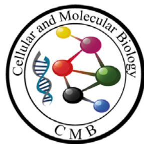

Cellular and Molecular Biology

E-ISSN : 1165-158X / P-ISSN : 0145-5680

www.cellmolbiol.org

C M B Association Publisher

Njavara rice (*Oryza sativa* Linn.) bran oil exerts anti-inflammatory effects through regulation of Notch-mediated T-cell receptor (TCR) activation in experimentally induced atherosclerosis

Lal Preethi Suja Sathyaseelan[1], Vidya Sabu[1], Antony Helen[2*]

[1] Research Scholar, Department of Biochemistry, Research Centre, University of Kerala, Kariavattom, Thiruvananthapuram Kerala 695581, India

[2] Department of Biochemistry, Research Centre, University of Kerala, Kariavattom, Thiruvananthapuram Kerala 695581, India

ARTICLE INFO

Original paper

Article history:
Received: May 8, 2022
Accepted: August 21, 2022
Published: September 30, 2022

Keywords:

Notch signaling, T cell activation, Njavara rice bran oil, Atherosclerosis, T cell signalosome complex

ABSTRACT

This study elucidated the molecular mechanism of the notch pathway in vascular health and the role of NjRBO as a nutraceutical for the modulation of notch-mediated $CD4^+$ Tcell activation in atherosclerotic rats. In this study, male Sprague–Dawley rats weighing 150–200g given standard diet formula were used. After the study duration of 60 days, in order to determine the nutraceutical effects of NjRBO, we sought to study the effects of treatment with NjRBO on notch pathway components in isolated splenic $CD4^+$ T lymphocytes. In the present study, Western blot analysis revealed that upon high-fat diet supplementation resulted in T cell activation evidenced by increased CD28 co-receptor and CD25 marker expressions. In consistent with the above findings, we analyzed the mRNA expression pattern of Notch1, cleaved notch fragment, Notch-1^{IC} and Hes1, which showed a consistent up-regulation upon T-cell activation. Immunofluorescence assay also revealed an increase in Notch 1 receptor expression. Up-regulation in the expression of TCR-activated signalosome complexes or CBM complex in the diseased showed an increase indicating that Carma1-Bcl10-Malt1 (CBM) is a crucial event for T- cell receptor-induced NF-κB activation. Additionally, NF-κB translocation was enhanced in causing a concomitant alteration in Th1, Th2 transcription factors, T-bet, GATA-3 and its respective cytokines, IFN-γ and IL-4. Accordingly, we present evidence that Notch-regulated TCR-mediated activation of $CD4^+$T-cell components was altered by NjRBO treatment, thereby revealing a novel role for the same in controlling TCR-mediated activation and inflammatory milieu.

Doi: http://dx.doi.org/10.14715/cmb/2022.68.10.4
Copyright: © 2022 by the C.M.B. Association. All rights reserved.

Introduction

Notch signaling is a fundamental cell-cell signal transduction system that controls several mechanisms essential for normal cellular development involving cell differentiation, activation and proliferation. Unlike other cell signaling pathways, its outcome remains complex through interactions with other signaling pathways, in conjunction with itself comprising several receptors and ligands, which makes its out-turn difficult to predict (1). This largely depends on the cell context in which the pathway gets activated. Notch interactions with its ligands, Delta or Jagged, cause proteolytic cleavage of the Notch intracellular domain (NICD) by presenilin (2). The intracellular fragment is then translocated to the nucleus which interacts with the transcriptional repressor, RBP-$J_κ$, converting it to a transcriptional activator (3) Notch/ RBP-$J_κ$ regulate the transcription of genes including basic-helix-loop-helix (b-HLH) transcription factors like hairy and enhancer of split 1 (Hes1) which in turn regulates cell differentiation (4). The contribution of Notch signaling to the molecular mechanism underlying atherosclerosis is limited. The early cellular events related to atheromatous plaque formation triggers complex immune responses, which eventually results in the activation of several cellular subtypes of both the innate and adaptive immune system. Recent studies reveal that notch modulates various aspects of atherosclerosis by controlling differentiation, activation and functioning of different cell types, including macrophage polarization (5,6) helper T cells and cytotoxic T-cells, B cells (7). However, notch interactions seem to regulate the functionality of immune cells in inflammatory diseases. Numerous studies have revealed that notch activation in CD4+ helper T cells is initiated by CD28-mediated TCR signaling in the presumptive absence of notch ligands (8,9,10,11,12,13). *In-vitro* and *in-vivo* studies have confirmed the differentiation of Th1 cells that occurs independently from RBP-J and has demonstrated that Notch triggers Th1 polarization through non-canonical signaling, which involves Notch1-dependent activation of the NF-κB pathway (14).

Mature T-cell activation requires the engagement of the T-cell receptor (TCR) by antigen presented through MHCs present on the surface of APCs. Also, a second co-stimulatory signaling through CD28-B7 mediated interaction is required for its sustained activation and functioning. Both TCR and CD28-mediated signals together result in the vigorous proliferation of T lymphocytes and ultimately drive them to produce effector cytokines that mediate immune responses (15). Usually IL-12 and IFN-γ

* Corresponding author. Email: helenabiochem@gmail.com

polarize $CD4^+$ T cells to the Th1 cell phenotype. Th1 cells express its lineage-specific transcription factor T-bet and secrete the signature cytokine IFN-γ, which provides protection against intracellular pathogens (16). Th2 cells involved in asthma and allergic reactions are induced by IL-4 and require the transcription factor GATA3 to secrete cytokines IL-4 and IL-13 (17).

TCR-mediated activation forms the fundamental process of the adaptive immune system and has been studied for decades. Engagement of the TCR complex is a prerequisite factor for the initiation of several signaling cascades, including the Inositol triphosphate (IP_3)-Ca^{2+}-nuclear factor of activated T cells (NFAT) pathway, Ras extracellular signal-related kinase (ERK)-AP-1 pathway, protein kinase C (PKC) θ-IκB kinase (IKK)-nuclear factor NF-κB pathway (15). Following T-cell activation, PKC-θ, which is a serine/threonine protein kinase, gets recruited to the immunological synapse (18). It plays a significant role in activating a range of signaling cascades, leading to the transcriptional regulation of T cells (19,20,21,15). Once activated, PKC phosphorylates a serine residue located in the membrane-associated guanylate kinase domain of the CARMA1 protein(22). CARMA1 is also called CARD11, which is a scaffold protein considered as a hallmark of IKK/NF-κB activation. CARMA1 constitutively associates with the plasma membrane and is recruited into lipid rafts following TCR stimulation (15). Subsequent recruitment of BCL10 and MALT1 results in the formation of the active CARMA1-BCL10-MALT1 signaling complex (23). This promotes NF-κB to translocate to the nucleus, thereby initiating the transcription of genes required for T-cell activation (24,25,26). Notch ligands regulate Th1 differentiation via signaling mediated through Notch receptors. Furthermore, Th1 cells produce its signature cytokine, IFN-γ, to promote the clearance of inflammatory antigens (27,28,29). $CD4^+$T-cell differentiation into Th1 and Th2 effector cells is driven by the master transcription factors T-bet and GATA3, respectively.

The increased understanding regarding how diet affects disease, along with higher healthcare costs, has evoked interest in food as a tool for the prevention of disease and health enhancement. Rice is the staple food variety of south India and is well-known for its medicinal properties. Among the studied ones, 'Njavara' (*Oryza sativa* L.,) is a staple medicinal rice that is endemic to south India, forms an essential part of the 'Panchakarma' (30) treatment procedure called as Njavarakhizhi and serves as an immune booster to relieve inflammatory ailments. Edible oil extracted from the germ and inner husk of rice, known as Rice bran oil (RBO), contains beneficial components like gamma-oryzanol, tocotrienols, B-complex vitamins and β-sitosterol (31). Several studies demonstrated that intake of rice bran oil (RBO) resulted in a decrease in plasma level of total cholesterol (TC) and low-density lipoprotein (LDL) in hyperlipidemia subjects (32,33,34,35). In addition, previous studies confirmed that consumption of a diet with NjRBO resulted in a decrease in atherosclerotic complications by regulating genes involved in metabolism (36) and in the modulation of inflammatory responses in hypercholesterolemic rats (37). Although evidence demonstrates the involvement of T cells in atherosclerosis and the significance of Notch signaling in its regulation, targeting Notch using therapeutic strategy in the form of dietary interventions like NjRBO in atherosclerosis has not been directly investigated. Herein, the study highlights the beneficial effects of NjRBO in modulating notch in the regulation of effector T-cell responses in experimentally induced atherosclerosis.

Materials and Methods

Extraction of Njavara rice bran oil

100g of stabilized Njavara rice bran (purchased from Njavara Eco farm, Palakkad, Kerala) was defatted using 800 ml of the petroleum diethyl ether solvent (60-80°C)(Merk Millipore) in a Soxhlet extractor for 16 hr. The extract was later evaporated on a rotary evaporator (Laborota 4000-Heidolph) at 70°C to remove the solvent and the crude extract obtained was used as Njavara rice bran oil (NjRBO) for the studies.

Animal experiments

Adult male Sprague–Dawley rats (150–200g) which were bred in the Department animal house, were used for the study. They were provided with controlled temperature (24–26°C), humidity (55–60 %) and photoperiod (12 h light–12 h dark cycle). Standard diet formula based on AIN-93M maintenance diet (38), containing 590g cornstarch, 200g casein, 15% soyabean oil and 10g α-cellulose fiber/kg diet, were fed to rats. Choline, cysteine, minerals and vitamins were added and tap water was provided *ad libitum*. All experiments were conducted as per the current institutional guidelines of the animal ethics committee (IAEC-KU-7/2015-'16-BC-AH (31)).

Experimental design

The animal grouping was as follows. Rats were divided into 4 groups of 6 rats each.

Group I: Normal (Standard diet given)

Group II: High-fat diet (HFD comprising 1.5% cholesterol and 0.5% choline)

Group III: HFD +NjRBO (100mg/kg body weight)

Group IV: HFD +Atorvastatin (10mg/kg body weight)

Njavara rice bran oil (NjRBO) crude extract was mixed along with AIN-93M standard diet formulations and given along with diet at a concentration of 100mg per kg body weight of animals.

Standard drug Atorvastatin at a dose of 10mg per Kg body weight was administered along with the standard diet. The duration of the study was 60 days.

Isolation of $CD4^+$T cells

$CD4^+$T lymphocytes were isolated from spleen single cell suspensions by the FITC-conjugated $CD4^+$ T-cell negative selection kit (Cat no: Catalog #19642, Stem Cell Technologies) by Easy-Sep purple magnet. Briefly, the spleen or lymph node was disrupted in the recommended medium consisting of PBS supplemented with 2 % Fetal Bovine Serum (FBS, Cat no: F2442, Sigma Aldrich) and 1mM EDTA (Sigma Aldrich). Aggregates were removed from the cell suspension by passing through 70 µm mesh nylon strainer and centrifuged at 120 x g for 10 minutes. The supernatant was removed and re-suspended the cells at $5x10^7$nucleated cells/ml in the recommended medium. $CD4^+$T cells were further purified by immunogenic negative selection.

Enzyme-Linked Immunosorbent assay

The presence of CD28, IFN-γ, and IL-4 was determined by Enzyme-Linked Immunosorbent Assay (ELISA) using specific antibodies. Briefly, $CD4^{+}$T cells were added to the microtiter plate cells. After incubation at 37°C for 2 h, the samples were washed to remove unbound particles. Secondly, IL-4(Cat no: ab 11524, Abcam), IFN-γ (Cat no: sc-57208, Santa Cruz) and CD28 (Cat no: ab 243228, Abcam) primary antibodies were added and incubated for 2hr. After 2hr, a secondary antibody IgG conjugated with HRP(Cat no: ab 7097, Abcam) was added and incubated for another 1h at 37°C. Finally, the substrate solution and stop solution were added and the reaction developed was measured with an ELISA reader at 450 nm. Results were expressed in OD units/mg protein.

Reverse transcription polymerase chain reaction (RT-PCR analysis)

Total RNA was extracted from isolated $CD4^{+}$T Cells using Trizol reagent (Cat no: 93289, Sigma Aldrich)(39) and its concentration was determined using nanodrop. cDNA was synthesized from the corresponding RNA using kits (Cat no: AB1453A, Thermo Scientific) as per the manufacturer's instructions. A volume of 5µg of the total RNA was used as a template in a reaction mixture comprising dNTPs, RNase inhibitors, Oligo DT and Reverse transcriptase. The reaction mixture was gently centrifuged and incubated for 5 min at 25˚C followed by 60 min at 42˚C for reverse transcription to take place. The reaction was stopped the following heating at 70˚C for 5 min. cDNA synthesis was accomplished using PCR Thermocycler (Eppendorf). Polymerase Chain Reaction (PCR) was performed to determine the gene expression of Notch1, PKC-θ, CARMA1, β- actin using a thermal cycler. cDNA 50-100ng was amplified using primers specific for Notch1, PKC-θ, CARMA1, GAPDH and β-actin following manufacturer's instructions. RT-PCR products were electrophoresed on 1.5% agarose gel and Ethidium bromide (EtBr) stained bands were visualized and quantified using gel documentation system (Bio-Rad) by the densitometric method. β-actin and GAPDH was used as internal standard and signals were expressed relative to their intensity in each sample.

Quantitative real time PCR (q-PCR)

RNA was extracted from $CD4^{+}$T cells using TRIZOL reagent (Cat no: 93289, Sigma Aldrich) following (39). cDNA was synthesized and q-PCR reactions were performed in a reaction volume of 20µl consisting of 4µl of cDNA, 1µl of forward (F) and reverse primer (R) each and 10µl of 2x using SYBR green Real-time PCR 185 kit (Cat no: A46112, Thermo Fisher Scientific) method following manufacturer's instructions. qPCR reaction was run in an Eppendorf Mastercycler after a pre-incubation at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 20 sec and annealing at 59°C for 15 sec. Expression levels were quantified using the comparative Ct (ΔCt) method using the formulae:

ΔCT = CT (a target gene) – CT (a reference gene).

Where ΔCt is the difference between the mean Ct value of triplicates of the sample and of the endogenous reference control.

Table 1. List of primers for RT-PCR.

S. No	Primer names	Primer sequences (5'-3')
1.	Notch1 FP	5′-CTGCAGTGACAACATTGATGACTGTGCC-3′
	Notch1 RP	5′-ACACTGACACTCGAAAGAGCCCAGTGTG-3′.
2.	PKC-θ FP	5′-CAATG GCCTT CTTAA GATCA AAA-3′
	PKC-θ RP	5′-CCTGA GAGATC GATGATC ACATAC-3′
3.	CARMA1 FP	5′-TTGTGGGAGAATGTGGAGTGT-3′
	CARMA1 RP	5′-TGCCCCTTGGTATGTAGAATG-3′
4.	GAPDH FP	5′-AACCACAGTCCATGCCATCAC-3′
	GAPDH RP	5′-TCCACCACCCTGTTGCTGTA-3′
5.	β-actin FP	5'-GGCTGTATTCCCCTCCATCG-3'
	β-actin RP	5'-CCAGTTGGTAACAATGCCATGT-3'

Table 2. List of primer sequences for qPCR.

S. No	Primer names	Primer sequences (5'-3')
1.	Notch1^{1C} FP	5′CACCCATGAC-CACTACCCAGTT3′
	Notch 1^{1C} RP	5′CCTCGGACCAATCA-GAGATGTT3′
2.	Hes1 FP	5′-ACCGGACAAACCAAAGACAGCCTCTG-3′
	Hes1 RP	5′-CTGCAGGTTCCGGAGGTGCTTCACTG-3′
3.	Bcl-10 FP	5′-GTTGTTCGTGGCTCC ATCTG-3′
	Bcl-10 RP	5′-ACAGATGAAGTGCTGAAACTTAG-3′
4.	MALT-1 FP	5′-CATGTTTGAGACCTTCAACAC-3′
	MALT-1 RP	5′-CCAGGAAGGAAGGCTGGAA-3′
5.	T-bet FP	5′-CGGCTGCATATCGTTGAGGT -3′
	T-bet RP	5′-GTCCCCATTGGCATTCCTC -3′
6.	GATA3 FP	5′-TCATTAAGCCCAAGCGAAGG -3′
	GATA3 RP	5′-GTCCCCATTGGCATTCCTC -3′

Immunofluorescence

Cells (5x10³cells per chamber) were seeded into culture slides (Axigen). The next day, cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde (Merk Millipore) for 10 min at room temperature, followed by permeabilization with 0.1% Sodium Citrate plus 0.1% Triton X-100 (Merk Millipore). The cells were further subjected to immunofluorescence staining with Notch1 (Cat no: sc-376403, Santa Cruz) (1:500) antibody for 2 h at room temperature. The cells were then washed with cold PBS three times for 3 min each and incubated with FITC-labelled secondary antibody (1:800) (Cat no: 7074, Cell signalling Technology) at room temperature for 1 h. Nuclei were counterstained for 5 minutes with 5 mM DAPI (Cat no: D9542, Sigma Aldrich). The cells were examined by fluorescence microscopy. Images were acquired using a (Zeiss, Life technologies) microscope.

Western blotting

Isolated $CD4^+$ T cells were lysed with the Protein Extraction Reagent (Merk, Millipore) at room temperature. After lysis, the cell debris was removed *via* centrifugation at 15,000 rpm for 10 min at 4°C. Supernatants were electrophoresed on SDS-10% polyacrylamide gel at 140 V for 1hr at room temperature. Proteins (10–50µg) were resolved and transferred to nitrocellulose membranes (Millipore) at 100 V for 1.5 h. After blocking the f non-specific sites, the membranes were treated with primary antibody (1:1,000 anti-rat CD25 (Cat no: Cat #PA5-117480, Invitrogen)and CD28 (Cat no: Cat no: ab 243228 Abcam) at 4°C overnight, followed by incubation with secondary antibody (1:2000 anti-rat IgG; Cat no: ab 6734, Abcam) for 1 h. The bands were detected using the enhanced chemiluminescence (ECL) detection reagents(Pierce™ ECL Western Blotting Substrate, Cat no: 32109).

Statistical analysis

Results were expressed as mean with standard error of mean using the statistical program SPSS/PC+, version 17.0 (SPSS Inc). Statistical evaluation was performed using one-way ANOVA, and the significance of results was analyzed using Duncans's test of $P < 0.05$.

Results

Njavara rice bran oil (NjRBO) decreases T-cell receptor (TCR) activation by lowering CD28 and CD25 expression

CD25 surface marker was detected to analyze the activation of $CD4^+$ T cells. It was found that HFD increased the expression of CD25 in isolated $CD4^+$ Tcells. Whereas dietary administration of NjRBO could mitigate the effect by lowering its protein level expression as detected by the western blot method (Fig 1A). CD28 is a co-stimulatory receptor expressed by activated $CD4^+$ T cells and its coordination with TCR is essential for T-cell activation, differentiation and survival, making the CD28 pathway a key checkpoint for controlling T-cell responses (40,41). $CD4^+$ T-cell co-receptor signaling molecule CD28 protein expression was demonstrated by the ELISA method on isolated $CD4^+$ T cells. Results showed that the mean levels of CD28 levels in T lymphocyte fraction in the control group was 0.408 units/mg protein; In HFD supplemented group, CD28 levels were elevated (0.9649 units/mg protein) than in the control. Whereas Njavara rice bran oil (NjRBO) down-regulated (Mean value of 0.523 units/mg protein) (Fig. 1C) resulted in a marked decrease in its co-receptor activity. This change was further confirmed using western blot analysis, as shown in Fig. 1B.

mRNA expression of notch components Notch1, $Notch1^{1C}$ and Hes1 were altered by NjRBO

To investigate the involvement of Notch signaling in peripheral T-cell activation and polarization, the mRNA expression levels of Notch l, its intracellular cleaved fragment $Notch1^{1C}$ and thymic differentiation factor, Hes l in $CD4^+$ T cells of normal and atherosclerotic rats were measured by RT-PCR and q-PCR. As shown in Fig 2, compared with the control group, mRNA expression in the atherosclerotic HFD group increased significantly for Notch l (mean value 19.20 ± 1.643, $p<0.05$), $Notch1(^{1C})$ (2.21 ± 0.22, $p<0.05$) and Hes1 (2.32 ± 0.189, $p<0.05$). Upon NjRBO supplementation revealed that lower expression of Notch1 (9.11 ± 1.32, $p<0.05$), $Notch1^{1C}$ (0.600 ± 0.12, $p<0.05$) and Hes1 (1.18 ± 0.22, $p<0.05$) compared to high-fat diet treated group. Hence, data showed that lowered Notch1, intracellular cleaved fragment $Notch1^{1C}$ and Hes1 expression was associated with supplementation of NjRBO in atherosclerotic rats. This showed a more effective response than atorvastatin treatment.

Njavara rice bran oil (NjRBO) decreases PKC-θ mRNA expression in $CD4^+$ T cells

In T-lymphocytes, PKC theta forms a connecting link for T-cell antigen receptor (TCR)-triggered activation of mature T cells (44). The results from the 1.5% agarose gel electrophoresis experiment revealed that PKC-θ

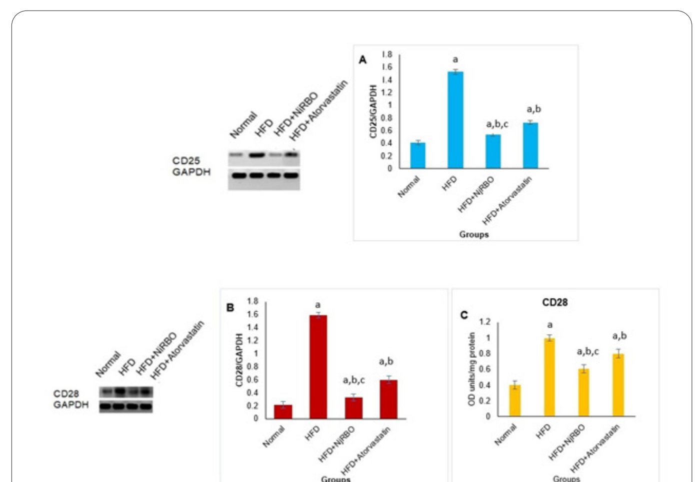

Figure 1. **A** Immunoblot result showing the protein expression of CD25 in whole-cell lysate by western blotting. Expression of GAPDH was monitored to ensure uniform protein loading in all lanes. **B** Immunoblot of CD28 protein levels (representative immunoblot from three independent experiments) in $CD4^+$ T-cell fraction, quantification of immunoblot band intensities for CD28 (normalized to GAPDH, n = 3). C CD28 (Cluster of Differentiation 28), co-stimulatory signaling protein expression was quantified on $CD4^+$ T cells using the ELISA method. Measurements were done in triplicate, expressed as units per mg protein. Group I - Normal, Group II - HFD, Group III - HFD + NjRBO, Group IV - HFD + Atorvastatin. Values expressed as mean± SEM in each group. a- Significant difference when compared with group I at p<0.05. b- Significant difference when compared with group II at p<0.05, c- Significant difference when compared with group IV at p<0.05

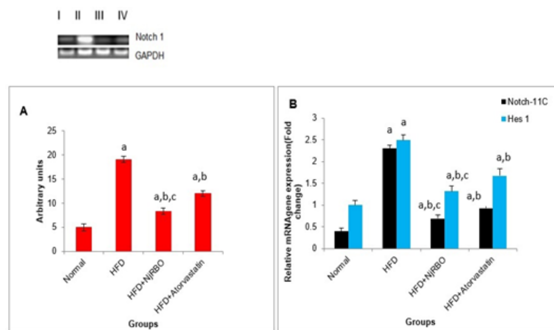

Figure 2. The expression of Notch 1, Notch1^{IC} and Hes1 is inhibited upon NjRBO oil supplementation **A** Gel image of the RT-PCR result of Notch 1 was analyzed in splenic CD4^{+} T lymphocytes and the arbitrary units were measured to evaluate the mRNA expression with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalization control **B** Relative mRNA expression of Notch1^{IC} and Hes1 in CD4^{+} T cells quantified using real-time PCR. Group I- Normal, Group II- HFD, Group III-HFD + NjRBO, Group IV- HFD+ Atorvastatin. Values expressed as mean± SEM in each group. a- Significant difference when compared with group I at p<0.05. b- Significant difference when compared with group II at p<0.05, c- Significant difference when compared with group IV at p<0.05.

was present at a higher level in activated T cells in HFD given group compared to NjRBO treated group, where its expression was diminished. (Fig 4). β-actin was used as a positive control.

Njavara rice bran oil (NjRBO) alters T-cell signalosome complex CARMA-1/Bcl-10/MALT-1 and lowers NF-κB mediated activation in CD4^{+} T lymphocytes

PKC-dependent signaling pathway comprises the triad of CARMA1 (scaffolding protein), Bcl-10 (small linker protein), and MALT-1 (an effector protein), also known as the CBM signalosome. The scaffold protein caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA-1) is a signaling protein that constitutes an essential component in TCR signaling in CD4^{+} T-lymphocytes (45). We investigated the role of CBM signalosome components Bcl-10/MALT-1/CARMA-1 in splenic lymphocytes by analyzing its expression through reverse transcriptase-polymerase chain reaction (RT-PCR) and q-PCR. RT-PCR showed lower density bands of CARMA-1 upon NjRBO (5.296 ± 0.47) consumption than in the HFD group (26.34 ± 0.343). Relative gene expression of MALT-1 and Bcl-10 as assessed through fold change and showed a decrease upon oil consumption (Fig 5A and Fig 5B). Nuclear localization of the $_{p}$65 NF- κB component is important in the progression of the disease. NF-κB translocation was studied by the ELISA technique in both cytosolic and nuclear fractions of T-cells. NF-κB was expressed in the nuclei of CD4^{+} T cells of the HFD of the HFD-treated group, but a decreased expression was shown upon treatment with NjRBO (Fig 5C). Hence our results suggest that T- cell signalosome component expression in CD4+T cells correlated closely with NF-κB expression and this activation could be decreased by NjRBO.

Njavara rice bran oil (NjRBO) regulates the expression of T-bet and GATA-3 mRNA in CD4+Tcells and its correlated cytokines IFN-γ and IL-4

CD4^{+} T cells are divided into the Th1 lineage characterized by the production of cytokines IFN-γ and TNF-α; and in contrast, Th2 cells based on the secretion of cytokines IL-4, IL-5, IL-13, and IL-10 (46). Quantitative RT-PCR (qRT-PCR) was used to demonstrate the relative expression levels of T-bet and GATA-3 mRNA in T cells. As shown in Fig 6A, CD4^{+} Tcells of HFD expressed higher levels of T-bet mRNA (0.512 ± 0.025) than of controls (0.129 ± 0.045) (P < 0.05) and lower levels of GATA-3 mRNA (0.091 ± 0.012) than of controls (0.321 ± 0.02) (P < 0.05) (Fig 6B), resulting in a much higher T-bet/GATA-3 ratio in HFD treated groups compared to controls (P < 0.05). Whereas NjRBO could alter this by showing

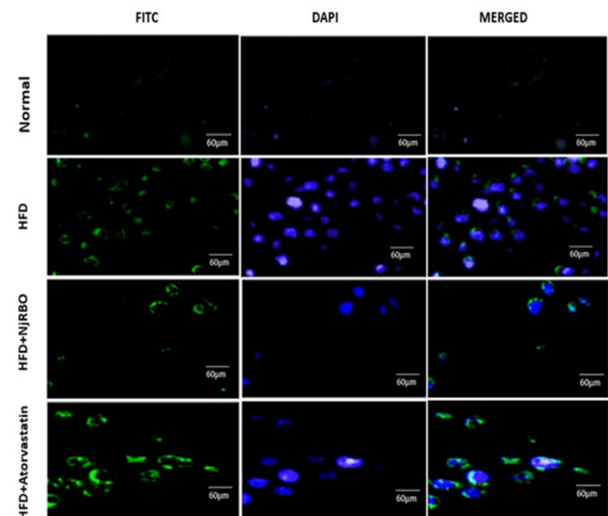

Figure 3. Representative images of immunofluorescent images staining for notch receptor (Notch1) in isolated CD4+T cells. DAPI staining is indicated in blue and notch staining is indicated in green. Scale bar =60µm.

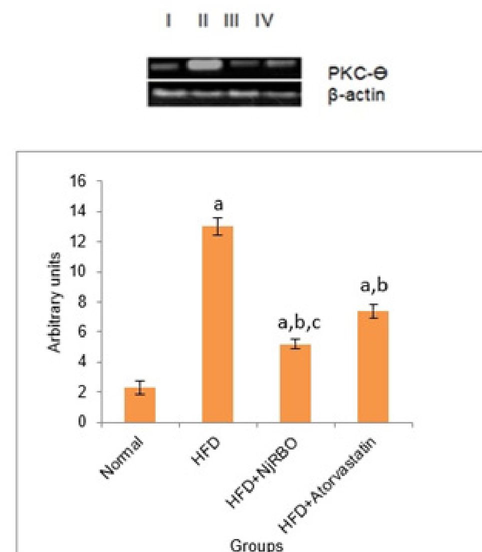

Figure 4. Expression analysis of protein kinase C θ (PKC-θ) in isolated T lymphocytes by RT-PCR. Total RNA was isolated from cell samples and reverse transcribed using random hexamers as primers and the product was run for PCR with PKC- θ specific primers. β-actin is used as a control. Groups were Group I- Normal, Group II- HFD, Group III- HFD + NjRBO, Group IV- HFD + Atorvastatin. Values expressed as mean± SEM in each group. a- Significant difference when compared with group I at p<0.05. b- Significant difference when compared with group II at p<0.05, c- Significant difference when compared with group IV at p<0.05.

down-regulated expression of Th1 specific transcription factor T-bet and an up-regulation in GATA3 expression. T-bet mRNA expression in CD4$^+$ Tcells was significantly correlated with its respective cytokine IFN-γ secretion (Fig 6C) ($P < 0.05$), which showed an increased protein level in HFD treated group. There was a significant down-regulation observed in cases with NjRBO treatment (1.74±0.23). IL-4 cytokine is selectively expressed by Th2 cells, hence mounting a model for Th2-specific gene expression. In order to determine whether NjRBO could alter IL-4 mediated differentiation and expansion of Th2 cytokine-producing in cells, protein levels were assessed, which showed an increase in its production (0.993 ±0.03) compared to the diseased (0.344±0.023) (Fig.6D).

Discussion

To the best of our knowledge, the findings in this study are novel as it highlights the need for the effectiveness of Njavara rice bran oil dietary intervention as an effective anti-atherogenic treatment option by regulating inflammatory notch-mediated T-cell adaptive response. Notch signaling has attractive potential as a therapeutic target candidate in atherosclerosis and the precise roles of the various notch members in T-cell development and activation need to be explored and should be utilized for novel treatment strategies. This novel signaling pathway has been extensively studied in T-cell development, its activation, and effector functions. TCR stimulation activates naive CD4$^+$ Tcells and causes its differentiation into multiple subsets of T-helper (Th) cells characterized by their specific cytokine profile(47,48). Even Though therapies that tend to activate/regulate notch signaling were known to improve T-cell-mediated immune response, the efficacy and safety of this approach need to be much studied. Herein the protective effects of NjRBO were evaluated by observing notch-regulated T-cell activation pathway components and related protein and mRNA expression.

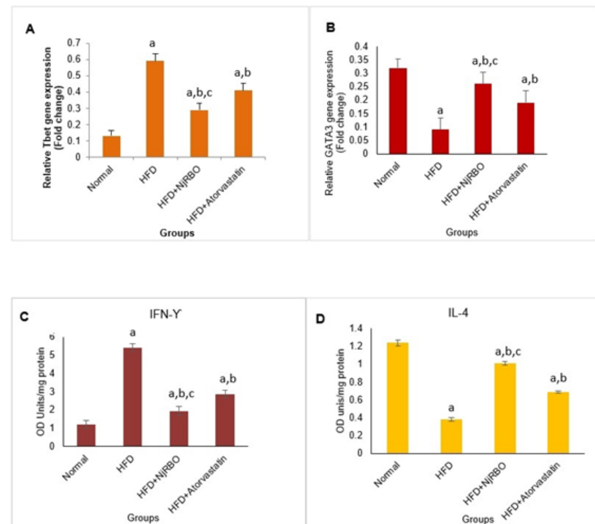

Figure 6. Th1/Th2 markers were quantitated by real-time PCR and ELISA techniques. **A** T-bet and **B** GATA 3 mRNA were measured via qPCR. Protein expression of cytokines **C** IFN-γ and **D** IL-4 measured by ELISA. Group I - Normal, Group II - HFD, Group III - HFD + NjRBO, Group IV -HFD + Atorvastatin. Values expressed as mean± SEM in each group. a- Significant difference when compared with normal at $p<0.05$. b- Significant difference when compared with HFD at $p<0.05$, c- Significant difference when compared with Atorvastatin at $p<0.05$.

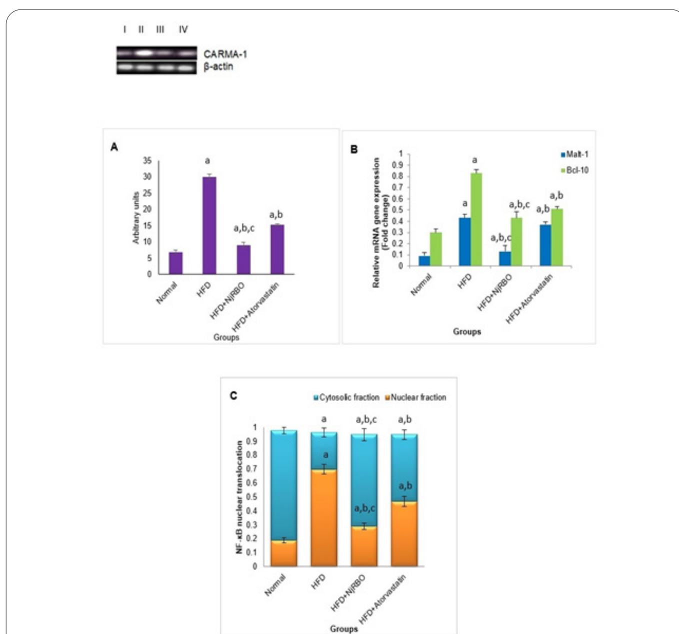

Figure 5. A Expression of CARMA1/Bcl10/MALT1 in isolated T lymphocytes by PCR. Total RNA was isolated from cell samples and reverse transcribed with primers specific for CARMA1. Values normalized to β-actin used as control. **B** Real time quantitative PCR showing mRNA levels of Bcl10/MALT1. Real-time q-PCR was performed with the SYBR GREEN PCR master mix. The amounts of Bcl10/MALT1 transcripts were normalized with GAPDH as control. The data are reported as the mean ±SD of four independent experiments. **C** NF-Kβ translocation rate was quantified in the CD4+T lymphocyte cytosolic and nuclear fraction by ELISA. Groups were Group I- Normal, Group II-HFD, Group III- HFD +NjRBO, Group IV- HFD + Atorvastatin. Values expressed as mean± SEM in each group. a- Significant difference when compared with group I at $p<0.05$. b- Significant difference when compared with group II at $p<0.05$, c- Significant difference when compared with group IV at $p<0.05$.

Stimulation of TCR promotes the surface expression of the transmembrane alpha chain of the IL-2 receptor (CD25) on T cells (49). CD25, a surface marker of CD4$^+$ T-cells, is upregulated upon T-cell activation and serves as a therapeutic target in inflammation. A prior investigation into the immunomodulatory properties of NjRBO in CD4$^+$ T cells demonstrated its ability to inhibit T-cell proliferation by showing a decreased expression of CD25 on activated CD4$^+$ T-cells. Its protein expression was analyzed by western blotting which showed a considerable decrease upon supplementation with NjRBO compared to the HFD-treated group. Peripheral T-cell activation requires T-cell engagement with its co-receptor CD28, which is controlled by T-cell specific kinase, PKC-θ. Previous studies describe PKC-θ as playing an essential role in activating the classical NF-κB signaling cascade (50). As previously reported, studies have confirmed that full-length CD28 expression is detected in activated T lymphocytes (51). In line with the above evidence, we demonstrated that both CD28 and PKC-θ protein expression in T cells showed a considerable increase upon HFD exposure which was mitigated by NjRBO. In the present study, though both atorvastatin and RBO significantly caused the reversal in inflammatory notch-regulated T-cell activation,

NjRBO supplementation caused a considerable decrease which was more pronounced that atorvastatin which may be intolerant to some patients. Hence these results revealed that despite the beneficial effect of atorvastatin in ameliorating dysregulation of T-cell functions, NjRBO treatment as a dietary nutraceutical caused a profound decrease in T-cell activation markers and the concomitant decrease in PKC-θ in the development of atherosclerosis.

We aimed to determine the Notch receptor activation during TCR-mediated signaling by monitoring Notch1 and its cleaved fragment, Notch1^{IC} expression at mRNA levels. There are numerous studies suggesting that Notch1 has an obligatory role in CD4^{+}T cell lineage induction (52,53). Elevated expressions of Notch1 and Notch1^{IC} could be linked to notch signaling. Hence in conclusion, we have determined that our results from gene expression studies and immunofluorescence imaging of Notch receptor, Notch1 revealed that a lowered expression pattern was shown upon NjRBO administration. In line with the above findings, then we evaluated whether Hes1 mRNA expression was induced following *in-vivo* notch signaling and revealed that expression levels of the Notch1-regulated genes Hes1 were up-regulated in the isolated splenic CD4^{+} T cells samples of high-fat diet (HFD) as determined by qPCR. In CD4^{+} T-cells, Notch1 can also become activated following receptor stimulation to generate the NICD, which migrates to the nucleus and induces gene transcription of effector genes of the pathway, Hes1. Since Hes1 transcriptional induction by the NICD controls critical aspects of thymic T-cell differentiation (54,55), our q-PCR results demonstrated that NjRBO administration resulted in decreased T- cell receptor-mediated induction of Hes1 gene.

The CBM signalosome complex plays a major pathophysiological role in T- lymphocytes and in adaptive immunity. T-cell activation requires the assembly of a signaling module composed of three proteins, CARMA1, Bcl10, and MALT1(56). So, we determined whether NjRBO treatment might mitigate the inflammatory effects of the CBM signalosome complex composed of CARMA-1, Bcl-10, and MALT-1 proteins within CD4^{+}T cells. Using quantitative RT-PCR, a finding that is reflected at the mRNA level was demonstrated, showing that the CBM signalosome can be considered as a treatment target by NjRBO, and the potential exists for exploring its food-based dietary approach as a novel treatment option for the prevention of atherosclerosis.

Next, we studied whether this protection correlated with levels of Th1 and Th2 specific transcription factors and inflammatory cytokines and that are strongly implicated in the pathogenesis of atherosclerosis. Naive T-helper (Th) cell differentiation towards Th1 or Th2 cells is regulated by the transcription factors T-box expressed in T-cells (T-bet) and GATA-binding protein-3 (GATA-3) (57-58). T-bet and IFN-γ factors which are well-known Th1-specific inflammatory markers showed the most significant reduction ($p < 0.05$) upon treatment with NjRBO. The cytokine milieu produced by activated CD4^{+} T cells is one of the crucial determinants for fate decision into different effector Th subtypes. An increase in Th1 differentiation followed by a concomitant decrease in Th2 cytokine production and differentiation was observed in the diseased high-fat diet (HFD) group. NjRBO oil supplementation maintained the cytokine levels by increasing IL-4 cytokine production and up-regulating Th2 immune response by elevated GATA3 at the protein level.

Conclusion

To date, no dietary nutraceuticals interfering with the Notch signaling are approved for inflammatory diseases like atherosclerosis. Based on the present preliminary investigations regarding this to the first of its kind, our study proposed that modulating the Notch signaling through NjRBO could offer a new therapeutic option in reducing the disease severity. NjRBO exerts potent regulatory effects in modulating notch-mediated T-cell activation by down-regulating several notch pathway components associated with atherosclerosis. Hence, the present study reinforces the use of NjRBO as an alternative natural hypolipidemic agent useful for the management of risk factors associated with atherosclerosis.

Ethics approval

All experiments were conducted as per the current institutional guidelines of the animal ethics committee (IAEC-KU-7/2015-’16-BC-AH (31)).

Acknowledgements

Funding

The authors declare that no funds, grants were received during the preparation of the manuscript.

Competing interest

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions

Aswathi IS assisted me in conducting writing, statistical analysis and conceptualization, Monisha Simon dealt with Data curation, Santhi Krishnan dealt with drafting of the manuscript and Jasmine Peter helped me to do analytical techniques.

References

1. Schnute B, Shimizu H, Lyga M, et al. Ubiquitylation is required for the incorporation of the Notch receptor into intraluminal vesicles to prevent prolonged and ligand-independent activation of the pathway. BMC Biol 2022; 20(1):65.
2. Jarriault S, Brou C, Logeat F, et al. Signalling downstream of activated mammalian Notch. Nature 1995; 377: 355–358.
3. Fazil M, Prasannan P, Wong BHS, et al. GSK3β Interacts with CRMP2 and Notch1 and Controls T-Cell Motility. Front Immunol 2021;12:680071.
4. Amira MB and Yasmen FM Notch Pathway and its Role in Cardiovascular System. Sys Rev Pharm 2020;11(10): 342-349.
5. Wang C, Ma C, Gong L, et al. Macrophage Polarization and Its Role in Liver Disease. Front Immunol 2021;12:803037.
6. Singla DK, Wang J, Singla R. Primary human monocytes differentiate into M2 macrophages and involve Notch-1 pathway. Can J Physiol Pharmacol 2017; 95:288–94.
7. Vieceli Dalla Sega F, Fortini F, Aquila G, et al. Notch Signaling Regulates Immune Responses in Atherosclerosis. Front Immunol 2019; 10:1130.
8. Thomas, Portia & Shanker, Anil. Notch as an Immunologic Basis of Cancer Disparities. Cancer health disparities 2019; 3. e1-e10.
9. Dongre A, Surampudi L, Lawlor RG, et al. Non-canonical Notch

signaling drives activation and differentiation of peripheral CD4+ T cells. Front Immunol 2014; 5:54.
10. Imam S, Dar P, Aziz SW, et al. Immune Cell Plasticity Allows for Resetting of Phenotype from Effector to Regulator with Combined Inhibition of Notch/eIF5A Pathways. Front Cell Dev Biol 2021; 9:777805.
11. Guy CS, Vignali KM, Temirov J, et al. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. Nat Immunol 2013; 14:262–70.
12. Mitra, Ankita & Shanthalingam, Sudarvili & Sherman et al. CD28 Signaling Drives Notch Ligand Expression on CD4+ T Cells. Frontiers in Immunology 2020; 11. 10.3389/fimmu.2020.00735
13. Steinbuck MP, Arakcheeva K, Winandy S. Novel TCR-mediated mechanisms of Notch activation and signaling. J Immunol 2018; 200:997–1007.
14. Christopoulos PF, Gjølberg TT, Krüger S, et al. Targeting the Notch Signaling Pathway in Chronic Inflammatory Diseases. Front Immunol 2021; 12: 668207.
15. Hwang JR, Byeon Y, Kim D, et al. Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. Exp Mol Med 2020; 52:750–761.
16. Szabo SJ, Kim ST, Costa GL, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000; 100(6):655-69.
17. Saotome M, Poduval DB, Nair R, et al. GATA3 Truncation Mutants Alter EMT Related Gene Expression via Partial Motif Recognition in Luminal Breast Cancer Cells. Front Genet 2022;13:820532.
18. Xie J, Han X, Zhao C, et al. Phosphotyrosine-dependent interaction between the kinases PKCθ and Zap70 promotes proximal TCR signaling. Sci Signal 2019; 12(577):eaar3349.
19. González-M N, Rodríguez-R C, A lcover A, et al. Sorting Nexin 27 Enables MTOC and Secretory Machinery Translocation to the Immune Synapse. Front Immunol 2022;12:814570.
20. Fu G, Hu J, Magnenat NN, et al. Protein kinase C η is required for T cell activation and homeostatic proliferation. Sci Signal 2011; 4(202):ra84.
21. Singleton KL, Roybal KT, Sun Y, et al. Spatiotemporal patterning during T cell activation is highly diverse. Sci Signal 2009; 2(65):ra15.
22. Shah K, Al-Haidari A, Sun J, et al. T cell receptor (TCR) signaling in health and disease. Signal Transduct Target Ther 2021; 6(1):412.
23. Blonska M and Lin X NF-kappaB signaling pathways regulated by CARMA family of scaffold proteins. Cell Res 2011; 21:55–70.
24. Sapski S, Beha N, Kontermann RE, et al. Influence of antigen density and immunosuppressive factors on tumor-targeted costimulation with antibody-fusion proteins and bispecific antibody-mediated T cell response. Cancer Immunol Immunother 2020; 69(11):2291-2303.
25. Pfeifhofer C, Kofler K, Gruber T, et al. Protein kinase C theta affects Ca2+ mobilization and NFAT cell activation in primary mouse T cells. J Exp Med 2003; 197(11):1525-35.
26. Sun Z, Arendt CW, Ellmeier W, et al. PKC-theta is required for TCR-induced NF-kappa B activation in mature but not immature T lymphocytes. Nature 2000; 404 (6776):402-7.
27. Hope JM, Dombroski JA, Pereles RS, et al. Fluid shear stress enhances T cell activation through Piezo1. BMC Biol 2022; 20(1):61.
28. Krueger PD, Goldberg MF, Hong SW, et al. Two sequential activation modules control the differentiation of protective T helper-1 (Th1) cells. Immunity 2021; 54(4): 687-701.e4.
29. Amsen D, Antov A, Flavell RA The different faces of Notch in T-helper-cell differentiation. Nat Rev Immunol 2009; 9:116–124.
30. K K, Bindu & Joshi, Mrudula & Karthik, Uma. Contribution of Kerala to ayurvedic literature in panchakarma with special reference to Panchakarmam Athava Sodhana Chikitsa: A Review. International Journal of Research in Ayurveda and Pharmacy 2018; 9: 30-34.
31. Lee, Ayoung & Ko, Juyeon & Ahn, et al. Potential Effects of Pigmented rice on Immunity: A Review Focusing on Anthocyanins, Gamma-oryzanol, and Arabinoxylan. Journal of Food and Nutrition Research 2021; 9: 26-31.
32. Vissers MN, Zock PL, Meijer GW, et al. Effect of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum lipoprotein concentrations in humans. Am J Clin Nutr 2000; 72 (6):1510–1515.
33. Mahdavi-Roshan M, Salari A, Vakilpour A, et al. Rice bran oil could favorably ameliorate atherogenicity and insulin resistance indices among men with coronary artery disease: post hoc analysis of a randomized controlled trial. Lipids Health Dis 2021;20(1):153.
34. Devarajan, Sankar & Singh, Ravinder & Chatterjee, et al. A blend of sesame oil and rice bran oil lowers blood pressure and improves the lipid profile in mild-to-moderate hypertensive patients. Journal of Clinical Lipidology 2015; 10. 10.1016/j.jacl.2015.12.011.
35. Talebi S, Bagherniya M, Atkin SL, et al. The beneficial effects of nutraceuticals and natural products on small dense LDL levels, LDL particle number and LDL particle size: a clinical review. Lipids Health Dis 2020; 19(1):66.
36. Yucheng Z, Xingrong J, Wenye C, et al. Rice bran attenuated obesity via alleviating dyslipidemia, browning of white adipocytes and modulating gut microbiota in high-fat diet-induced obese mice. Food Funct 2020; 11:2406.
37. Pushpan CK, Salini V, Sindhu G, et al. Attenuation of atherosclerotic complications by modulating inflammatory responses in hypercholesterolemic rats with dietary Njavara rice bran oil. Biomedicine and Pharmacotherapy 2016; 83:1387-1397.
38. Surono IS, Wardana AA, Waspodo P, et al. Effect of functional food ingredients on gut microbiota in a rodent diabetes model. Nutr Metab (Lond) 2020;17:77.
39. Jaeschke A, Harvey NR, Tsurkan M, et al. Techniques for RNA extraction from cells cultured in star PEG-heparin hydrogels. Open Biol 2021;11(6):200388.
40. Esensten JH, Helou YA, Chopra G, et al. CD28 co-stimulation: From Mechanism to Therapy. Immunity 2016; 44(5):73-88.
41. Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in immunotherapy. Blood 2018; 131(1):58-67.
42. Marrocco V, Fiore P, Benedetti A, et al. Pharmacological Inhibition of PKCθ Counteracts Muscle Disease in a Mouse Model of Duchenne Muscular Dystrophy. EBioMedicine 2017; 16:150-161.
43. Fields PE, Kim ST, Flavell RA. Cutting edge: Changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. J Immunol 2002; 169:647–650.
44. Murphy KM and Reiner SL. The lineage decisions of helper T cells. Nat Rev Immunol 2002; 2 (12):933-44.
45. Mullen AC. High FA, Hutchins AS, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science 2001; 292(5523):1907-10.
46. Xi, Yang & Jingying, Dai & Chenglong, et al. Epigenetic Therapy Promotes the Ratio of Th1/Th17 Lineage to Reverse Immune Evasion and Treat Leukemia Relapse Post-Allogeneic Stem Cell Transplantation in Non-APL AML Patients. Frontiers in Molecular Biosciences 2021; 7. 10.3389/fmolb.2020.595395.
47. Hernandez R, Põder J, LaPorte KM, et al. Engineering IL-2 for immunotherapy of autoimmunity and cancer. Nat Rev Immunol 2022; 10.1038/s41577-022-00680-w.
48. Lin X, Mahony OA, Mu Y, et al. Protein Kinase C-θ Partici-

pates in NF-κB activation Induced by CD3-CD28 co-stimulation through Selective Activation of I-κB Kinase β. Molecular and cellular biology 2000; 20:2933-40.

49. Glinos DA, Soskic B, Williams C, et al. Genomic profiling of T-cell activation suggests increased sensitivity of memory T cells to CD28 co-stimulation. Genes Immun 2020; 21:390–408.
50. Gloor, Andrea Daniela & Berry, Gerald & Goronzy, et al. Age as a risk factor in vasculitis. Seminars in Immunopathology 2022; 10.1007/s00281-022-00911-1.
51. Osborne BA, Minter LM. Notch signaling during peripheral T-cell activation and differentiation. Nat Rev Immunol 2007; 7(1):64-75.
52. Wendorff AA, Koch U, Wunderlich FT, et al. Hes1 is a critical but context-dependent mediator of canonical Notch signaling in lymphocyte development and transformation. Immunity 2010; 33(5):671-84.
53. Wong GW, Knowles GC, Mak TW, et al. HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCRβ-selected mouse thymocytes. Blood 2012; 120(7):1439-48.
54. McAllister-Lucas LM, Jin X, Gu S, et al. The CARMA3-Bcl10-MALT1 signalosome promotes angiotensin II-dependent vascular inflammation and atherogenesis. J Biol Chem 2010; 285(34):25880-25884.
55. Liu L, Deng YQ, Jiao WE, et al. Maggot extracts promote regulatory T cell differentiation by upregulating Foxp3 in allergic rhinitis. Am J Transl Res 2021;13(12):13540-13554.
56. Zhu Y, Ding H, Wang Z, et al. Effect of epigallocatechin-3-gallate on the activity of alpha-glucosidase in vitro. Wei Sheng Yan Jiu 2010; 39(2):168-71, 176.
57. Nowroz F, Roy T, Haque M, Ferdous J, Noor R, Mondal G. Yield and Grading of Potato (Solanum tuberosum L.) as Influenced by Different Mulch Materials. Agrotech Ind Crops 2021; 1(1): 1-10. doi: 10.22126/etic.2021.6209.1001
58. Schmitt N, Ueno H. Regulation of human helper T cell subset differentiation by cytokines. Curr Opin Immunol 2015; 34:130-6.