ORIGINAL RESEARCH

Cytokine and transcription factor analysis on activation of T lymphocytes of peripheral blood

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Abstract

Background: The immune system is a highly controlled network that enables the emergence of antigen-specific defensive mechanisms against outside antigens and tolerance to self-antigens.

Objective: Isolation of CD3+ T cells from PBMCs of healthy individuals and analyse T cell proliferation, secretion of IFNgamma, IL-2, IL-4 and IL-17, and expression of Tbet, GATA-3 and RORgammaT post activated and culture of CD3+ T cells

Material And Method: The study was conducted in the department of oral pathology and microbiology, Dr. D Y Patil Dental College and Hospital Pimpri Pune. Blood samples were collected from 5 healthy individuals. The study parameters included isolation of peripheral blood mononuclear cells. Activation of CD3+ T cells and analysis of T cell proliferation, secretion of cytokines and gene expression. The study was approved by the college ethics committee.

Result: 1.68% proliferation CD4+CD69+ t cells are seen without activation and with activation 30.78% proliferation of CD4+CD69+ t cells are observed. In case of CD4+CD25+ t cells 3.56% proliferation seen without activation and 29.01% cells are seen with activation. When cytokine secretions were analysed IL2 secretion was increased and downregulation was seen with IL4, IL17, IFNγ.

Key Words: CD3+T cells, immunomodulation, cytokines, GATA 3, Tbet, RORγt

Introduction

Innate and adaptive defences make up the mammalian immune system, which guards against infections while preventing dangerous overactivation[1]. Recognizing antigens and coordinating immune responses, T and B cells are important participants[1,2]. When activated improperly, as in autoimmunity or allergy, the host can suffer serious consequences[2].

A subset of T lymphocytes known as CD4+CD25+ regulatory T cells (Tregs) suppresses unwarranted immune responses to preserve immunological homeostasis[3]. They have a crucial role in conditions including diabetes, colitis, and cancer by preventing autoimmunity through cell-to-cell contact and cytokines like TGF-β4. Systemic autoimmune diseases result from the depletion of these cells, highlighting their critical regulatory function[5].

C-type lectindomain-containing type II enzyme responsible cellular functions group includes CD69 as a member6. It is among the first indicators produced by NK cells, macrophages, neutrophils, eosinophils, T and B lymphocytes, after activation[7]. A helpful marker for assessing the cytotoxic function of NK cells is CD69[8]. Although the specific function of CD69 expression on immune cells is still unknown, mounting experimental data suggests that CD69 may control the homing and migration of activated lymphocytes as well as patterns of cytokine release[7,8].

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Cytokines

The pluripotent cytokine interferonγ (IFNγ), which belongs to the wider family of factors known as interferons and is called for its capacity to prevent the development of live viruses[9,10]. IFNγ's numerous biological effects are linked to cancer, autoimmune disorders, allergies, obesity, and pregnancy[11,12,13].

One of the families of polypeptides that mediates interactions between leukocytes is interleukin-2 (IL-2)[14,15,16]. In the course of an immune response, T cells secrete IL-2, which is

essential for the development of naive T cells into effector T cells[17,18].

It has been demonstrated that IL4 is essential for several biological processes. Antigen- presenting cells are encouraged to proliferate and differentiate by this cytokine.

Six structurally related cytokines make up the IL-17 family: IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F[19]. Even though Th17 cells often thought to be the main producer of IL-17, it has been demonstrated that CD8+ cells, sometimes known as "Tc17," can also produce this cytokine[20,21].

Gene expression

Transcriptional regulator T-bet, sometimes referred as Tbx21, is a crucial Immunological signaling pathway[22]. Northern blot study of various organs revealed that the lung, thymus, and spleen are the primary sites where T-bet is expressed[23,24].

A transcription factor called GATA3 is essential for the growth and differentiation of lymphocytes, breast tissue, and hair follicles[25]. GATA binding protein 3 (GATA3) is one of six transcription factors that make up a family. Promoting cell differentiation in a number of tissues and organs, such as the skin, trophoblasts, parathyroid glands, urothelial system, kidneys, and central nervous system, is another regulatory role of GATA3[26].

RORγt is expressed by lymphoid and myeloid cells in the immune system, and it is elevated during Th17 development[27,28]. RORgamma t controls the synthesis of IL-17 in the periphery and directs the development of pro-inflammatory T helper 17 (T(H)17) cells, which are crucial in inflammatory diseases and autoimmunity[29,30].

Materials and Methods

This study has been conducted in Dr. D Y Patil Dental College and Hospital Pimpri, Pune. Samples of peripheral blood from 5 healthy individual collected from Department of Oral Pathology and Microbiology (Ref.No.DYPDCH/EC/648/53/2021). Further study such as t cell activation, culture and cytokine analysis has been conducted in Logical Life Sciences Pvt Ltd, Pune.

a. Sample collection

Written consent was obtained from the healthy donors. 10 mL of blood sample was drawn from 5 healthy donors in lithium heparinized tubes. Tubes were mixed well and stored at 40C until the PBMCs isolation procedure was initiated.

b. Peripheral blood mononuclear cell isolation

The samples were underlaid as a 1:1 volume with Ficoll-Paque medium (HISEP LSM 1073) equal to the sample volume. The Samples were centrifuged at 2500 rpm for 30 minutes at room temperature. After centrifugation, PBMCs were harvested present in a buffy layer from Ficoll- Paque medium layers into a fresh tube. Then cells were washed with PBS and centrifuged at 2000 rpm for 5 minutes at 4^0c for two times and the supernatant was discarded. The cell pellet was resuspended in 2 ml TexMACS™ medium (CAT.NO- 130-097-196), from that 10 ul cells were taken and diluted to 500 ul PBS and cell count analysis was performed on (Miltneyi Biotec's MACSQuant 10 - flow cytometer). The cell count was calculated as per dilution factor = cell count x50x 2000

c. **T cell activation**

APCs were prepared in a ratio of 1:1 of activation beads and antibodies from Miltneyi Biotec's T cell expansion kit (CAT NO- 130-091-441). The T cell activation was carried out in 24 well culture plates. First, each donor's cells were added as per cell count. APCs were added in a ratio of 1:2 to that of the cells. TexMACS™ medium was added concerning a final volume of 1 ml. Culture plates were incubated in a CO2 incubator at room temperature for 15 hours.

d. Analysis of surface activation markers using flow cytometry

After incubation samples were centrifuged at 2000 rpm for 5 minutes at 4^0C, from the supernatant 50 µl was taken for cytokine analysis. The samples were divided into tubes and labeled with CD69, CD25. including control. In each tube 1 µl of CD3 (Anti-human REAfinity PE -CAT.NO. - 130-113-139) was added. The 1.5 µl of each CD69 (Anti-human REAfinity APC -CAT.NO. - 130-112-614) CD25 (Anti-human REAfinity APC -CAT.NO. - 130-113-284) antibodies were added as per labeled tubes. Later antibody addition, samples were incubated at 4^0C for 30 minutes, wrapped with aluminum foil. After incubation samples were adjusted up to 200 ul with PBS and analyzed on flow cytometry at appropriate channels.

e. Cytokine analysis

Cytokine bead array was performed by using miltenyi biotec's MACSplex cytokine 12 multi assay kit. (CAT.NO -130-099-169). The 50 µl sample was taken into a 1.5 ml polystyrene tube. The 15 µl capture beads were Resuspended in the sample for at least 30 secs and it was vortexed before use for well mixing and incubated for 2 hr at room temperature in dark. After 2hr incubation 0.5 ml of MACSPlex Buffer was added to each tube. And centrifuged at 3000×g for 5 minutes. The supernatant was carefully aspirated by leaving 20 ul in the tube. The MACSPlex Capture Bead pellet was resuspended in each tube by adding 0.5 mL of MACSPlex Buffer and pipetted up and down and centrifuged at 3000×g for 5 minutes. The supernatant was carefully aspirated by leaving 20 ul in the tube. The 15 ul of detection reagent was added to each tube and incubated for 1 hr. 0.5 ml of MACSPlex buffer was added to each tube and centrifuged at 3000 x g for 5 minutes. The samples were analyzed by miltneyi biotec's MACs Quant flow cytometer at PE and FITC channels.

f. Gene expression

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

T cells were seeded in a 24 titer well plate for 48 hrs at 37°C with continuous humidified 5% CO2 supply. Post 48 hrs of co-culture total RNA was isolated using PureLink™ Total RNA Blood Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Total RNA was used for cDNA synthesis using cDNA synthesis kit (Applied Biosystems), according to the manufacturer's instructions (2 µg of RNA; 1 cycle 48° C/30 min; 95° C/5 min) and stored at -20° C. The quantification of mRNA expression levels for GATA- 3, T-BET and ROR-YT was carried out on QuantStudio™ 5 Real-

Time PCR System using specific primers (IDT). Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) was used for real-time monitoring of amplification (2 µl of template cDNA; 40 cycles: 95 ° C/ 15 s; 60° C/ 1 min) with appropriate primers. Expressions of target genes were normalized to human GAPDH using the ΔΔCt method. The cycle threshold (CT) values for each gene were corrected using the mean CT value. RT-qPCR data were quantified using the 2-ΔΔCt method and presented as relative gene expression normalized to the average CT for the human GAPDH gene.

The primers used are shown in table below;

Gene Name	Sequence
GAPDH -F	GTCTCCTCTGACTTCAACAGCG
GAPDH -R	ACCACCCTGTTGCTGTAGCCAA
T-BET - F	ATTGCCGTGACTGCCTACCAGA
T-BET R	GGAATTGACAGTTGGGTCCAGG
ROR-YT - F	5 -TCACCTGTG AGGGGTGCAAG-3
ROR-YT - R	5 -GTTCGG TCAATGGG GCAGTT-3
GATA-3 F	ACCACAACCACACTCTGGAGGA
GATA-3 R	TCGGTTTCTGGTCTGGATGCCT

Results

In our study we have collected blood from healthy donor. Then the peripheral blood mononuclear cells were isolated. Post activation of T cells analysis of surface marker is done using flow cytometry. 1.68% proliferation CD4+CD69+ t cells are seen without activation and with activation 30.78% proliferation of CD4+CD69+ t cells are observed (Table 1). In case of CD4+CD25+ t cells 3.56% proliferation seen without activation and 29.01% cells are seen with activation (Table 1). When cytokine secretion is analysed IL2 secretion was increased that is 35.09773776pg/ml. downregulation is seen with IL4 that is - 57.30639098pg/ml, IL17 - 5.544207317pg/ml, IFNγ -88.12034384pg/ml (Figure 1). Expression of transcription factor T- bet was increased that shows ct value 1.42710691, for GATA 3 it is 1.426221933 and when RORγt gene expression were seen it shows ct value 1.338674106 (Figure 2).

Discussion

The human body defends the organism against a number of dangerous pathogens whilst still limiting unwarranted or overly enthusiastic immune reactions that might be damaging to the host.

Lukheeram et al in 2011[1] reviewed that a key component of the adaptive immune system, CD4 T cells are necessary for producing a regulated and efficient immune response to infections. Through unique phenotypes and cytokine patterns, they affect both innate and adaptive immunity. Their specific responsibilities are further highlighted by recently discovered subtypes such as Tfh and Th9. Technological developments are exposing epigenetic modifications that contribute to their uniqueness. Future therapeutic uses could benefit from this expanding understanding. Takeuchi Y et al in 2016[2] reviewed that CD4- regulatory T cells (Tregs) are essential for immunological homeostasis and self-tolerance, but they can potentially promote tumor growth by inhibiting antitumor responses. They are now recognized as prospective targets in cancer immunology and significant participants in tumor immunity. Their whole function in the cancer treatments of today is yet unknown, though. Our study's observation of Tregs' changed expression of transcription factors points to functional flexibility that could affect their activity in tumor settings. This is in line with the need to comprehend Treg behavior better in order to create cancer immunotherapies that work.

Table 1: CD4+CD69+ t cell proliferation and CD4+CD25+ t cell proliferation in percentage with activation and without activation

	CD4+CD69+ t cell proliferation in %	CD4+CD25+ t cell proliferation in %
Without Activation	1.68	3.56
With Activation	30.78	29.01

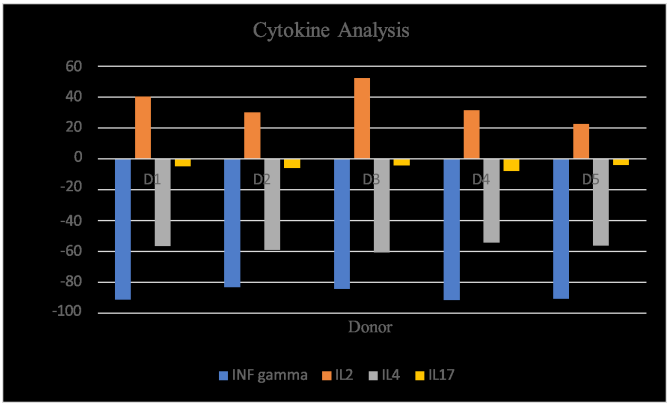

Fig. 1: Cytokine analysis of INF gamma, IL2, IL4, IL17

Fig. 2: Expression of transcription factors

Thornton et al in 2000[14] reviewed CD4+CD25+ regulatory T cells (Tregs) are important suppressors of autoimmune responses, requiring TCR activation and cell contact but inhibiting T cell proliferation by blocking IL-2 production and acting without APC involvement. The transcription factors T-bet, GATA3, and RORγt are master regulators of Th1, Th2, and Th17 cells, respectively. Our study found an increase in these transcription factors, which may indicate a shift toward effector T cell differentiation, potentially opposing Treg-mediated suppression. Levings MK et al in 2001[4] demonstrate that, human $CD4^+CD25^+$ regulatory T cells cannot multiply outside of peripheral circulation suggests that they are reliant on outside cues. Their decreased CD40 ligand activity suggested that their activation potential was limited. These Tregs did, however, show markedly elevated CTLA-4 expression, indicating improved suppressive activity. In our study transcription factors T-bet, GATA3, and RORγt were found to be overexpressed in these cells. This points to a change in the transcriptional profile that might connect Th1, Th2, and Th17-associated pathways to regulatory function.

Kak et al in 2018[9] reviewed an immune cytokine that controls gene expression and offers antimicrobial defense is interferon-gamma (IFNγ). Comprehending its function in immunological dysregulation, autoimmune diseases, and infections is essential. Examining IFNγ tolerance or resistance can provide insight into intricate disease processes. According to this study, their downregulation in healthy PBMCs might be an indication of tolerance mechanisms or early immunological modulation. Mojic et al in 2018[10] concluded that In immunocompetent hosts, IFNγ is essential for anti-tumor action and tumor behavior shaping. Additionally, it affects pathways that could result in a tumor microenvironment that suppresses the immune system. In healthy PBMCs, downregulation of IFNγ might be a sign of early immunological modulation associated with tumor tolerance. This raises the possibility of consequences for resistance mechanisms and the advancement of cancer. Teunnisen et al in 199831 Contributing to cutaneous inflammation and chronic dermatoses, activated T cells' production of IL-17 and IFNγ is crucial in inducing keratinocytes to release proinflammatory cytokines. IL-17 has a strong effect on keratinocyte behavior, much like the well-known stimulant IFNγ. Both cytokines increase the production of cytokines and surface chemicals, which intensifies the skin's inflammatory response. Our study's cytokine analysis of healthy people's PBMCs revealed downregulated levels of IL-17 and IFNγ, suggesting a non-inflammatory immune profile. Under typical circumstances, this indicates a lower likelihood of chronic skin irritation.

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