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Research Article

Exploring the Impact of Leucocyte Ig-like Receptor B1 on TNIP1 Protein Expression in Immune Effector Cells

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ABSTRACT

Background: Leucocyte Immunoglobulin-like Receptor B1 (LILRB1), also known as Immunoglobulin-like Transcript 2 (ILT2) or CD85j, is an inhibitory receptor that plays a crucial role in regulating immune effector cell activity. LILRB1 exerts its inhibitory function through Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM)-mediated signaling, which dampens immune responses and prevents excessive inflammation.

TNFAIP3-Interacting Protein 1 (TNIP1) is a key negative regulator of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, a transcription factor that plays a pivotal role in immune and inflammatory responses. TNIP1's regulatory function is essential for maintaining immune homeostasis and preventing autoimmune diseases.

Objective: The objective of this study is to investigate the impact of LILRB1 stimulation on TNIP1 protein expression levels in various immune effector cells, including:

- T cells (CD4+ and CD8+ T cells),- B cells

Monocytes, Myeloid cells (dendritic cells and macrophages)

Understanding the relationship between LILRB1 and TNIP1 may provide valuable insights into the mechanisms governing immune regulation and potentially identify novel therapeutic targets for autoimmune and inflammatory diseases [1].

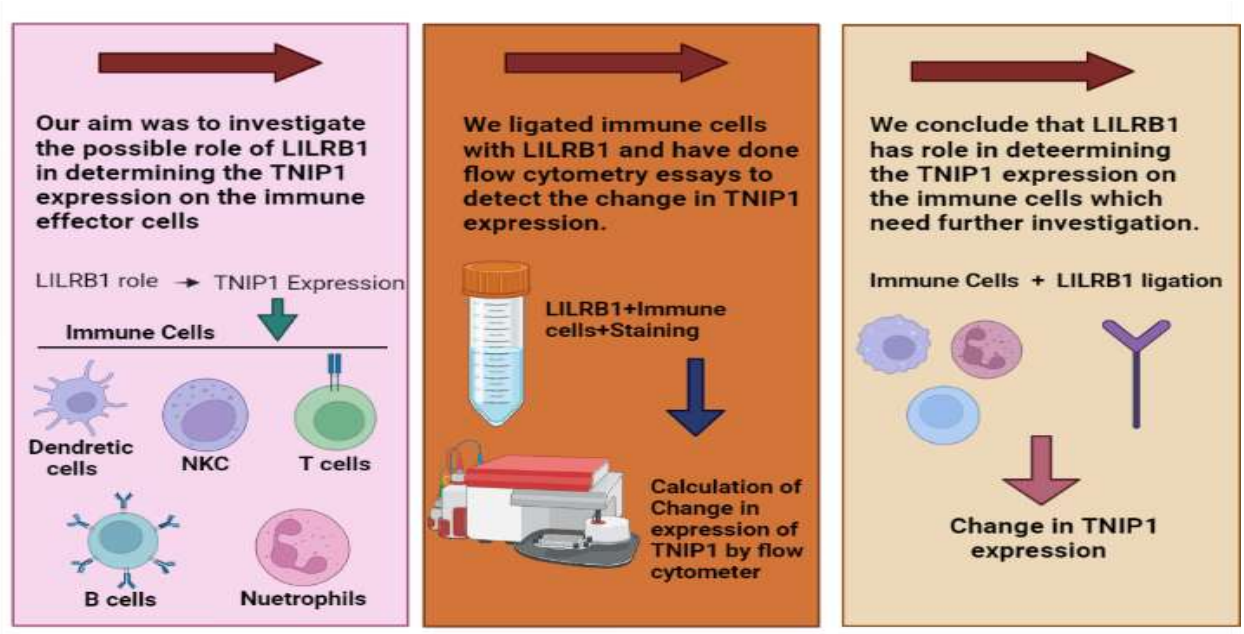
Methods: Peripheral blood samples were collected from healthy donors and leukocytes were isolated using RBC lysis buffer. Cells were incubated with LILRB1 antibody, TNIP1 antibody, and lineage-specific markers (CD8, CD14, CD15, and CD19) at different time intervals (0 h, 2 h, 4 h, and 24 h).

Results: LILRB1 stimulation significantly increased TNIP1 expression in CD8+ T cells (average PE 681,294.72; ~8× control) and CD15+ myeloid cells (average PE 100,659.1; ~1.2× control). CD14+ monocytes showed high expression at baseline but declined with increasing incubation time (average PE 193,264.75; ~2× control). In contrast, CD19+ B cells exhibited the lowest TNIP1 expression (average PE 69,010.5), with values decreasing below control levels at 24 h. Overall, expression patterns varied by cell type and incubation period, indicating differential regulatory mechanisms.

Conclusion: It is concluded that LILRB1 stimulation modulates TNIP1 expression in a cell-specific and time-dependent manner, enhancing expression in T cells and myeloid cells while reducing it in monocytes and B cells over prolonged incubation.

Keywords: LILRB1, TNIP1, immune effector cells, flow cytometry, inhibitory receptor, immune regulation

Graphical Abstract:



INTRODUCTION

When our body is exposed to infections, it responds by a complex group of signals produce by receptors. Immunoglobin (Ig) family play very important role in the immune system as

immunomodulatory receptors, adhesion molecules, costimulatory proteins and antigen receptors. One of the subtypes of Ig receptor is leukocyte Ig like receptors (LILR) that play its role in both adaptive and innate immunity and also, known as myeloid inhibitory receptors. Leucocyte Ig-like receptor B1 (LILRB1) is a subtype family of LILR which is expressed within a broad range of immune cells [1]. LILRB1 plays crucial role in the modulation of the immune system, and it is expressed within B cells, dendritic cells, monocytes, natural killer cells (NK) and T cells. It also plays very important role in the several disorders such as cancers, cardiovascular diseases and different infections [2]. For example, several studies have shown that Cytomegalovirus (CMV) infection is associated with the expression level of LILRB1 [3]. TNIP1 is a protein that acts as a negative regulator of the NF-kB signaling pathway and this pathway plays vital role in the immune and inflammatory responses [4]. Moreover, there are many disorders such as glioma and gastric carcinoma that are associated with the polymorphism in TNIP1 gene [5]. A study has investigated that due to increase in the TNIP1 mediated by LILRB1, immunosuppressive monocytes infiltrating tumour associated lymph nodes and dendritic cells have an immature phenotype. Also, it reduces the performance of antigen presenting cell. It decreases the level of costimulatory molecules and histocompatibility, poor phagocytosis, reduce cytokine secretion function and decrease in the response of T lymphocyte response. Moreover, level of TNIP1 can be decreased by using small interfere nucleic acid and it increases the performance of dendritic cells and immunosuppressive monocytes. Also, increase the performance of antigen presenting cells and cell surface expression [6]. LILRB1 is expressed with variety of cancers such as metastatic breast cancer, triple negative breast cancer, Sezary cells (a type of cutaneous T cell lymphoma), B cell lymphoma, acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML) and gastric cancer cell lines [7]. LILRB1 is expressed in various type of cells and expression level can vary with the cell type. In human beings LILRB1 is expressed by the monocytes and B cells. Also, expression in monocytes is higher than the B cells. Increase in LILRB1 expression can inhibit the response of B cells and some conditions can increase this expression level such as malaria which can interfere with TNF-α signaling and disrupt the effective immune response against infections. HLA-G is another factor that contributes in the inhibition of tumour growth by interacting with the LILRB1 [8]. Also, a study indicates that there is a possibility HLA-G interact with LILRB1 to treat B cell malignancies [9]. Natural killer NK cells that are also known as cytotoxic lymphocytes has very low expression level of LILRB1 but it can vary in different individuals. Moreover, ligand binding of LILRB1 can stop the activation of LILRB1 expressing NK cells, in result it leads to immune evasion by Plasmodium falciparum and tumor [10]. LILRB1 can also inhibit the antibody dependent cellular cytotoxicity of natural killer cells mediated by cetuximab in the triple negative breast cancer [11]. One of the studies also suggest that LILRB1 helps natural killer cells to stop the HIV replication [12]. LILRB1 has very low expression level in T cells, and it is limited to predominantly memory [13]. The expression level of LILRB1 can increased during differentiation of T cells and expressed only on CD8-T cells. A study investigated and find out people who were infected with cytomegalovirus (CMV) had much higher expression level of LILRB1 in the T cells, B cells, natural killer cells, and in monocytes than normal healthy people. Polymorphism in LILRB1 can is also one of the possibilities of different expression level of LILRB1 in different individuals [14].

Objective

- To investigate the role of Leucocyte Ig-like receptor B1 (LILRB1) in regulating the expression level of TNIP1 protein in immune effector cells, and to determine how this interaction contributes to the modulation of immune signaling and homeostasis.

METHODOLOGY

This was an experimental in vitro study conducted at Amiri Hospital, Kuwait from October 2023 to October 2024. Peripheral blood samples were collected from healthy volunteers after obtaining informed consent. Leukocytes were isolated, stained with fluorescently conjugated antibodies, and analyzed using flow cytometry to quantify TNIP1 expression in relation to LILRB1 status.

Sample Collection

Peripheral blood was collected under aseptic conditions by a trained phlebotomist. EDTA-coated plastic tubes were used to prevent coagulation. The order of draw was maintained to avoid cross-contamination. Samples were stored at 4°C and processed promptly, avoiding freeze–thaw cycles to minimize analyte degradation (World Health Organization, 2010). Each participant was identified, consented, and labeled tubes were prepared. A suitable antecubital vein was selected and cleaned with 70% isopropyl alcohol and 2% chlorhexidine gluconate. Venipuncture was performed using a 16-gauge needle, and blood was drawn into EDTA tubes. After collection, the tourniquet was released, pressure was applied at the puncture site, and samples were inspected for complications before storage.

Isolation of White Blood Cells (WBCs)

Leukocytes were isolated using the red blood cell (RBC) lysis buffer method, which improves the efficiency of flow cytometry by reducing erythrocyte contamination. The bulk lysing technique (Lyse–Stain–Wash, LSW) was employed due to its ability to process larger volumes and reduce cell loss compared to tube lysing. RBC lysis was performed using an ammonium chloride-based buffer prepared in the laboratory according to standardized protocols (Kalina et al., 2012; O'Donahue et al., 2021).

Preparation of RBC Lysis Buffer

A 10× stock solution was prepared with NH_4Cl (80.2 g), $NaHCO_3$ (8.4 g), and disodium EDTA (3.7 g) in 900 mL distilled water. The pH was adjusted to 7.4, and the volume was made up to 1000 mL. A fresh 1:10 working solution was prepared for each experiment.

PBS Preparation

PBS was prepared using NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g) per liter of distilled water, pH adjusted to 7.4. PBS was used for washing and resuspension to maintain cell integrity. Blood samples were mixed with RBC lysis buffer at a 1:5 ratio and incubated for 10 minutes. After centrifugation at 300×g, the supernatant was discarded. If RBCs remained, the lysis step was repeated. The leukocyte pellet was washed twice with PBS and resuspended in RPMI medium for further use. Leukocyte suspensions (1×10^6 cells per tube) were incubated with TNIP1 primary antibody, followed by PE-conjugated goat anti-mouse IgG secondary antibody. Non-

specific binding was blocked with mouse serum. Cells were then stained with lineage-specific antibodies (CD3, CD4, CD8, CD15, CD19, and CD59, FITC conjugates). After fixation in permeabilization buffer, cells were stored at 4°C until flow cytometry analysis.

Table 1: Recipe for the PBS water

Component	Amount	Concentration
Potassium Phosphate Monobasic (mw: 136.09 g/mol)	0.245 g	0.0018 M
Sodium Phosphate Dibasic (mw: 141.96 g/mol)	1.44 g	0.01 M
Potassium Chloride (mw: 74.55 g/mol)	0.2 g	0.0027 M
Sodium Chloride (mw: 58.44 g/mol)	8 g	0.137 M

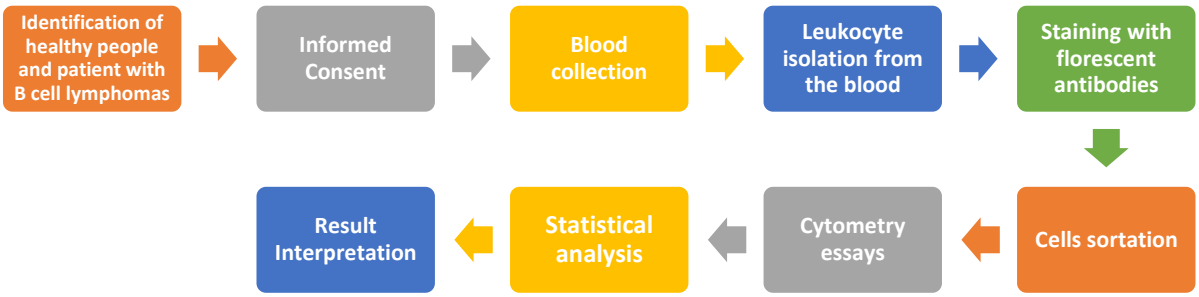
To make 10 litre stocks of 10x PBS we can add 800 g sodium chloride to the 8-litre solution of distilled water or any suitable container. Add 20 g of potassium chloride to the solution then add 144 g sodium phosphate dibasic then add 24 g of potassium phosphate monobasic. After mixing that, and filled the bottle up to 10 L. Then pH should be maintained at 7.4 by using the HCL or NaOH. While making buffer solutions, pH should always be measured by using the pH meter.

Florescent Antibodies and Flow Cytometry

After the isolation of leukocytes, they are processed for the fluorescence activation cell sorting (FACS). Specific conjugating antibodies are used to florescent the target population of cells. Some of the florescent antibodies that are used in this experiment are given in the table 1. For example, to target the CD8 T cells and CD4 T cells we can use CD8, CD7, CD8 and CD3 (fluorescently labelled) antibodies. To label the antibodies, take a small tube (eppendorf) containing $1x10^6$ cells and washed them with 1 mL PBS solution and centrifuge it for 3 to 4 minutes at 300g. Then discard the supernatant by using pipette and resuspend the pellet. Add 2 µL of TNIP1 and leave it for 10 minutes. Then wash the tube with 1 ml PBS and centrifuge it at 12000 RPM. Discard the supernatant and add 2 µL of Goat anti-mouse IgG-PE and leave it in the dark for 20 minutes. Again, wash it with 1 ml PBS and centrifuge it at 300g. Add 10 µL of mouse serum and leave it for 20 minutes again in the dark. Again, wash it with 1 ml PBS and centrifuge at 300g and discard the supernatant. Add 2 µL of Lineage-specific CD marker antibody-FITC (CD8, CD15, CD19, CD59) and leave it in the dark for 20 minutes. Again, wash it 1 ml PBS and centrifuge at 300g. Resuspend the pellet with 50 ml fixation buffer (permeabilization buffer). Mix thoroughly and store them at 4C for the cytometry analysis. We can analyse the processed samples with flow cytometer (such as by using such as BD LSR II flow cytometer).

Table 2: Showing the target cell population and their specific antibodies (Zola et al., 2005).

Cell Type	Florescent Antibodies
CD8 T cells and CD4 T cells	CD3, CD8, CD4CD7
B Cells	CD19, CD20, CD22
Myeloid cells	CD15, CD117, CD45, CD34
Natural Killer Cells	CD57, CD161, CD56



Statistical Analysis

Data were analyzed using SPSS version 26.0. Continuous variables (e.g., mean fluorescence intensity of TNIP1) were expressed as mean ± standard deviation (SD). Comparisons between LILRB1-positive and LILRB1-negative subsets were made using independent t-test. A p-value < 0.05 was considered statistically significant.

RESULTS

We took the several blood samples and then we had added the LILRB1 antibodies to ligate TNIP1 and then we added specific CD marker (such as CD8, CD14, CD15, CD19 and CD56) and we added specific TINP1 antibodies and we incubate them for specific time (0h, 4h, 6h and 24 hours). Then we had done the flow cytometry analysis to determine the TINP1 level in the specific subset cells. I have selected some samples as examples to describe the data, each sample is selected from each time frame of 0h, 2h, 4h and 24h.

The left side of the graph it is showing the data for forward scatter (indicate the size of the cells) and side scatter (indicate the granularity and complexity of cells). We added the CD19 marker so the cells in inside the gating (P2) are supposed to be B cells. The right graph is showing the the FITC florescence and the same gating applied in FITC histogram too.

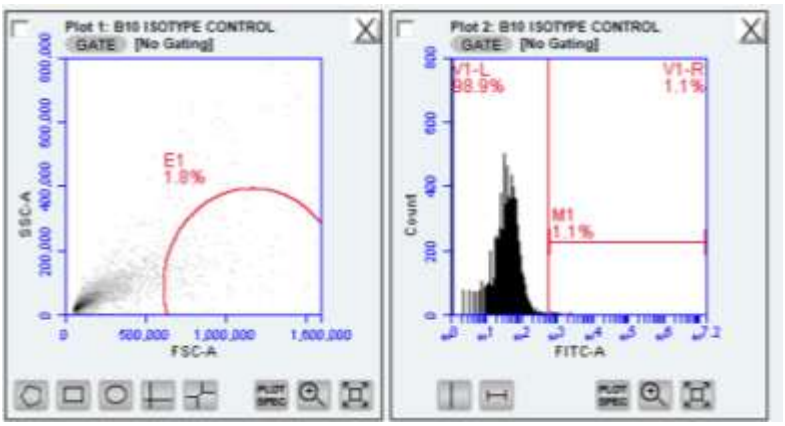


Figure 1: Showing the isotype control. We can compare it with rest of the samples to determine the TINP1 level in these samples. The mean PE value for the control is 83,102.57

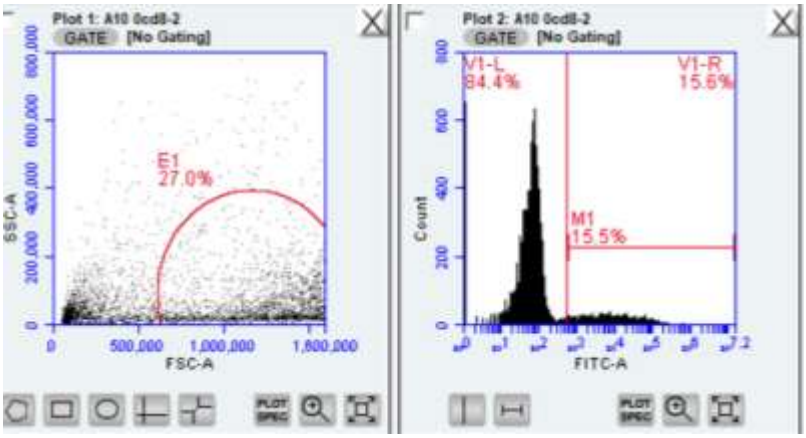


Figure 2: This is showing the results for the sample (containing CD-8 marker) with 0h incubation time. The mean PE value for this result is 2104385.03

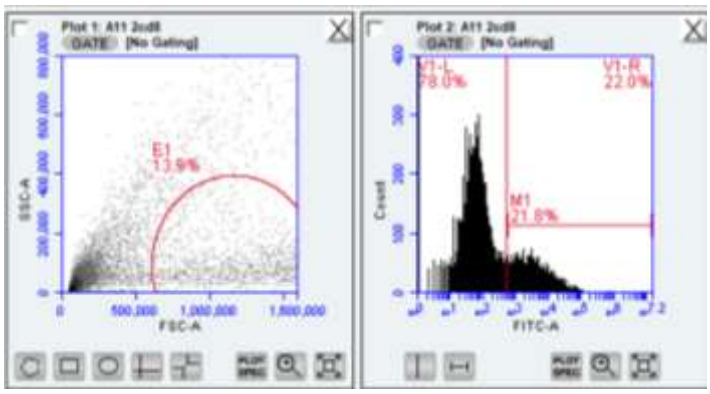


Figure 3: This is showing the results for the sample (containing CD-8 marker) with 2h incubation time. The mean PE value for this result is 1297155.94

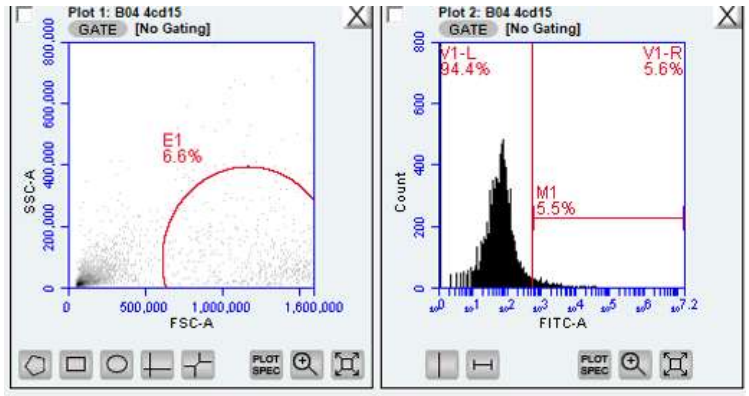


Figure 4: This is showing the results for the sample (containing CD-15 marker) with 4h incubation time. The mean PE value for this result is 415392.03.

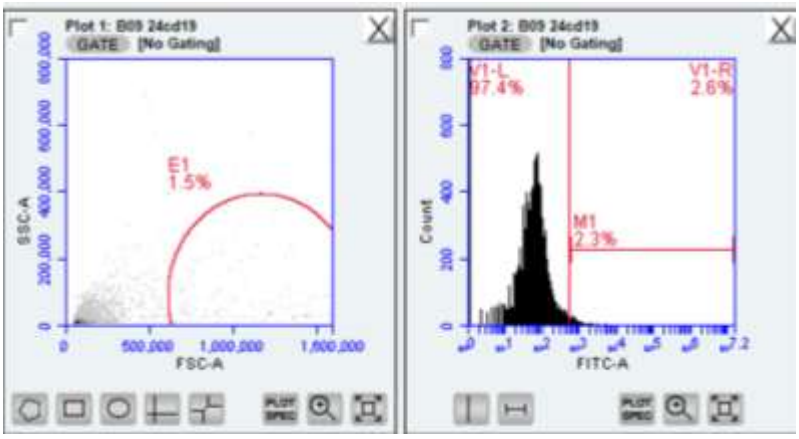


Figure 5: This is showing the results for the sample (containing CD-19 marker) with 24h incubation time. The mean PE value for this result is 12961.59

The table showing the results for each marker showing the mean PE values and incubation time with LILRB1. For the table 1.1 which is showing the results for CD-15 marker. The average mean PE value for all the samples is 100659.1 which is more than the mean PE value of control that is 83102.57. The lowest value in the table is 7192 and the highest value is 415392.03 (5 times higher than the control) which is a very huge jump. The sample 6 having highest value was incubated for 4h and sample 5 with lowest value was incubated for 2h. Samples 1, 2, 3 and 4 were incubated for 0h do not have much differences in mean PE values and these values are less than the control means PE value which suggest that LILRB1 did not stimulate the TNIP1 at 0h and expression was minimal at this time. Moreover, sample with 4h incubation indicating that LILRB1 stimulate the TINP1 production in that 4h time period therefore it is suggesting high expression of TNIP1 on the myeloid cells.

Table 3: Showing results for samples containing CD-15 that were analysed using flow cytometry.

Sample No.	Incubation Time	CD Marker	Mean PE value
1	0 h	CD 15	76608.41
2	0 h	CD 15	14775.05
3	0 h	CD 15	78630.62
4	0 h	CD 15	11356.49
5	2 h	CD 15	7192
6	4 h	CD 15	415392.03
		Average Mean PE	**100659.1**

Table 4 showing the results for CD-8 marker. The average mean PE value for all the samples is 681294.72 which is almost 8 times higher than the control mean PE value which is indicating overall good TNIP1 expression on the T cells. The highest value in the table is 2104385.03 of sample 2 which is almost 25 times higher than control value and the least value is 4805.92 of sample 6 which is extremely low as compared to highest value. The results in this table are highly variable so maybe indicating the gene switch off and on by the LIRB1 on the T cells as sample 2 at 0h incubation showing 2104385.03 mean PE value and sample 7 that was incubated for 4h only

showing 570157. There can be other several reasons for this high variability such as genetic differences in individual and also, environmental factors.

Table 4: Showing results for samples containing CD-8 that were analysed using flow cytometry.

Sample No.	Incubation Time	CD Marker	Mean PE value
1	0 h	CD 8	79633.86
2	0 h	CD 8	2104385.03
3	0 h	CD 8	91451.11
4	2 h	CD 8	1297155.94
5	2 h	CD 8	621474.24
6	2 h	CD 8	4805.92
7	4 h	CD 8	570157
		Average Mean PE	**681294.72**

Table 5 is showing the results for the CD-19 marker. The average mean PE value for all the samples is 69010.5 which is lowest compare to all other CD markers average PE values and also less than the control value. The highest value in this table is 189429 of sample 3 which is approximately 2 times higher than the control. The lowest value in the table is of sample 4 and it was incubated for 24 hours. The mean PE values in this table are decreasing with the time of incubation except sample 3 which is indicating that maybe the higher duration with LILRB1 switching off the gene for the TNIP1 production on the B cells. To validate this argument more samples need to be analysed but these results conclude that TNIP1 expression is lowest in the B cells as compared to other CD markers.

Table 5: Showing results for samples containing CD-19 that were analysed using flow cytometry.

Sample No.	Incubation Time	CD Marker	Mean PE value
1	0 h	CD 19	67727.60
2	2 h	CD 19	5924.49
3	4 h	CD 19	189429
4	24 h	CD-19	12961
		Average Mean PE	**69010.5**

This table 6 showing the results for the CD-14 marker. The average mean PE of all values in the table is 193264.75 which is 2 times higher than the control value. Sample 2 has the highest mean PE value of 566280.29 with 0h incubation and the lowest value 69719 is of sample 4 with 4h incubation. The PE values are decreasing with the increasing duration of time. The sample 2 with highest value have oh incubation time and sample 3 with second highest PE value was incubated for 2h and the lowest value of sample 4 was incubated for 4h. This is indicating that maybe LILRB1 decreasing the TNIP1 expression on the monocytes with increasing time. More samples needed to validate this indication. There can be other factors too for this decreasing order such as individual genetic differences.

Table 36: Showing results for samples containing CD-14 that were analysed using flow cytometry.

Sample No.	Incubation Time	CD Marker	Mean PE value
1	0 h	CD 14	13423.74

2	0 h	CD 14	566280.29
3	2 h	CD 14	123636
4	4 h	CD-14	69719
		Average Mean PE	**193264.75**

Other than these above results, we also tried to determine the level of TNIP1 different individual without LILRB1 because we ran out of LILRB1 reagent. For this purpose, we took 5 blood samples of different individual and then we used 5 different CD markers for specific subsets (such as CD8, CD14, CD15, CD19 and CD56). So, we used 5 different blood samples for each CD marker and 1 control. So, total we processed 30 samples including 5 controls without containing any antibodies and 25 overall samples containing antibodies of our interest. Also, we added the TINP1 antibodies in every sample to determine the level of TINP1 in each subset.

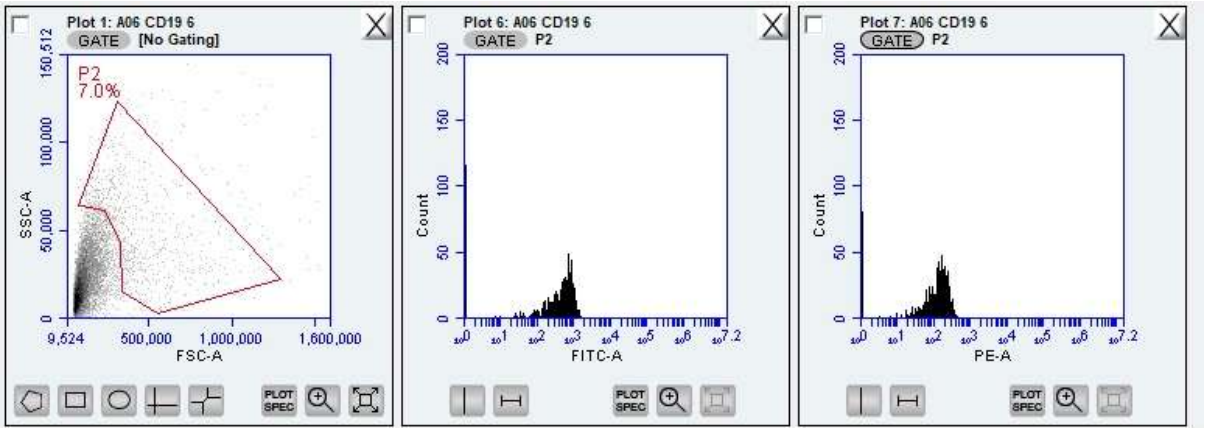


Figure 6: This graph is showing the results of flow cytometry analysis for the one sample containting CD19 marker. For this sample the mean PE value is 127.28..

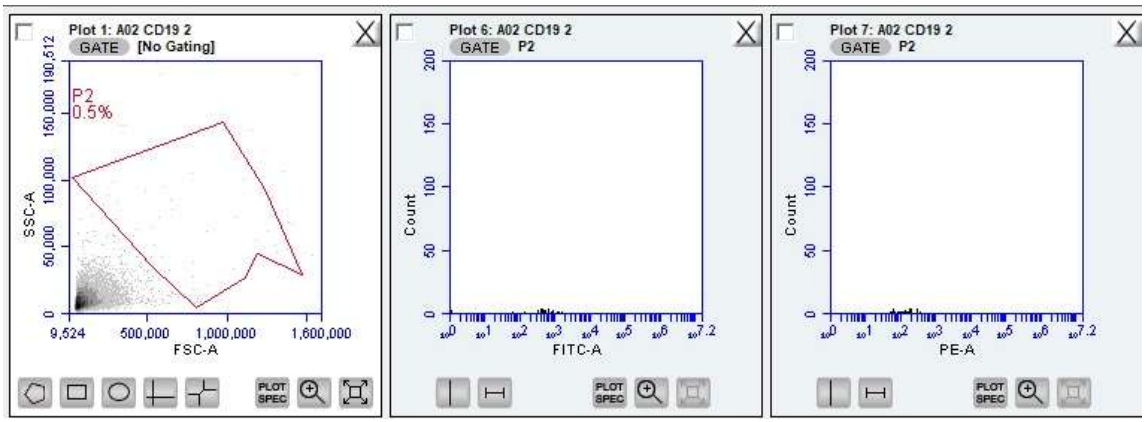


Figure 7: Showing the results for the sample 2 containing CD19 marker (B cells). The mean PE value for this sample is 146.98.

DISCUSSION

Now, coming to our objective, which was role of Leucocyte Ig-like receptor B1 on the expression level of TNIP1 Protein in Immune Effector Cells. Our results suggest that LILRB1 can influence the expression level of TNIP1. We cannot exactly say that it effects the TNIP1 expression in

increasing or in decreasing order because TNIP1 expression can vary individual to individual and also response against LILRB1 ligation can be different for different CD markers in different individuals. Overall, we know that high expression of TNIP1 is directly responsible for the negative regulation of NF-kB signaling pathway which play very important role in the immune system and as well as for inflammatory responses [15]. So, our aim was to investigate, does LILRB1 determine the expression of TNIP1 protein on the immune cells and then if it does, it can be the underlying reason of weak immune system (because many diseases such as B cell lymphomas are associated with poor anti-tumor immune responses) lead by high TNIP1 expression [16]. So, we used five CD markers (CD 8, CD 15, CD 19, CD 56 and CD 14) to analyze the expression level of TNIP1 after the ligation of LILRB1 immune effectors cells (T cells, B cells, natural killer cells, monocytes and dendritic cells). According to the results, the highest expression of TNIP1 was found on the $CD8^+$ T cells which has the average mean PE value of 681294.72 which is 8 times higher than the control PE value. This increase in the mean PE value suggests that ligation of LILRB1 can increase the TNIP1 protein expression on the cells. T cells plays very important role in suppressing and stopping the tumor and also, in the inflammatory responses. During inflammatory response, interferon gamma (IFNy) is produced by the recruited $CD8^+$ T cells [17]. Kim et al (2019) studies have shown that blockade of LILRB1 can enhance the activation of $CD8^+$ T cells and promote the cell death program by activating the cell death protein 1 (PD1) and this research concludes that increase expression of LILRB1 act as a negative regulator of human $CD8^+$ T cells. LILRB1 prevent CD8 T cells from proper functioning by blocking the cytolytic activity of T cells. Our study is showing that ligation of LILRB1 increases the expression of TNIP1 on the T cells so there is a possibility that this TNIP1 can also be contributing in the negative regulation of $CD8^+$ T cells or preventing the T cells from working effectively to suppress tumors [18]. Also, one more point is to be noted, table 3.2 showing the mean PE values for CD 8 marker, it is indicating that at first LILRB1 is increasing the TNIP1 expression but as we increasing the incubation time of cells with LILRB1, the TNIP1 expression is decreasing, one of the reasons for this shift is maybe due to gene switching off but more samples and results are needed to validate these arguments.

TNIP1 work as a negative regulator of NF-kB pathway and TNIP1 acts as a compressor of ligand bound retinoic acid receptor (RAR) and peroxisome proliferator activated receptor (PPAR) [19]. Addition to this it can stop EGF-R induced nuclear translocations [20]. Oshima et al (2009) has shown that knockout of TNIP1 gene in the mice may not change the phenotype in the same way as inverse of over expression studies. They found that cells in the absence of TNIP1 had the same levels of NF-kB dependent gene expression similar to wild type cells. Natural killer cells play vital role in the apoptosis of the cells and overexpression of TNIP1 play very important role in the inhibition of the apoptosis [21]. There are many shortcomings in this research, the reagents, time and resources were very limited and there is a doubt that some of our samples had more LILRB1 than other samples because maybe some of the colleagues had used more LILRB1 than it was suggest. Also, when we ran out of LILRB1, we tried to just calculate the TNIP1 expression without LILRB1 and also, we did not use the permeabilization buffer that is important for the cell's maintenance and their proper functioning but instead of permeabilization buffer we used simple PBS So, these points raise the question on the quality of the samples. In conclusion, we can say that LILRB1 has a role to play in the regulation of TNIP1 and more samples and studies are needed to validate these points. Our samples and results are very limited and this is why, we cannot exactly say how much LILRB1 contribute in the regulation of LILRB1. We needed more resources and

more time to actually investigate the deeper role LILRB1 can play in the expression of TNIP1 but our results provide an indication that there is a great potential in new findings as there are studies that suggest that many types of cancers that are linked with the TNIP1 and LILRB1 expression levels so more focus and research needed in this area.

CONCLUSION

It is concluded that LILRB1 stimulation significantly modulates TNIP1 expression in immune effector cells in a subset- and time-dependent manner. CD8+ T cells and CD15+ myeloid cells demonstrated strong upregulation of TNIP1, indicating a pronounced inhibitory effect, whereas CD14+ monocytes showed early induction followed by a decline, and CD19+ B cells exhibited overall suppression with prolonged incubation. These results suggest that the LILRB1–TNIP1 axis plays a pivotal role in regulating immune balance, with differential impacts depending on the lineage of immune cells.

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