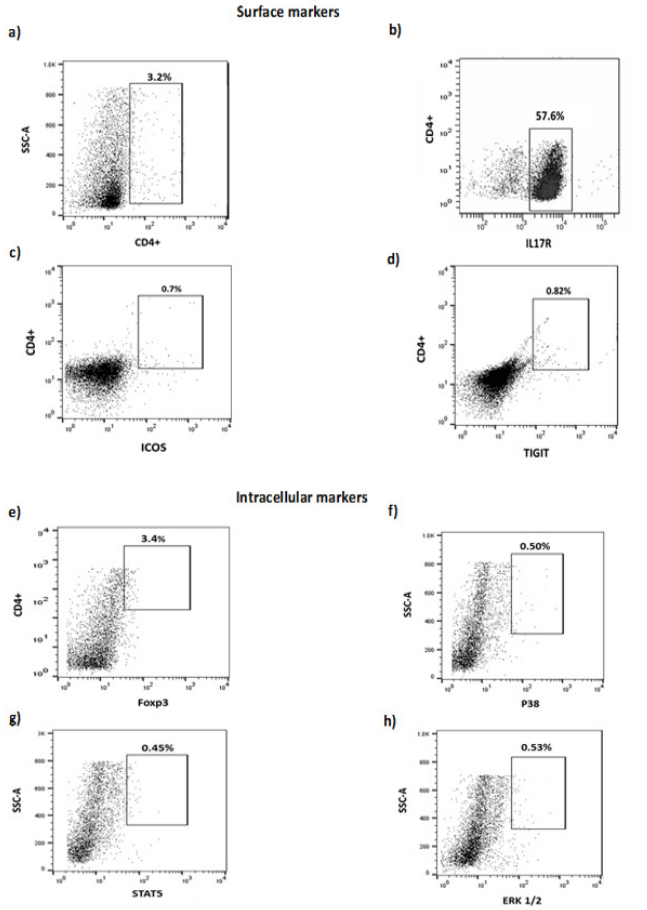

Figure 3: Dot plot representing the surface and molecular markers in $CD4^{+}IL17R^{high}$ cells after in vitro cultured treated with GA-AgNP in the presence of IL-2 and TGF-β. Dot plot shows $CD4^{+}$ ILR^{high} cells stained with FITC-anti- mouse CD4, VioBlue-anti ICOS, VioBlue-anti mouse IL17R and PE-anti mouse TIGIT for surface markers and APC- anti Foxp3, alexa fluor-anti STAT5, alexa fluor-anti p38 and alexa fluor-anti ERK1/2 for intracellular markers to determine the profile of isolated $CD4^{+}CD25^{-}$ $IL17R^{high}$ cells. Figure b) shows IL17 receptors are highly expressed in these cells following treatment. While other co stimulatory markers are not differentially expressed as compared to control group. Data shown are representative of three independant experiments. n=5

changed after 120 hr in vitro culture treated with IL-2 as compared to control group (62.9 ± 13.1 % vs 60 ± 0.2 %). In contrast, addition of GA-AgNP in $CD4^{+}IL17R^{high}$ cells supplemented with TGF-β and IL-2 in vitro culture resulted in significant reduced of CD4 expression in these cells (5.8 ± 6.0 %, $p < 0.005$). However, although the levels of IL17R expression was reduced in these cells, the changes was not significant as compared to control. These cells also expressed low levels of inhibitory TIGIT and stimulatory ICOS proteins respectively similar with the control group. In contrast, although not significant, transcriptional protein Foxp3 was slightly induced in these cells after supplemented with IL-2 and TGF-β. Similarly, STAT5 protein was not significantly phosphorylated in $CD4^{+}IL17Rhigh$ cells as compared to unstimulated cells in control group. But MAPK pathway proteins, p38 and ERK1/2 showed to be phosphorylated

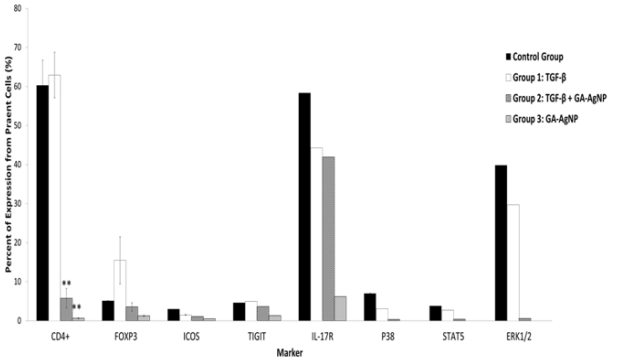

Figure 4: Mean of cellular and pathway profiling of parent cells after treatment. The mean of 15d-PGJ_2, AgNP and ctrl AgNP were compared with iTreg by using Kruskal Wallis test. Treatment groups 15d-PGJ_2, AgNP and ctrl AgNP showed lower percent of cellular and signaling pathway profiling compared to control group except Foxp3 in control $CD4^{+}$ group is lower than 15d-PGJ_2 .A significant difference of $p \leq 0.01$ was denoted as **, respectively. Error bars represents Standard Error Mean (SEM). n= 5

at lower levels although not significantly different as compared to control group.

In $CD4^{+}IL17R^{high}$ treated with GA-AgNP and supplemented with IL-2 and TGF-β, the levels of CD4 expression were significantly reduced (2: 5.8 ± 6.0 %, $p <0.005$) as compared to control (60 ± 0.2 %). However, levels of IL17 receptor were not differentially expressed in these cells as compared to control group. TIGIT and ICOS proteins remained expressed at low levels similar to control group. Similarly, Foxp3 expression was not significantly changed as compared to control group. In addition, although statistically not significant, STAT5 protein was suppressed at lower levels than control group. P38 and ERK1/2 phosphorylated proteins were also reduced as compared to control but at insignificant levels.

Meanwhile, $CD4^{+}IL17R^{high}$ cells treated with GA-AgNP without TGF-β showed to have the lowest levels of CD4 expression(0.63 ± 0.3 %) as compared to control group (60 ± 0.2 %) with significant level of $p<0.005$. Similarly, addition of GA-AgNP also significantly reduced IL17 receptor expression in these cells ($p<0.005$). Expression levels of TIGIT and ICOS were also reduced as compared to control group although not significant. Phosphorylated levels of STAT5 and p38 were also reduced in these cells treated with GA-AgNPs. Although not significant, phosphorylated ERK1/2 proteins were reduced further as compared to control group (0.0 ± 0.0 % vs 44.3 ± 0.2 %).

DISCUSSION

Activation of IL-17R on Th17 cells will lead to the release of pro-inflammatory cytokines IL17A, IL17F, IL21 and IL22 to further remove pathogenic substances (28).

However, excessive Th17 response can lead to adverse inflammatory reaction (8, 13). This will be counteract by immune regulator such as $CD4^+CD25^+Foxp3^+$ T-regulatory cells. These cells are responsible for self and non-self-discrimination and avoid adverse autoimmune reaction (14, 29).

Current study identified the synergistic effect of biosynthesized GA-AgNP in inducing anti-inflammatory properties of $CD4^+IL17R^{high}$ cells isolated from healthy non obese resistance (NOR) mouse model, thus we tested the potential effect of GA-AgNP in inducing Foxp3 expression in these cells together with ICOS and TIGIT receptors. ICOS are co-stimulatory receptors expressed on activated $CD4^+$ and $CD8^+$ cells. These receptors are analogous to CD28 and CTLA-4 activities (30-32). While TIGIT molecules opposed the effect of co-stimulatory receptors, mainly expressed by T-regulatory cells and dendritic cells (33, 34). In addition, to further dissect the molecular pathways involved, we measured the phosphorylation levels of signalling molecules involved in IL-2 pathway as well as MAPK pathways of p38 and ERK1/2 proteins. We also measured changes on IL17 receptor expression in these cells to indicate the immunomodulatory effect of GA-AgNP in these cells in healthy condition.

Our findings showed that treatment with GA-AgNP reduced expression levels of CD4 and IL17 receptors in $CD4^+IL17$ cells. However, addition of TGF-β did not altered the expression of IL17 receptors in these cells. In addition, although not significant, $CD4^+IL17R^{high}$ cells treated with GA-AgNP have lower levels of phosphorylated ERK1/2 proteins. Contrary to a previous report on the effect of TGF-β in inducing Foxp3 expression in $CD4^+CD25^-$ cells (35), our results showed that the collateral use of GA-AgNP on $CD4^+IL17R^{high}$ cells for 120hr in vitro in the presence of TGF-β does not promote the expression of Foxp3 protein in these cells. Also, addition of GA-AgNP in cells supplemented with TGF-β reduced the expression levels of TIGIT and ICOS receptors and phosphorylated proteins of MAPK pathway p38 and STAT5 proteins in $CD4^+IL17R^{high}$ cells.

Our preliminary data on the effect of GA-AgNP in modulating the pro-inflammatory properties of $CD4^+$ cells from healthy model may suggest the potential effect exerted by these nanomaterials in modulating the markers of stimulated $CD4^+$ cells during adverse inflammatory reaction. We proposed that these biosynthesized nanoparticles are capable of altering the role of IL-2 and TGF-β cytokines. These cytokines are essential and intersected at MAPK pathways (36, 37). In addition, low levels of Foxp3 expression in cells treated only with TGF-β is in contrast with previous study by Fantini and colleagues (2014) that showed similar concentration of TGF-β induced iTreg cells form $CD4^+$ T cell population (37,38). Discrepancy in these findings could be due to different incubation period used in both studies as current study exposed cultured cells to the cytokines at a shorter period. This is because current study tested the effect of GA-AgNP in 120 hr in vitro time frame while previous studies investigated the effect up to ten days without the addition of AgNP.

Current results reported that there is a significant decreased of CD4 expression levels but no significant reduced in IL17 receptors in stimulated $CD4^+IL17R^{high}$ cells following treatment with GA-AgNP supplemented with IL-2 and TGF-β. This may suggest that GA-AgNP augmented the effect of these cytokines to promote stability of IL17-expressed cells (39, 40). In fact, it is suggested that GA-AgNP can trigger plasticity of $CD4^+$ cells into non-$CD4^+$ due to the pleotropic role of TGF-β. This cytokine plays a dual role as pro-inflammatory and anti-inflammatory activities, depending on the secondary cytokine produced in the cell vicinity (41, 42). Thus, current study suggested that the addition of GA-AgNP triggers pro-inflammatory effect of TGF-β and IL-2 cytokines in $CD4^+IL17R^{high}$ cells and is highly capable of inducing plasticity in $CD4^+$ to non-$CD4^+$ cells from healthy mouse model. This is in line with previous reports by Murphy et al (2016) and Zhao et al (2016) where they concluded that conversion of $CD4^+$ cells into non-$CD4^+$ cells as a results of interaction between external factors such as cytokines and nanomaterials are necessary to regulate homeostasis (43, 44). This process is primarily mediated by activation of OX40 protein receptor. This receptor and its ligand OX40L are expressed on the activated CD4 and CD8 T cells (45). Also, Zhao and colleagues (2009) reported that inhibition of Fas/FasL will induce conversion of non-$CD4^+$ cells from $CD4^+$ cells (40). As a result, transcription factors STAT5, p38 and ERK1/2 were not phosphorylated, indicating no activation of MAPK pathway as demonstrated in current findings.

CONCLUSION

Taken collectively, current findings may contribute to the preliminary data on the effect of biosynthesized AgNP from Garcinia Atroviridis extract on immune modulatory components of normal cells from healthy model. Given the role of TGF-β as pleotropic cytokine, we proposed that the addition of GA-AgNP in TGF-β/IL2 environment triggered the plasticity of $CD4^+$ cells into different population. Further studies are necessary to identify the molecular mechanisms involved. Current findings may serve as a basis in investigating the immunotoxicity of nanomaterial used in therapeutic bio products.

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REFERENCES

1. Agha-Mohammadi S, Lotze MT. Immunomodulation of cancer: potential use of selectively replicating agents. The Journal of clinical investigation. 2000;105(9):1173-6.
2. Holtan SG, Creedon DJ, Haluska P, Markovic SN, editors. Cancer and pregnancy: parallels in growth, invasion, and immune modulation and implications for cancer therapeutic agents. Mayo Clinic Proceedings; 2009: Elsevier.
3. Naidoo J, Page D, Wolchok J. Immune modulation for cancer therapy. British journal of cancer. 2014;111(12):2214.
4. Waldmann TA. Effective cancer therapy through immunomodulation. Annual review of medicine. 2006;57.
5. Wohlfert EA, Nichols FC, Nevius E, Clark RB. Peroxisome proliferator-activated receptor γ (PPARγ) and immunoregulation: enhancement of regulatory T cells through PPARγ-dependent and-independent mechanisms. The Journal of Immunology. 2007;178(7):4129-35.
6. Awasthi A, Riol-Blanco L, Jдger A, Korn T, Pot C, Galileos G, et al. Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. The Journal of Immunology. 2009;182(10):5904-8.
7. de Lafaille MAC, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Immunity. 2009;30(5):626-35.
8. Wei L, Laurence, A., Elias, K. and O'Shea, J. IL-21 Is Produced by Th17 Cells and Drives IL-17 Production in a STAT3-dependent Manner. Journal of Biological Chemistry. 2007. 282(48), pp.34605-34610.
9. Yang X, Chang, S., Park, H., Nurieva, R., Shah, B., Acero, L., Wang, Y., Schluns, K., Broaddus, R., Zhu, Z. and Dong, C. , . Regulation of inflammatory responses by IL-17F. . The Journal of Experimental Medicine. 2008 (205(5), .):pp.1063-75.
10. Bettelli E, Oukka M, Kuchroo VK. T H-17 cells in the circle of immunity and autoimmunity. Nature immunology. 2007;8(4):345.
11. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annual review of immunology. 2009;27:485-517.
12. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology. 2010;129(3):311-21.
13. Peck A, Mellins ED. Precarious balance: Th17 cells in host defense. Infection and immunity. 2010;78(1):32-8.
14. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nature immunology. 2005;6(11):1142.
15. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299(5609):1057-61.
16. Horwitz DA, Zheng SG, Gray JD, Wang JH, Ohtsuka K, Yamagiwa S. Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease. In Seminars in immunology 2004 Apr 1 (Vol. 16, No. 2, pp. 135-143). Academic Press.
17. Zheng SG, Wang JH, Koss MN, Quismorio F, Gray JD, Horwitz DA. CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-β suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. The Journal of Immunology. 2004 Feb 1;172(3):1531-9.
18. Khatiravan, V., Ravi, S. & Ashokkumar, S. Synthesis of silver nanoparticles from Melia dubia leaf extract and their in vitro anticancer activity. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2014, 130.
19. Garvita, S., Piyoosh, K. B., Shailesh, K. S., Rajeshwar, P. S., Madhu, B. T. & Ashok, K. Green synthesis of silver nanoparticles using cell extracts of Anabaena doliolum and screening of its antibacterial and antitumor activity. Journal of Microbiology and Biotechnology. 2014, 24, 1354-1367.
20. Rajan, R., Chandran, K., Harper, S. L., Yun, S.-I. & Kalaichelvan, P. T. Plant extract synthesized silver nanoparticles: An ongoing source of novel biocompatible materials. Industrial Crops and Products. 2015, 70, 356-373.
21. Castillo PM, Herrera JL, Fernandez-Montesinos R, Caro C, Zaderenko AP, Mejнas JA, Pozo D. Tiopronin monolayer-protected silver nanoparticles modulate IL-6 secretion mediated by Toll-like receptor ligands. 2008: 627-635.
22. Wong KK, Cheung SO, Huang L, Niu J, Tao C, Ho CM, Che CM, Tam PK. Further evidence of the anti-inflammatory effects of silver nanoparticles. ChemMedChem: Chemistry Enabling Drug Discovery. 2009 Jul 6;4(7):1129-35.
23. Haase H, Fahmi A, Mahltig B. Impact of silver nanoparticles and silver ions on innate immune cells. Journal of biomedical nanotechnology. 2014 Jun 1;10(6):1146-56.
24. Alsarhan A, Sultana N, Al-Khatib A, Kadir MR. Review on some Malaysian traditional medicinal plants with therapeutic properties. Journal of Basic and Applied Sciences. 2014 Apr 18;10:149-59.
25. Tan WN, Lim JQ, Afiqah F, Nik Mohamed Kamal NN, Abdul Aziz FA, Tong WY, Leong CR, Lim JW. Chemical composition and cytotoxic activity of Garcinia atroviridis Griff. ex T. Anders. essential oils in combination with tamoxifen. Natural product research. 2018 Apr 3;32(7):854-8.
26. Tan WN, Wong KC, Khairuddean M, Eldeen IM, Asmawi MZ, Sulaiman B. Volatile constituents of the fruit of Garcinia atroviridis and their antibacterial and anti-inflammatory activities. Flavour and Fragrance Journal. 2013 Jan;28(1):2-9.

27. Tan WN, Khairuddean M, Wong KC, Khaw KY, Vikneswaran M. New cholinesterase inhibitors from Garcinia atroviridis. Fitoterapia. 2014 Sep 1;97:261-7.27 Tan WN, Wong KC, Khairuddean M, Eldeen IM, Asmawi MZ, Sulaiman B. Volatile constituents of the fruit of Garcinia atroviridis and their antibacterial and anti-inflammatory activities. Flavour and Fragrance Journal. 2013 Jan;28(1):2-9.
28. Korn T, Oukka M, Kuchroo V, Bettelli E. Th17 cells: effector T cells with inflammatory properties. InSeminars in immunology 2007 Dec 1 (Vol. 19, No. 6, pp. 362-371). Academic Press.
29. Sakaguchi S. Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. Nature immunology. 2005 Apr;6(4):345.
30. Mages HW, Hutloff A, Heuck C, Bьchner K, Himmelbauer H, Oliveri F, Kroczek RA. Molecular cloning and characterization of murine ICOS and identification of B7h as ICOS ligand. European journal of immunology. 2000 Apr;30(4):1040-7.
31. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature. 1999 Jan;397(6716):263.
32. Watanabe M, Hara Y, Tanabe K, Toma H, Abe R. A distinct role for ICOS-mediated co-stimulatory signaling in CD4+ and CD8+ T cell subsets. International immunology. 2005 Jan 24;17(3):269-78.
33. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nature immunology. 2009 Jan;10(1):48.
34. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, Levine Z, Beiman M, Dassa L, Achdout H, Stern-Ginossar N. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. Proceedings of the National Academy of Sciences. 2009 Oct 6:pnas-0903474106.
35. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-β signaling in the development of natural CD4+ CD25+ Foxp3+ regulatory T cells. Nature immunology. 2008 Jun;9(6):632.
36. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+ CD25$^-$ naive T cells to CD4+ CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. Journal of Experimental Medicine. 2003 Dec 15;198(12):1875-86.
37. Walker MR, Kasprowicz DJ, Gersuk VH, Bиnard A, Van Landeghen M, Buckner JH, Ziegler SF. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+ CD25–T cells. The Journal of clinical investigation. 2003 Nov 1;112(9):1437-43.
38. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-β induces a regulatory phenotype in CD4+ CD25$^-$ T cells through Foxp3 induction and down-regulation of Smad7. The Journal of Immunology. 2004 May 1;172(9):5149-53.
39. de Rham C, Ferrari-Lacraz S, Jendly S, Schneiter G, Dayer JM, Villard J. The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors. Arthritis research & therapy. 2007 Dec;9(6):R125.
40. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. Immunity. 2009 May 22;30(5):646-55.
41. Ruscetti F, Varesio L, Ochoa A, Ortaldo J. Pleiotropic effects of transforming growth factor-β on cells of the immune system. Annals of the New York Academy of Sciences. 1993 Jun;685(1):488-500.
42. Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA. Anti-inflammatory and pro-inflammatory roles of TGF-β, IL-10, and IL-22 in immunity and autoimmunity. Current opinion in pharmacology. 2009 Aug 1;9(4):447-53.
43. Murphy A, Casey A, Byrne G, Chambers G, Howe O. Silver nanoparticles induce pro-inflammatory gene expression and inflammasome activation in human monocytes. Journal of Applied Toxicology. 2016 Oct;36(10):1311-20.
44. Zhao X, Sun G, Sun X, Tian D, Liu K, Liu T, Cong M, Xu H, Li X, Shi W, Tian Y. A novel differentiation pathway from CD4+ T cells to CD4$^-$ T cells for maintaining immune system homeostasis. Cell death & disease. 2016 Apr;7(4):e2193.
45. Croft M, So T, Duan W, Soroosh P. The significance of OX40 and OX40L to T-cell biology and immune disease. Immunological reviews. 2009 May;229(1):173-91.