Chapter IV

Results

4. Results:

4.1. Effect of folate treatments (concentration and form) on NK92 cell activation status and mTOR pathway expression in mono-culture conditions:

4.1.1. Effect of folate concentration and form on NK92 cell viability:

NK92 cells were mono-cultured in different concentrations (0.02 mM, 0.2 mM, 2 mM) of folate (FA or 5-MTHFA) in addition to no folate treatment group (NoF) for 72hrs to assess the effect of folate concentration on NK92 cell viability. The viability was assessed using flow cytometry based on the intensity of FSC (forward scatter) and SSC (side scatter) gating parameters. Additionally, microscopic observation was conducted to evaluate NK92 cell granularity and aggregation.

Our findings showed that NK92 cells cultured in the 2 mM folate concentration exhibited increased granularity and cell cluster disaggregation compared to other treatments (Figure 8). However, the 2 mM concentration of "5-MTHFA" form had a more severe impact on NK92 cell viability than the FA form and seemed to be lethal (Figures 8 and 9). Therefore, the 2 mM concentration of folate was excluded from further experiments.

NK92 cell viability in the other folate concentrations (0.02 mM and 0.2 mM) of both forms as well as in the folate-deprived media (NoF) was more than 70%. Therefore, these folate treatments were selected for pursuing further experiments (Figure 10).

Among the selected folate treatments, NK92 cells grown in high folate concentration (0.2 mM) had the lowest viability among all the other treatments ($p < 0.0001$). However, the viability in "5-MTHFA" form was lower than that in the cultures with FA form of the same concentration (Figures 8, 9 and 10).

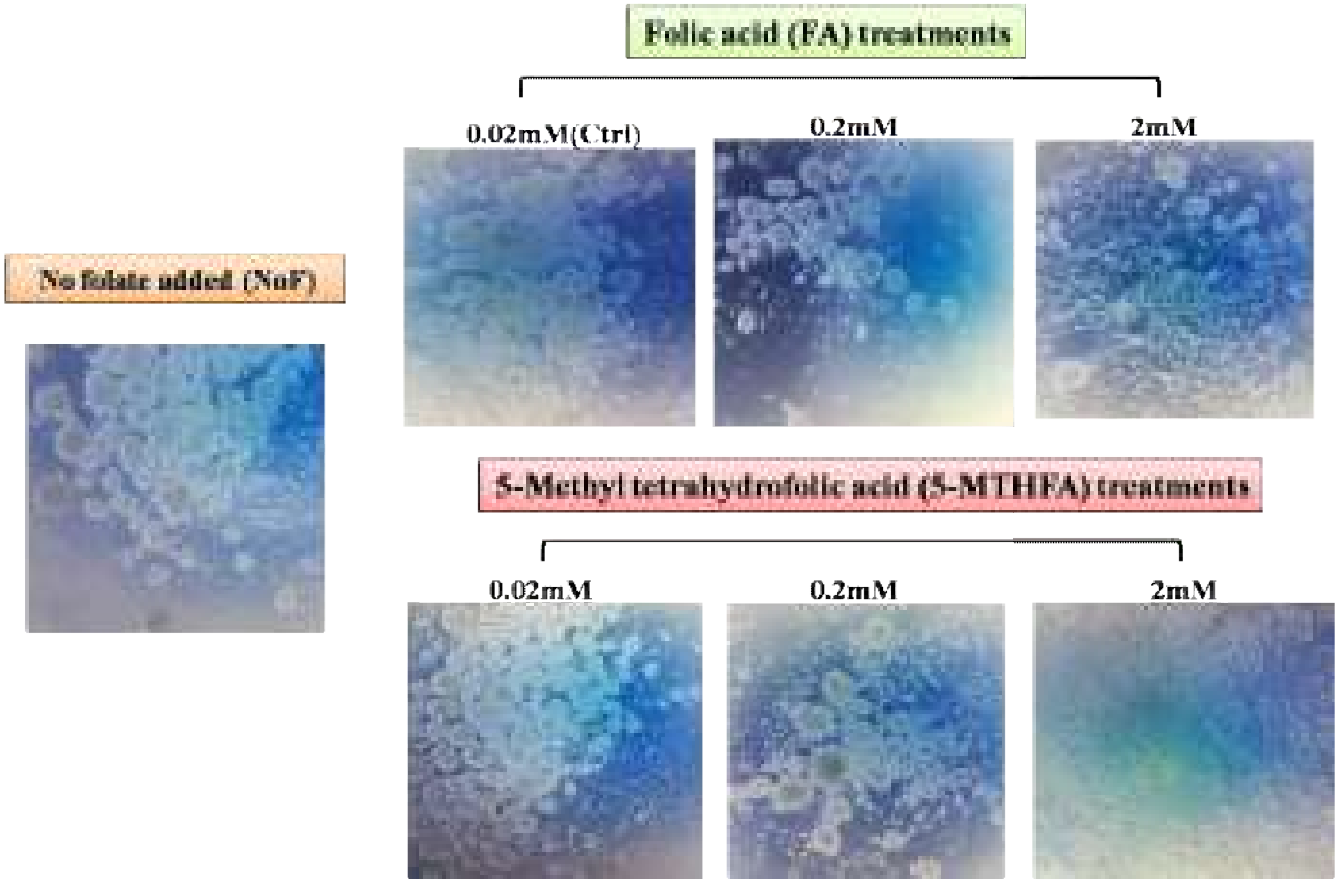

Figure (8): Morphological Changes in NK92 Cell Culture under Different Folate Treatments after 72hrs.

The provided microscopic images, captured at 10X magnification, reveal profound morphological changes in NK92 cell culture under different folate treatments. The images demonstrate that as the folate concentration increases, the cell clusters tend to disaggregate, and the cells exhibit signs of cellular death, accompanied by an increased granularity. Notably, the extremely high concentration (2 mM) of 5-MTHFA exhibits a more pronounced toxic effect compared to the same concentration of folic acid (FA).

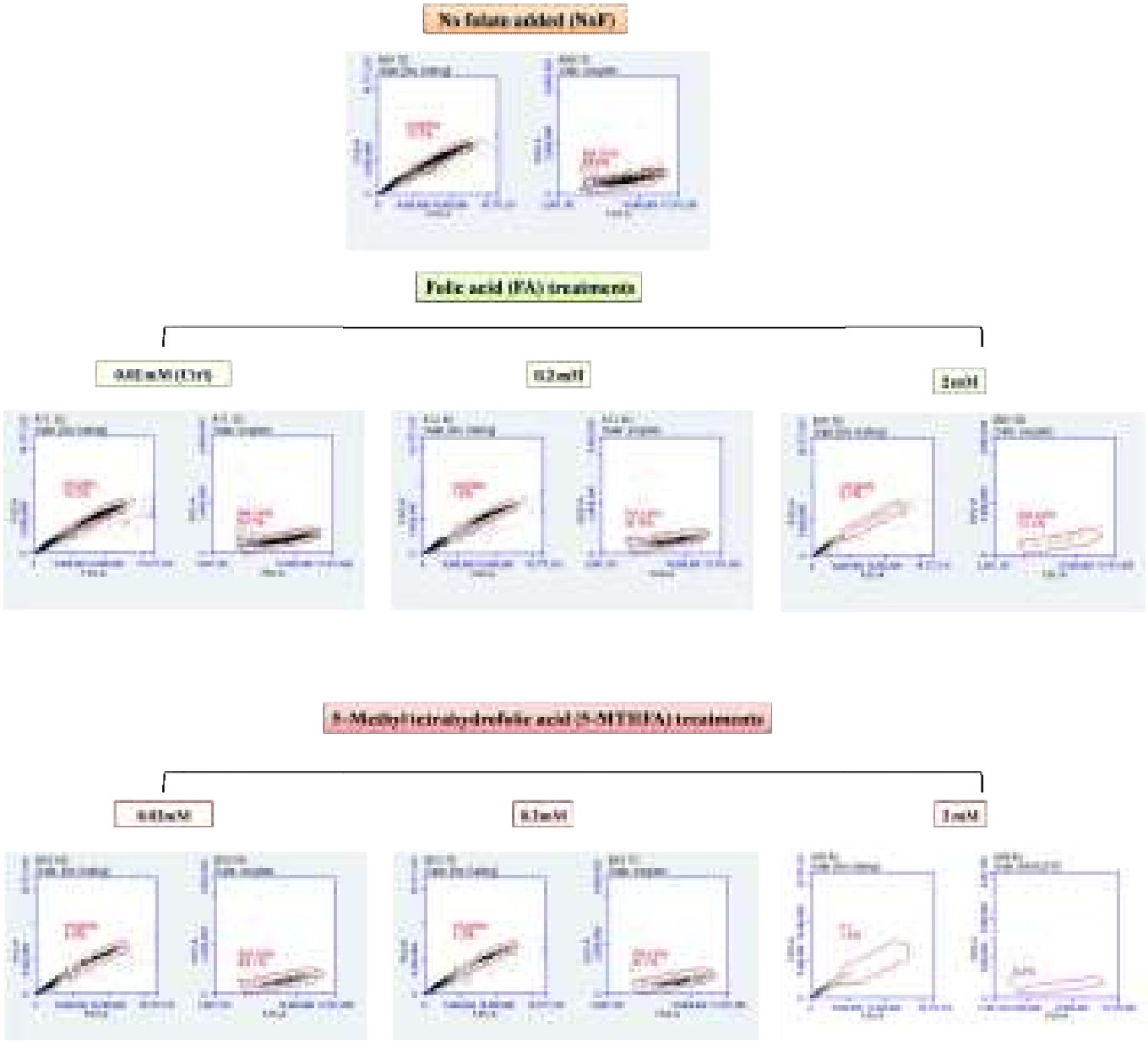

Figure (9): Representative flow cytometry plots showed the effect of folate treatments on the viability of NK92 cells in mono-culture after 72hrs.

The plots revealed noticeable changes in the intensity of singlets and the live cell population, particularly with the extremely high folate concentration (2 mM). Notably, the extremely high concentration (2 mM) of 5-MTHFA led to the absence of the Live cell population.

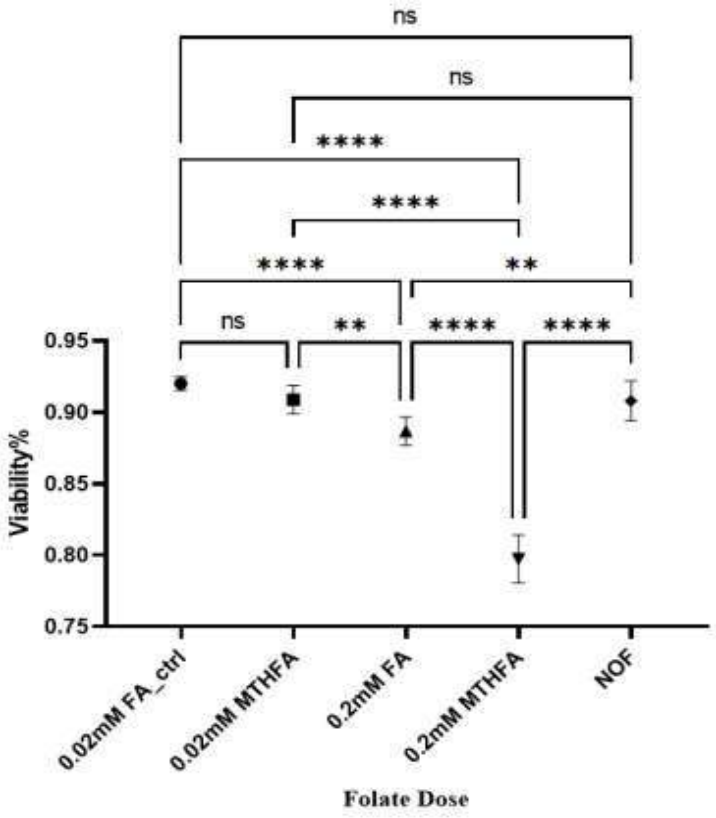

Figure (10): Symbol graph represents the difference in mono-cultured NK92 cell viability between the selected folate treatments after 72hrs.

The graph shows that the cell viability in high concentration (0.2 mM) of 5-MTHFA was the lowest among the other folate treatments.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test. The significance levels: $^{**}p < 0.01$; $^{****}p < 0.0001$; ^{ns} not significant.

4.1.2. Effect of folate concentration and form on NK92 activation status:

The flow cytometry analysis showed a significant increase in the MFI values for CD56 in all folate treatment groups during the first 48hrs of culturing compared to the control treatment condition (0.02 mM of FA) ($p < 0.0001$) (Figure 11A). However, after 72hrs, the CD56-MFI values and the percentage of CD56+ NK92 cells were comparable to those of the control treatment except for the 5-MTHFA treatments in both concentrations (0.02 mM, 0.2 mM) where the CD56-MFI values and the percentage of CD56+ NK92 cells were significantly higher compared to the control treatment (Figure 11A and Figure 12A).

Additionally, we observed that the MFI values for IFNγ remained comparable throughout the 72hrs except for the cells cultured in 0.2 mM of 5-MTHFA which exhibited higher levels of IFNγ expression compared to the cells cultured in all other treatment conditions ($p < 0.0001$) (Figure 11B). However, the percentage of IFNγ+ cells decreased throughout the 72hrs in all the treatment conditions, including the control treatment, although the cells cultured in 0.2 mM of 5-MTHFA maintained the highest percentage of IFNγ+ cells compared to the other treatments ($p<0.05$) (Figure 12B).

Further, we observed that FA over-supplementation (0.2 mM) and folate deficient (NoF) conditions significantly up-regulated the *IFNγ* gene expression ($p < 0.05$) (Figure 13). *GZMB* gene expression was significantly down-regulated in the NK92 cells treated with 0.2 mM of 5-MTHFA ($p < 0.05$) (Figure 13). However, no significant change in the expression of *PRF1* gene was observed between different folate statements (Figure 13).

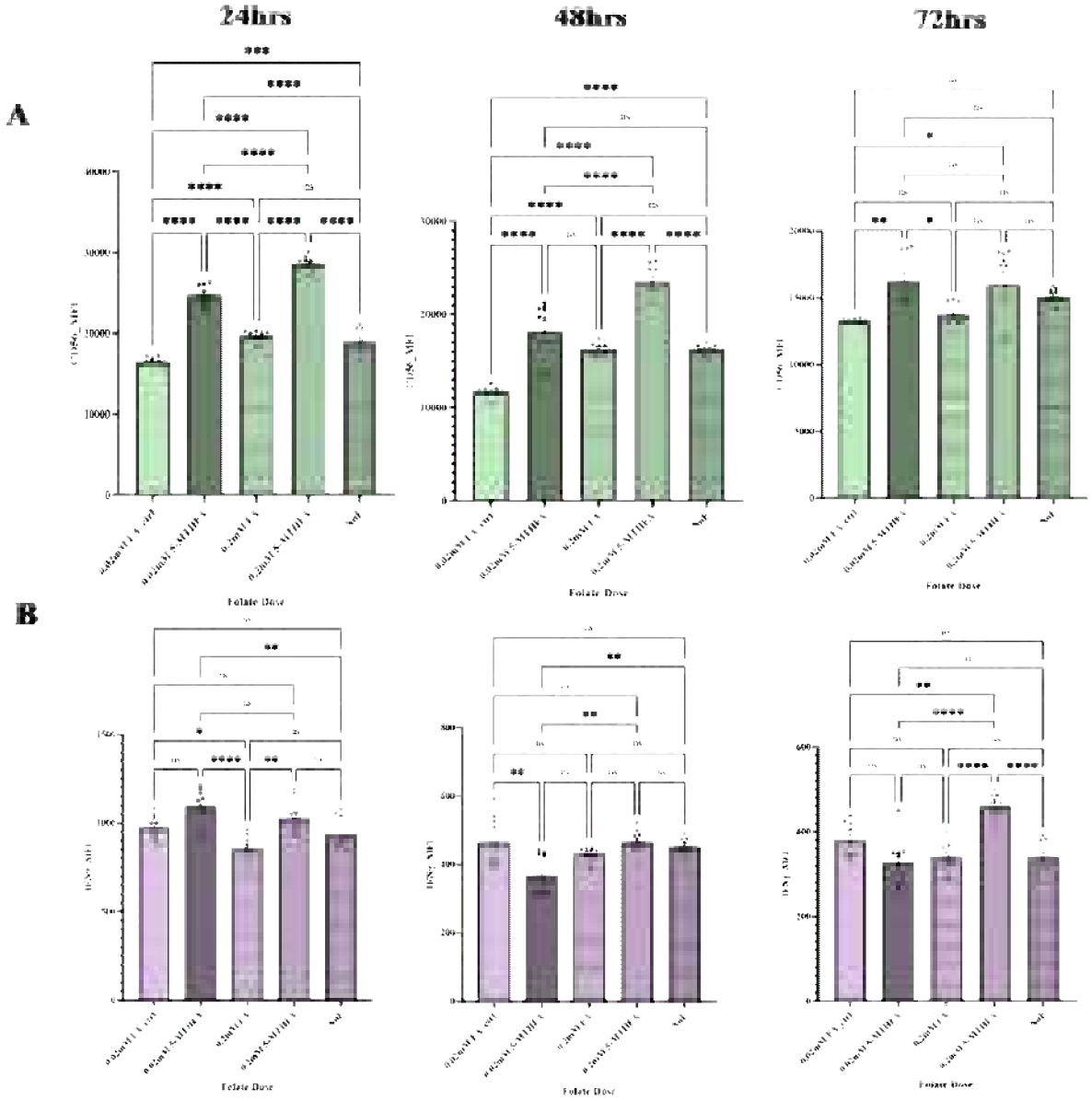

Figure (11): Column graphs representing the effect of folate treatments on the expression levels (represented by MFI) of A). CD56 and B). IFNγ in mono-cultured NK92 cells after 24 hrs, 48 hrs and 72 hrs.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences in MFI values between the treatments are indicated as follows: $*p < 0.05$; $**p \leq 0.005$; $***p < 0.001$; $****p < 0.0001$; ^{ns} not significant.

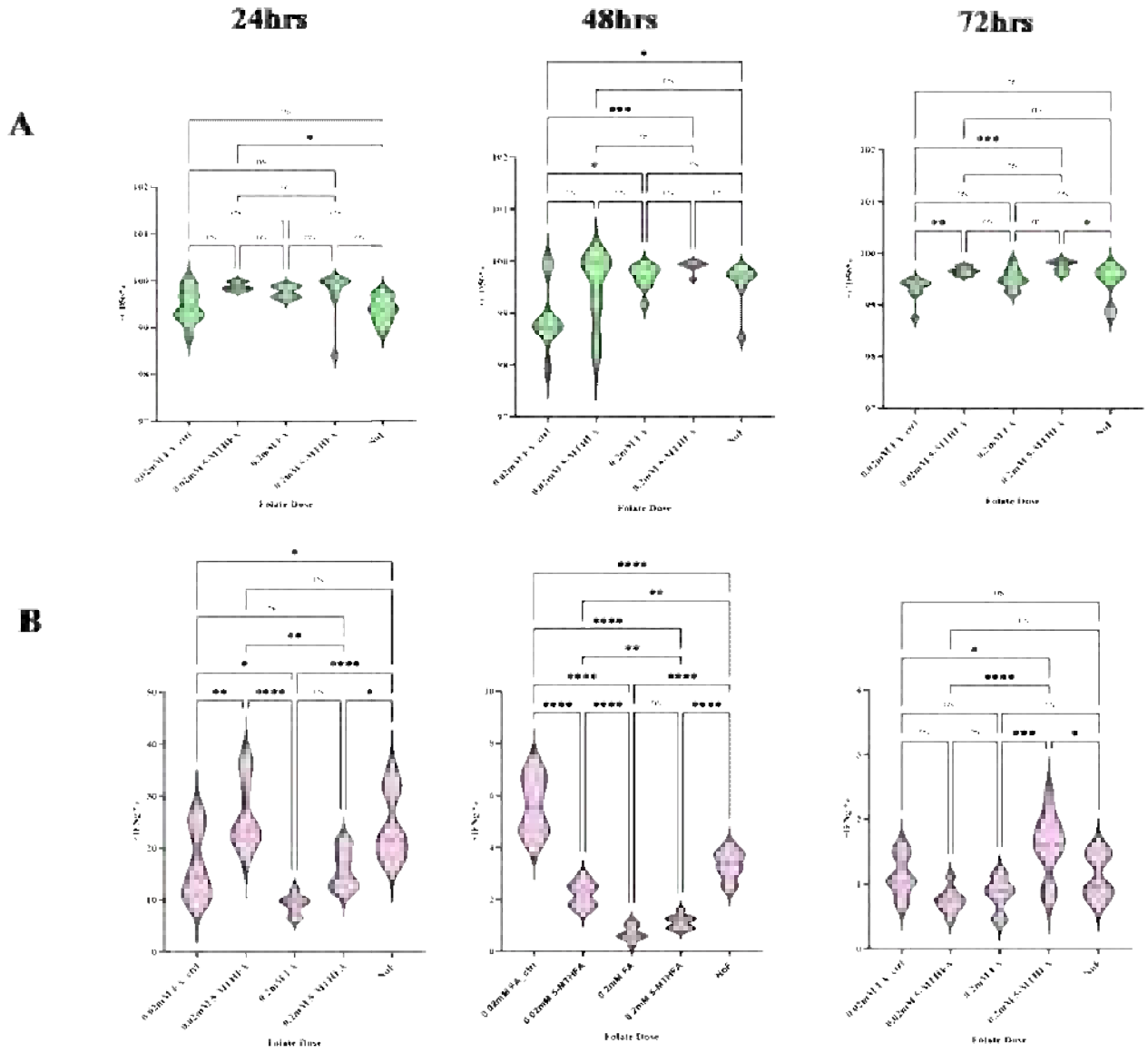

Figure (12): Violin plots depicting the effect of folate treatments on the percentage (%) of positive cells expressing A). CD56 and B). IFNγ, in mono-cultured NK92 cells after 24 hrs, 48 hrs and 72 hrs.

The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values. *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; ** $p \leq 0.005$; ***$p < 0.001$; ****$p < 0.0001$; ^{ns} not significant.

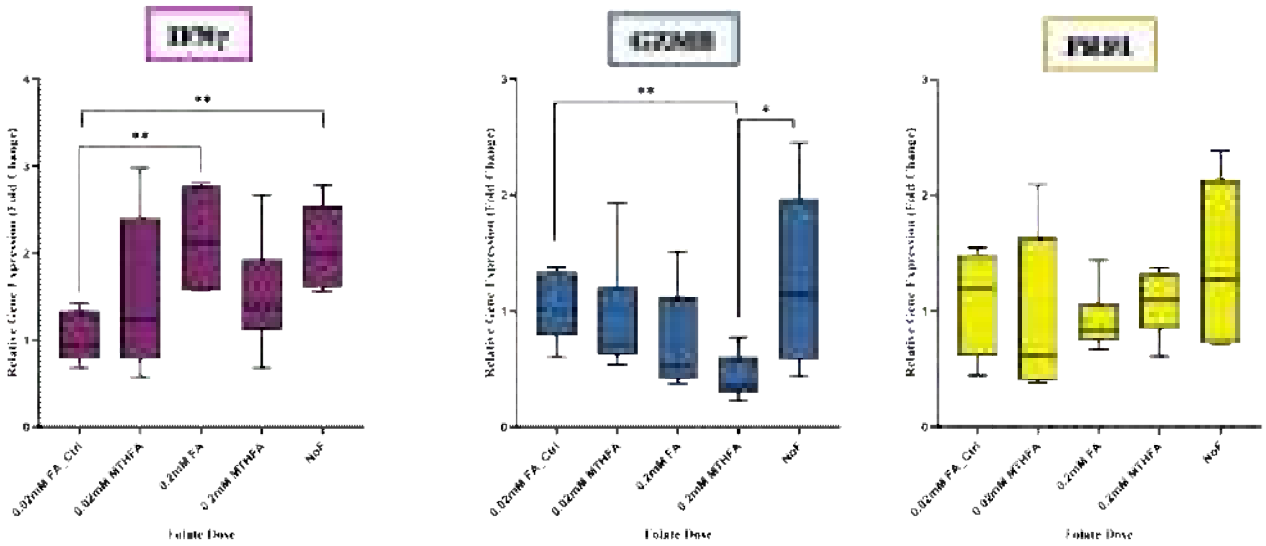

Figure (13): Box plots showing the effect of folate treatments after 72 hrs on the relative gene expression of NK cell function-related genes including *IFNγ*, *GZMB* and *PRF1*.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: $*p < 0.05$; $**p \leq 0.005$.

4.1.3. Effect of folate concentration and form on the expression of key genes in Folate metabolism of NK92 cells:

The RT-PCR analysis revealed a significant down-regulation of *MTHFR* gene in the folate-deficient cells group (NoF) compared to the other folate treatment groups ($p < 0.05$). Further, *DHFR* gene expression was significantly up-regulated in the cell group treated with over-supplementation of FA (0.2 mM) ($p < 0.05$), while the expression level of *RFC* gene in over-supplemented folate groups (0.2 mM of FA and 5-MTHFA) was significantly lower than the control group ($p < 0.05$) (Figure 14).

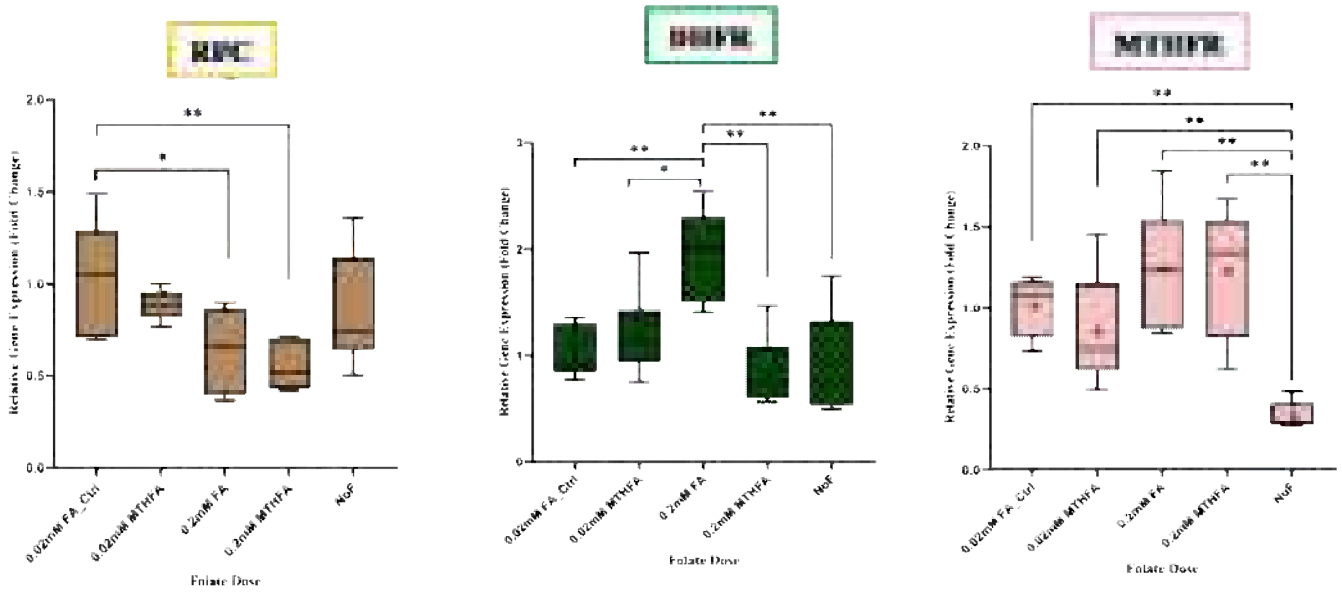

Figure (14): Box plots showing the effect of folate treatments after 72 hrs on the relative gene expression of folate metabolism-related genes including *RFC*, *DHFR* and *MTHFR*. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$.

4.1.4. Effect of folate treatments on the expression of key genes in the mTOR signalling pathway of NK92 cells:

We observed that NK92 cells grown in folate-deficient media (NoF) had the lowest *mTOR* gene expression level compared to the other group treatments ($p < 0.05$) (Figure 17). The expression levels of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2* and *AMPKα1* genes were significantly down-regulated in the cell groups supplemented with a high dose of folate (0.2 mM of FA and 5-MTHFA) ($p < 0.05$) (Figures 17 and 18). The *ATF3* and *ATF4* genes showed a modest decrease in the expression in the FA over-supplemented cell group ($p < 0.05$) (Figure 18). The master regulator of proliferation, *MYC* gene, did not display a significant change in the expression in all different folate treatment groups (Figure 18). The expression levels of *SREBP1* and *HIF1α* genes increased 2-fold and 4-fold, respectively in the FA over-supplemented cell group ($p < 0.05$) (Figure 17).

The flow cytometry analysis showed a significant elevation in the MFI values of HIF1α across the 72 hrs period in NK92 cells in all folate treatment groups compared to the cells grown in the control condition (Figure 15). Among the five different treated groups of NK92 cells, those treated with a high dose of folate (0.2 mM) exhibited the highest MFI values of HIF1α (Figure 15). However, the most substantial increase was observed in the group of cells treated with 0.2 mM of 5-MTHFA ($p < 0.0001$) (Figure 15). The percentage of cells expressing HIF1α in all treatment groups was comparable after 72 hrs (Figure 16).

Further, the ELISA analysis showed that the protein levels (represented by the arbitrary units) of SREBP1 in the high dose of FA and folate-deprived groups were higher (9.859 ± 1.579 and 9.385± 1.034, respectively) than that in the control group (8.615 ± 2.188) (Figure 19).

In addition, ELISA analysis for phosphorylated proteins in the mTOR pathway showed that phosphorylated P70S6K at the Thr389 site in the high dose of FA and folate-deprived groups were higher (8.789 ± 1.646 and 8.408 ± 1.086, respectively) than that in the control group (7.240 ± 1.792). However, statistical analysis (Kruskal-Wallis test) revealed that these differences were not statistically significant ($p > 0.05$) (Figure 19). The levels of phosphorylated Raptor at the Ser863 and Ser792 sites were comparable as well in all folate treatments ($p > 0.05$) (Figures 19 and 30).

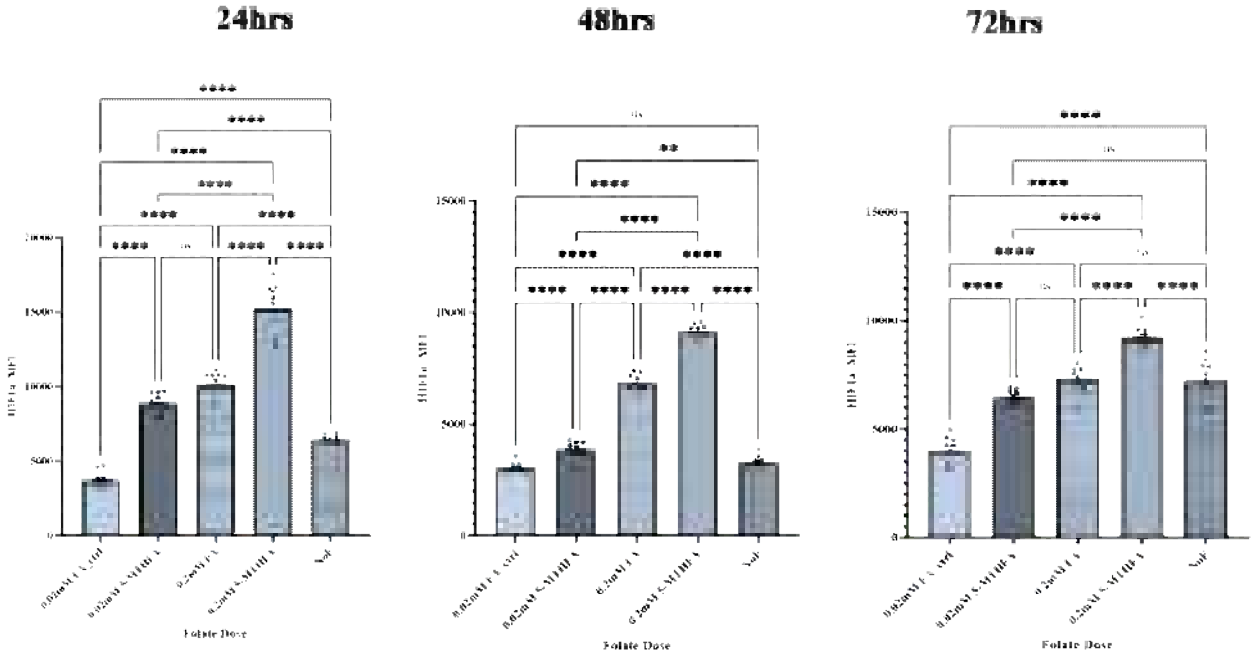

Figure (15): Column graphs represent the effect of folate treatments on the expression levels (represented by MFI) of HIF1α in mono-cultured NK92 cells for 24 hrs, 48 hrs and 72 hrs.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test. The significant differences in MFI values between the treatments are indicated as follows: $^{**}p \leq 0.005$; $^{****}p < 0.0001$; ^{ns} not significant.

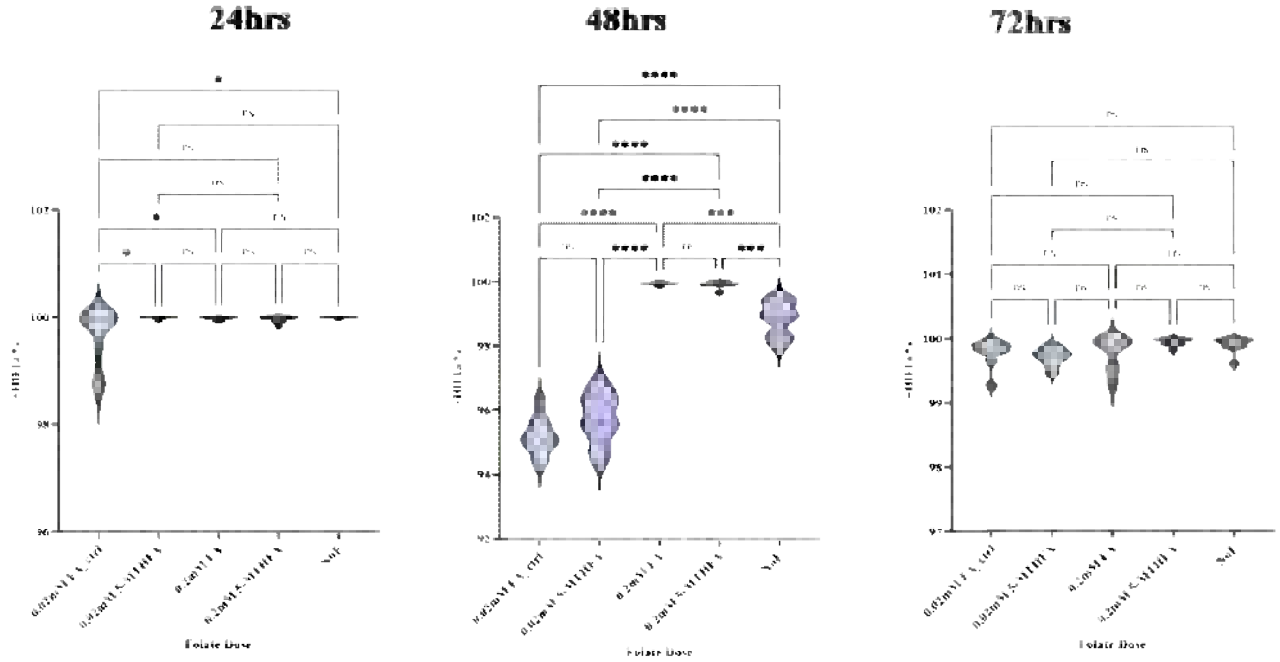

Figure (16): Violin plots represent the effect of folate treatments on the percentage (%) of positive cells expressing HIF1α in mono-cultured NK92 cells for 24 hrs, 48 hrs and 72 hrs.

The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$; ^{ns} not significant.

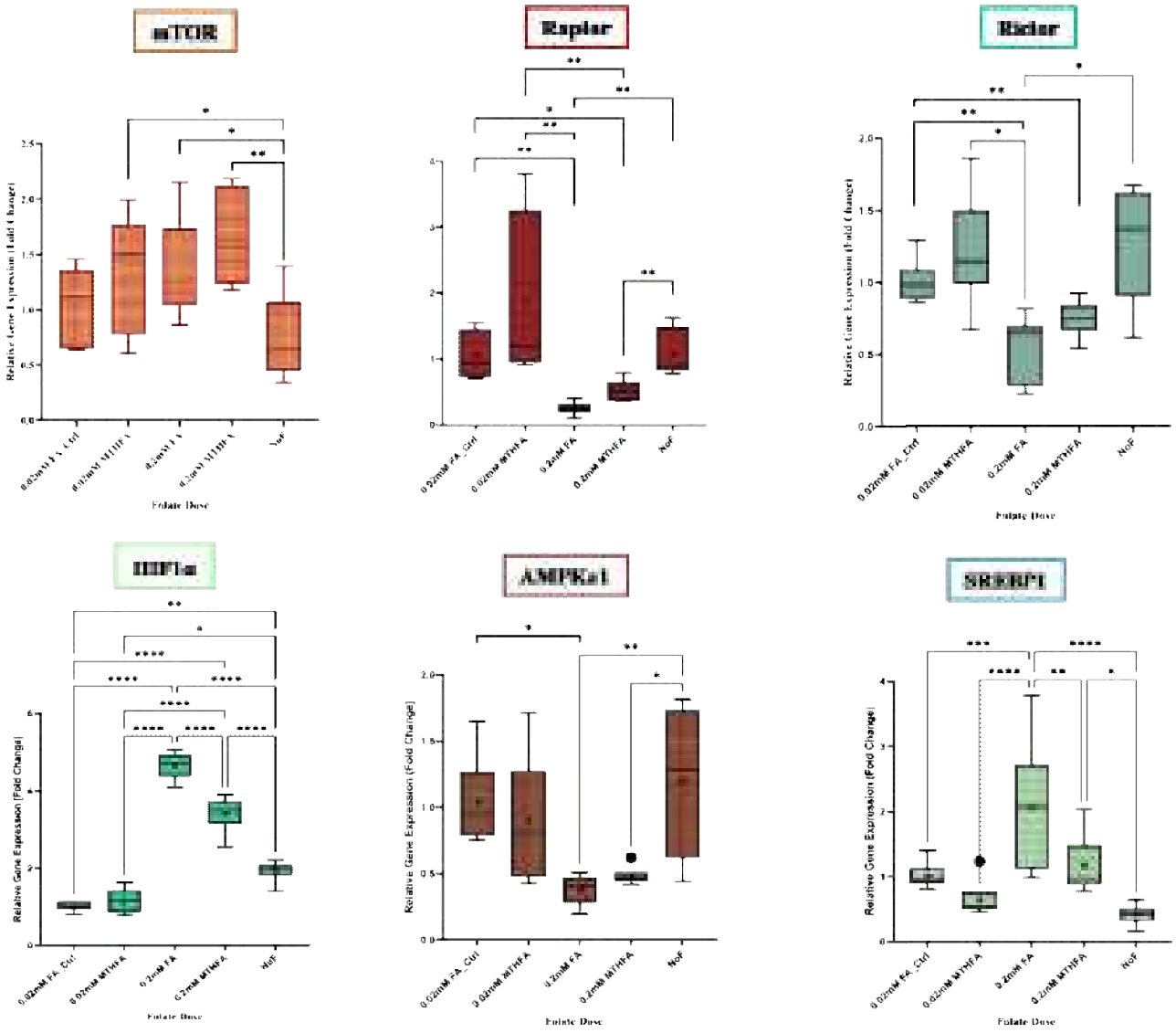

Figure (17): Box plots showing the effect of folate treatments after 72 hrs on the relative gene expression of mTOR pathway-related genes including *mTOR, Raptor*, *Rictor*, *HIF1α AMPKα1* and *SREBP1*.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ****$p < 0.0001$.

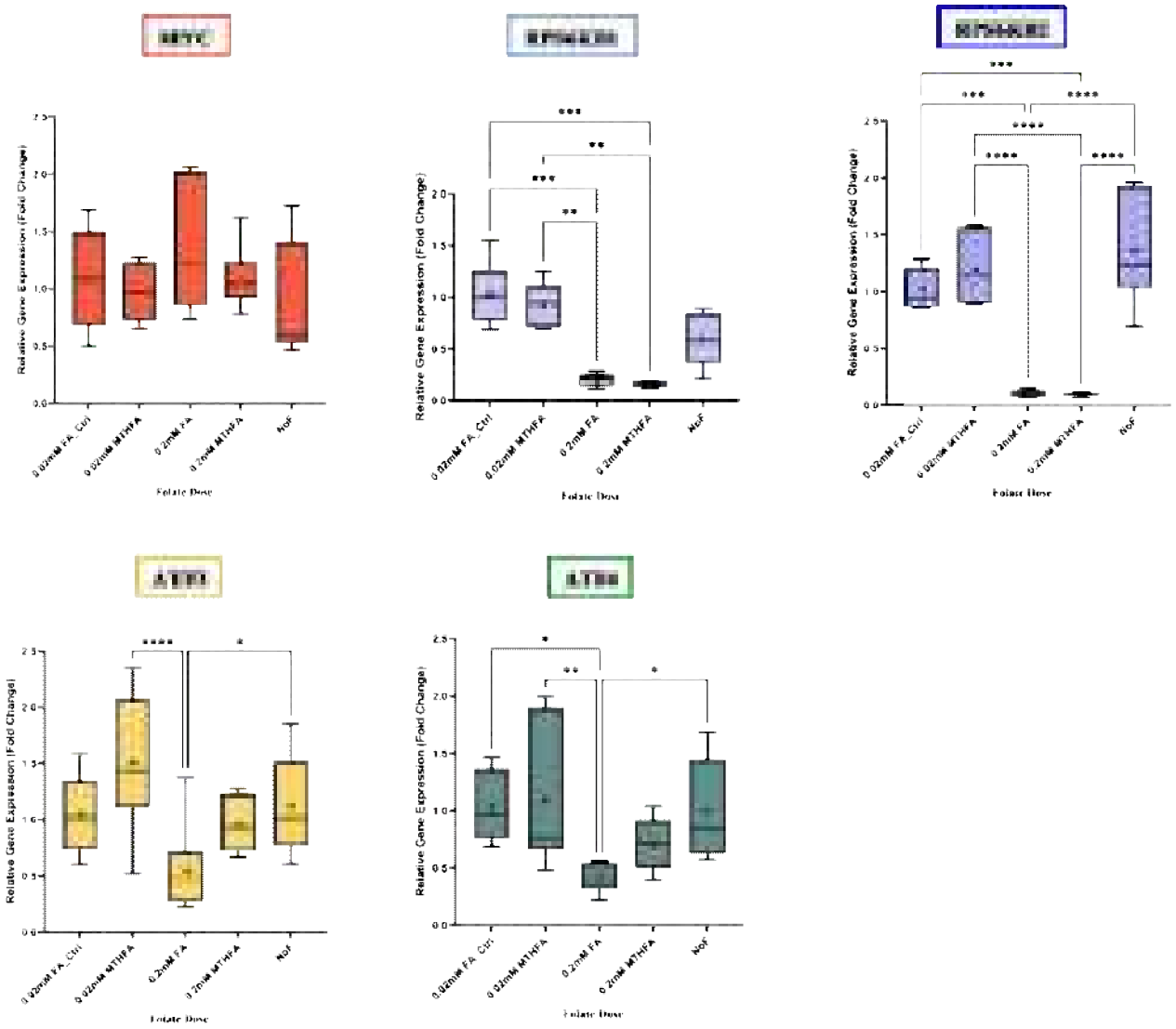

Figure (18): Box plots showing the effect of folate treatments after 72 hrs on the relative gene expression of mTOR pathway-related genes including *MYC*, *RPS6KB1*, *RPS6KB2*, *ATF3* and *ATF4*.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ***$p < 0.001$; ****$p < 0.0001$.

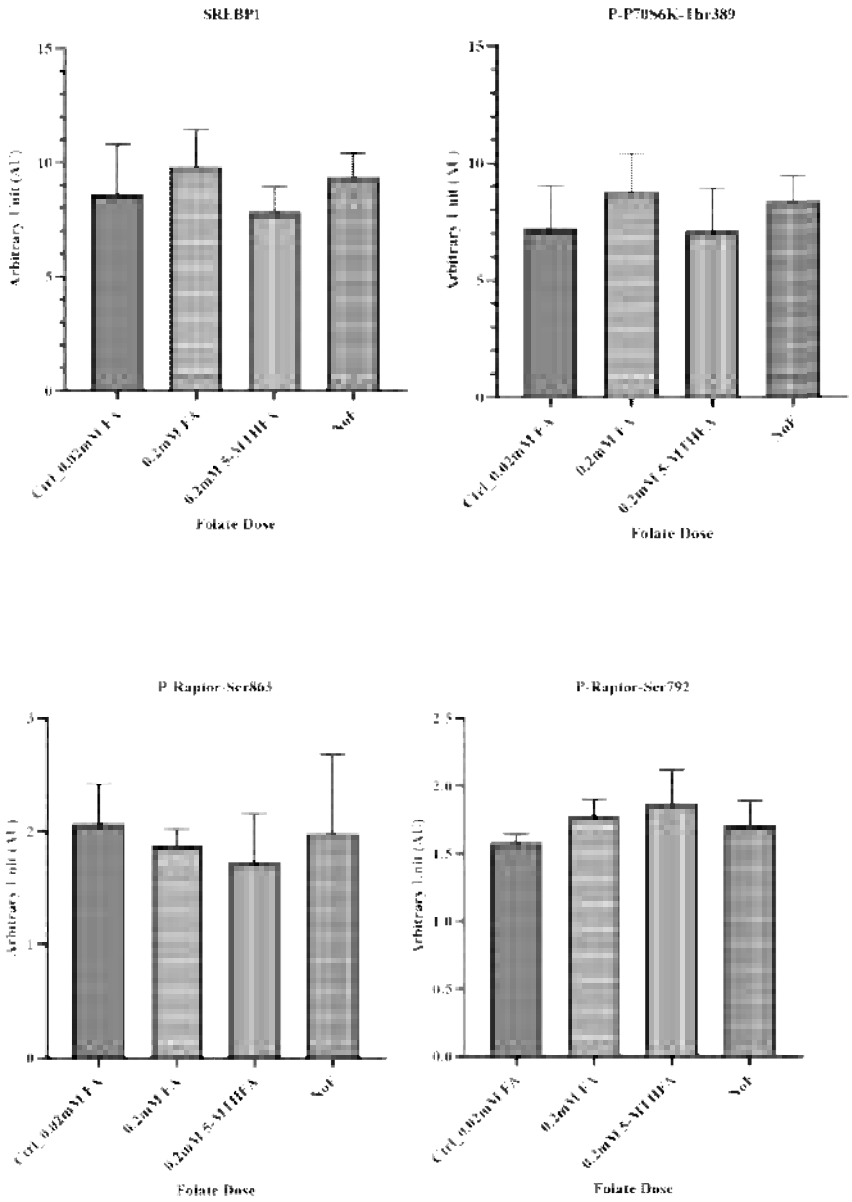

Figure (19): Bar plots showing the effect of folate treatments on the protein levels (represented by the arbitrary units; AU) of SREBP1, and the phosphorylated proteins including p-P70S6K-Thr389, p-Raptor-Ser863, and p-Raptor-Ser792 in mono-cultured NK92 cells after 72hrs. Data presented as mean ± SEM (n = 6). Statistical analysis was conducted by one-way ANOVA test.

4.2. Effect of succinate treatments on NK92 cell activation status and mTOR pathway expression in mono-culture conditions:

4.2.1. Effect of succinate treatments on NK92 cell viability:

The cell viability of NK92 cells mono-cultured for 72 hrs showed a significant decrease in all concentrations of succinate (50, 100, and 500 µM) compared to the control group ($p < 0.0001$) in a dose-independent manner (Figure 20).

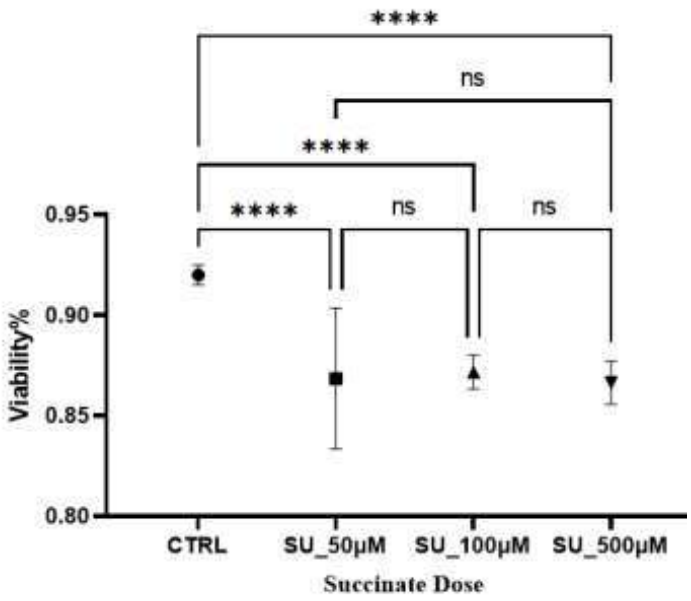

Figure (20): Symbol graph depicting the effect of succinate treatments on the viability of mono-cultured NK92 cells after 72hrs

The graph shows that the cell viability decreased in all succinate treatments.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test, Significance levels: ****$p <$ 0.0001; ^{ns} not significant.

4.2.2. Effect of succinate treatments on NK92 cell activation status:

The MFI values for CD56 were notably higher in NK92 cells cultured in succinate-supplemented media of all concentrations (50, 100, and 500 µM) after 24 hrs and 48 hrs. However, after 72 hrs, the MFI of CD56 decreased compared to the non-succinate control group (Figures 21A, 22A). It is worth mentioning that the control group exhibited consistent MFI values for CD56. Further, there were no important changes in the percentage of NK92 cells expressing CD56 (Figures 21A, 22A).

The MFI values of IFNγ were significantly lower in all succinate-treated groups compared to the control group. After 72 hrs, the MFI values of IFNγ were lower in cell cultures with high succinate concentration (500 µM) compared to the control group ($p < 0.05$). Additionally, the percentage of NK92 cells expressing IFNγ was lower in succinate-treated groups throughout the 72-hr time course compared to the control group (Figures 21B, 22B).

Further, high succinate treatment (500 µM) increased the *IFNγ* gene expressions while 100 µM and 500 µM of succinate increased *GZMB* gene expression ($p < 0.05$). However, no remarkable change in the expression of *PRF1* gene was observed in all succinate-treated groups compared to the control group ($p > 0.05$) (Figure 23).

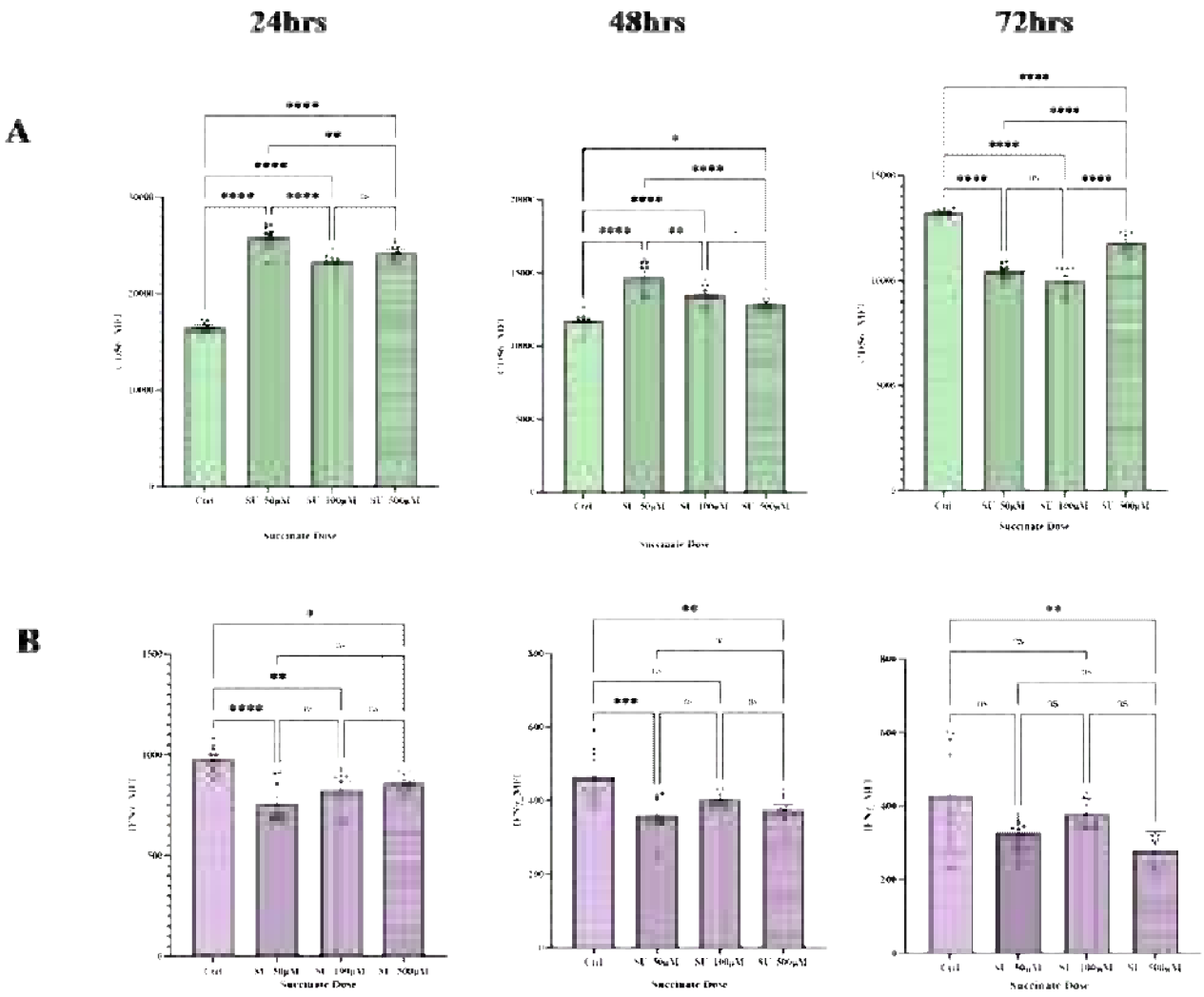

Figure (21): Column graphs showing the effect of succinate treatments on the expression levels (represented by MFI) of A). CD56 and B). IFNγ in mono-cultured NK92 cells after 24 hrs, 48 hrs and 72 hrs.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test. The significant differences in MFI values between the treatments are indicated as follows: $*p < 0.05$; $**p \leq 0.005$; $***p < 0.001$; $****p < 0.0001$; ^{ns} not significant.

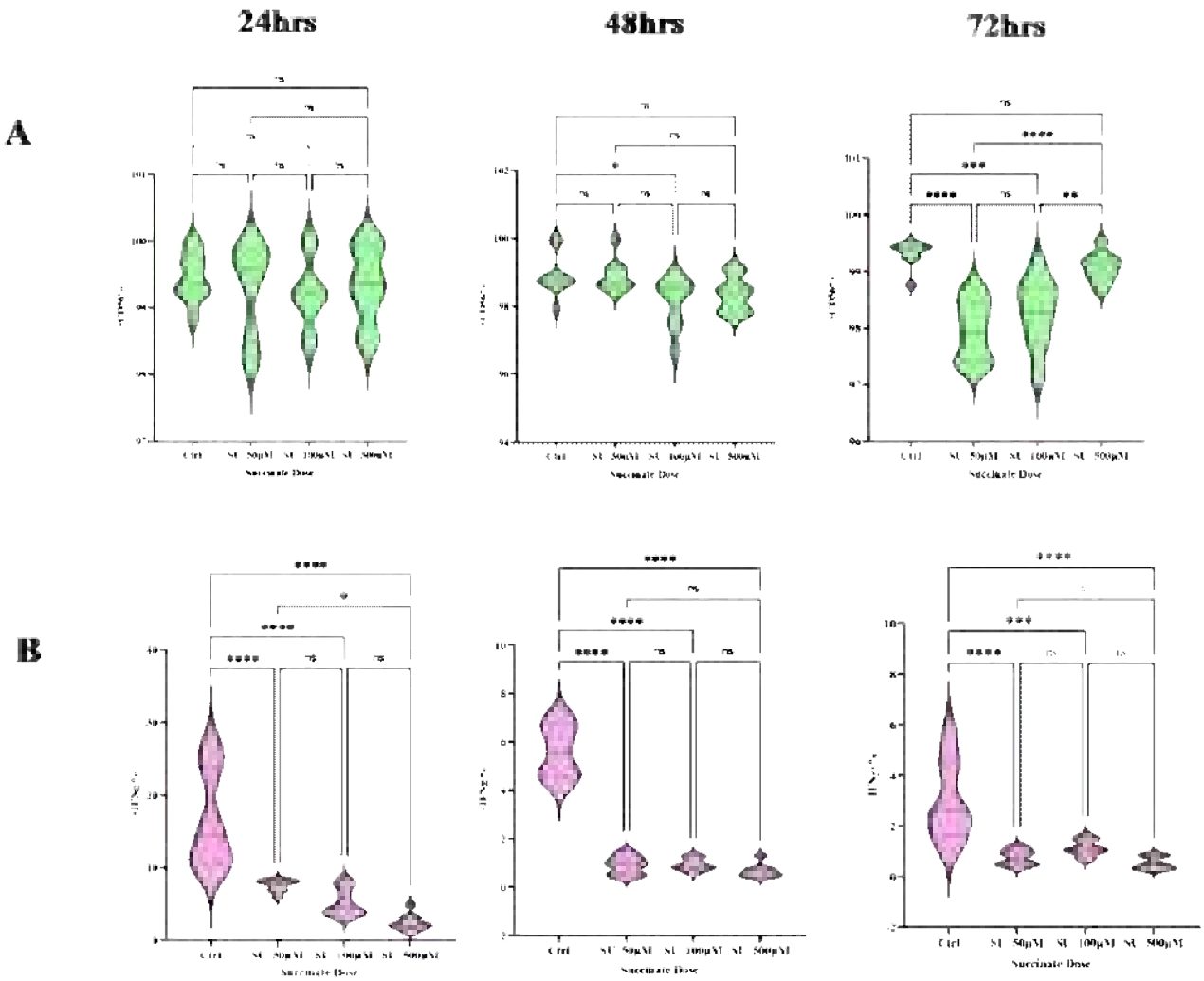

Figure (22): Violin plots depicting the effect of succinate treatments on the percentage (%) of positive cells expressing A). CD56 and B). IFNγ, in mono-cultured NK92 cells after 24 hrs, 48 hrs and 72 hrs.

The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values. *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: $*p < 0.05$; $**p \leq 0.005$; $***p < 0.001$; $****p < 0.0001$; ^{ns} not significant.

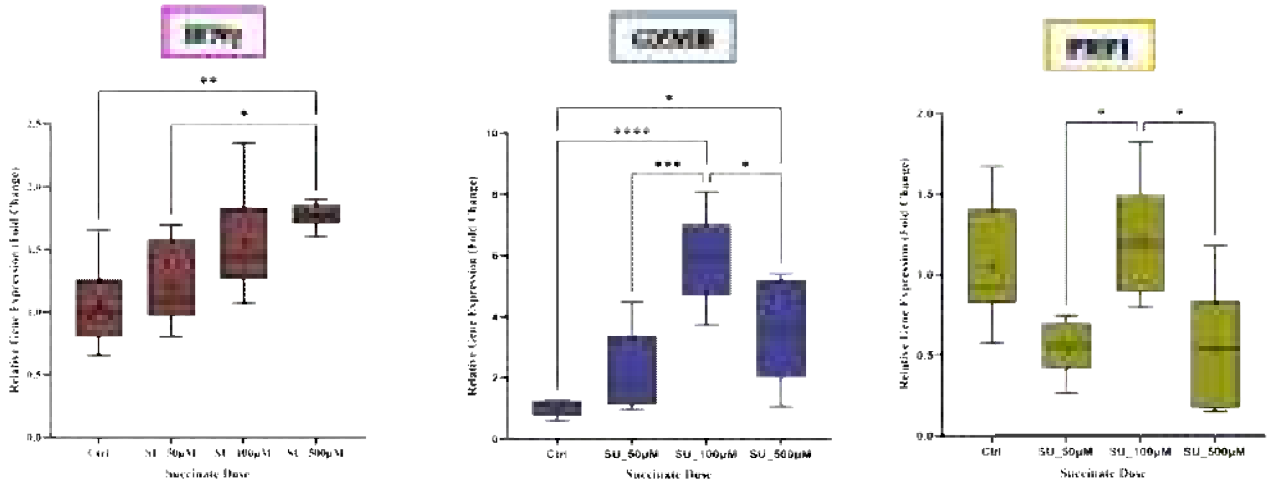

Figure (23): Box plots showing the effect of succinate treatments after 72 hrs on the relative gene expression of NK cell function-related genes including *IFNγ*, *GZMB* and *PRF1*.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ***$p < 0.001$; ****$p < 0.0001$.

4.2.3. Effect of succinate treatments on the expression of key genes in Folate metabolism of NK92 cells:

A notable down-regulation of *MTHFR* and *RFC* genes in the succinate-treated groups compared to the control group ($p < 0.0001$) (Figure 17). In contrast, *DHFR* gene expression was significantly up-regulated in the cell groups treated with high concentrations of succinate (100µM, and 500µM) compared to the control group ($p < 0.05$) (Figure 24).

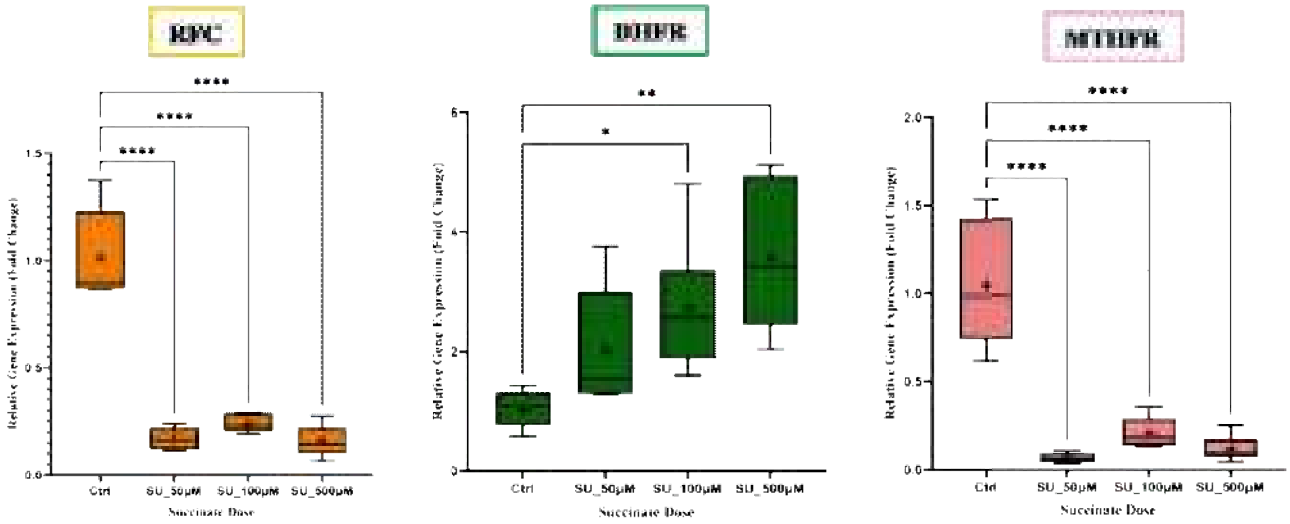

Figure (24): Box plots showing the effect of succinate treatments after 72 hrs on the relative gene expression of folate metabolism-related genes including *RFC*, *DHFR* and *MTHFR*. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ****$p < 0.0001$.

4.2.4. Effect of succinate treatments on the expression of key genes in the mTOR signalling pathway of NK92 cells:

We observed a noteworthy decrease in the expression levels of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2*, *AMPKα1*, *SREBP1* and *ATF3* genes (p <0.0001) (Figures 27 and 28). The expression levels of *mTOR* and *ATF4* genes in the succinate-treated groups and control group were comparable. The *MYC* gene displayed a significant increase in the expression in the group treated with 100µM of succinate ($p < 0.05$) (Figure 28). *HIF1α* gene notably exhibited a 6-fold increase in expression ($p < 0.0001$) in high succinate concentration-treated groups (100 µM, and 500 µM) (Figure 27). Besides, succinate treatments significantly increased the MFI values for HIF1α after 24hrs and 48hrs (Figure 25). Notably, this increase was observed after 72hrs in the low succinate concentrations (50µM and 100µM) ($p < 0.05$), but not in the higher concentration (500µM) compared to the control group (Figures 25 and 26). It is worth mentioning that the control group exhibited consistent MFI values for HIF1α throughout the 72 hrs (Figure 25). Further, there were no important changes in the percentage of NK92 cells expressing HIF1α (Figures 25 and 26).

Further, the ELISA analysis showed that the protein levels of SREBP1 (represented by the arbitrary units) in the succinate treatments (50 µM and 500 µM) were (8.155 ± 1.401 and 9.777 ± 1.094, respectively) comparable to that in the non-succinate control group (8.615 ± 2.188) (Figure 29). Additionally, ELISA analysis for phosphorylated proteins in the mTOR pathway showed that phosphorylated P70S6K at the Thr389 site in succinate treatment groups (50µM and 500µM) were higher (8.231 ± 1.775 and 8.228 ± 1.422, respectively) than that in the control group (7.240 ± 1.792). However, statistical analysis (Kruskal-Wallis test) revealed that these differences were not statistically significant ($p > 0.05$). The levels of phosphorylated Raptor at the Ser863 and Ser792 sites in succinate treatments were comparable to that in the control group ($p > 0.05$) (Figures 29 and 30).

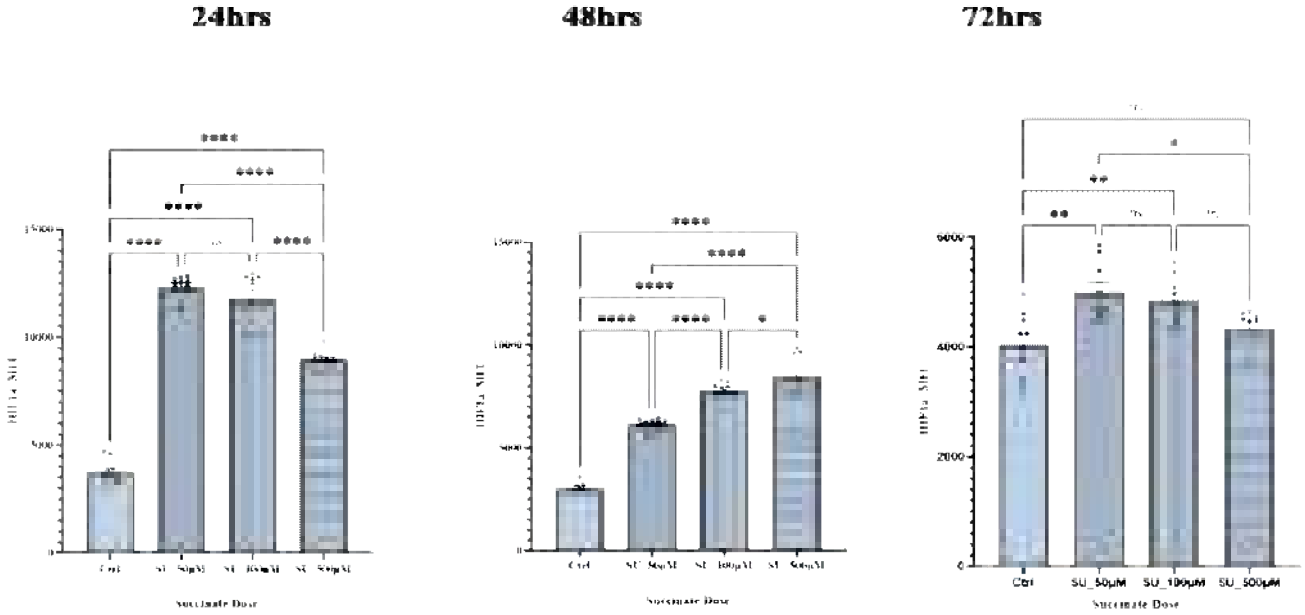

Figure (25): Column graphs represent the effect of succinate treatments on the expression levels (represented by MFI) of HIF1α in mono-cultured NK92 cells for 24 hrs, 48 hrs and 72 hrs.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test. The significant differences in MFI values between the treatments are indicated as follows: $*p < 0.05$; $**p \leq 0.005$; $****p < 0.0001$; ^{ns} not significant.

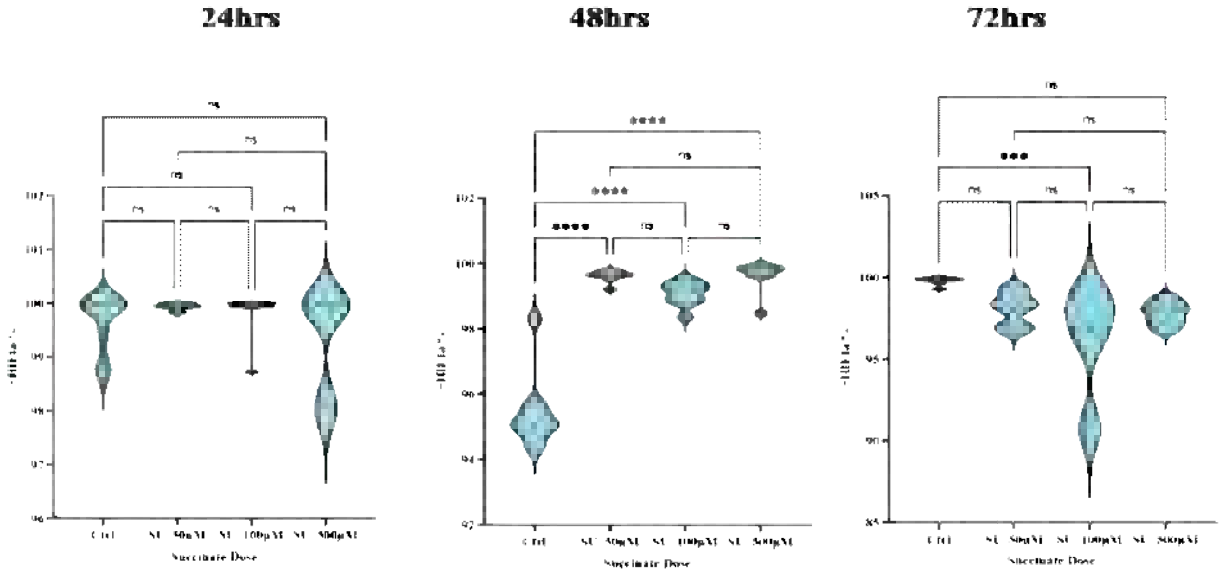

Figure (26): Violin plots represent the effect of succinate treatments on the percentage (%) of positive cells expressing HIF1α in mono-cultured NK92 cells for 24 hrs, 48 hrs and 72hrs.

The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values. *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: ***$p < 0.001$; ****$p < 0.0001$; ^{ns} not significant.

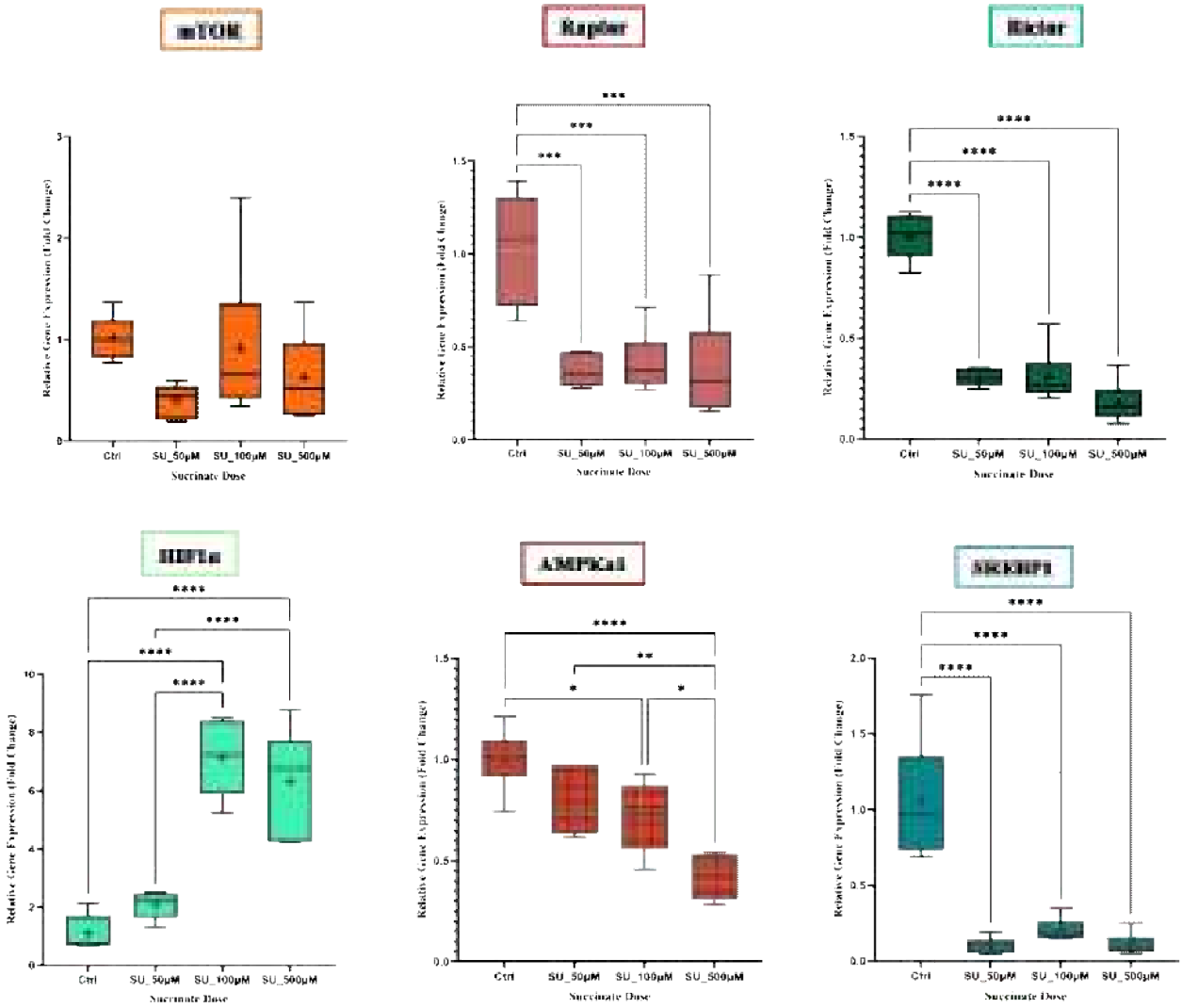

Figure (27): Box plots showing the effect of succinate treatments after 72 hrs on the relative gene expression of mTOR pathway-related genes including *mTOR, Raptor*, *Rictor*, *HIF1α AMPKα1* and *SREBP1*.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; ** $p \leq 0.005$; ***$p < 0.001$; ****$p < 0.0001$.

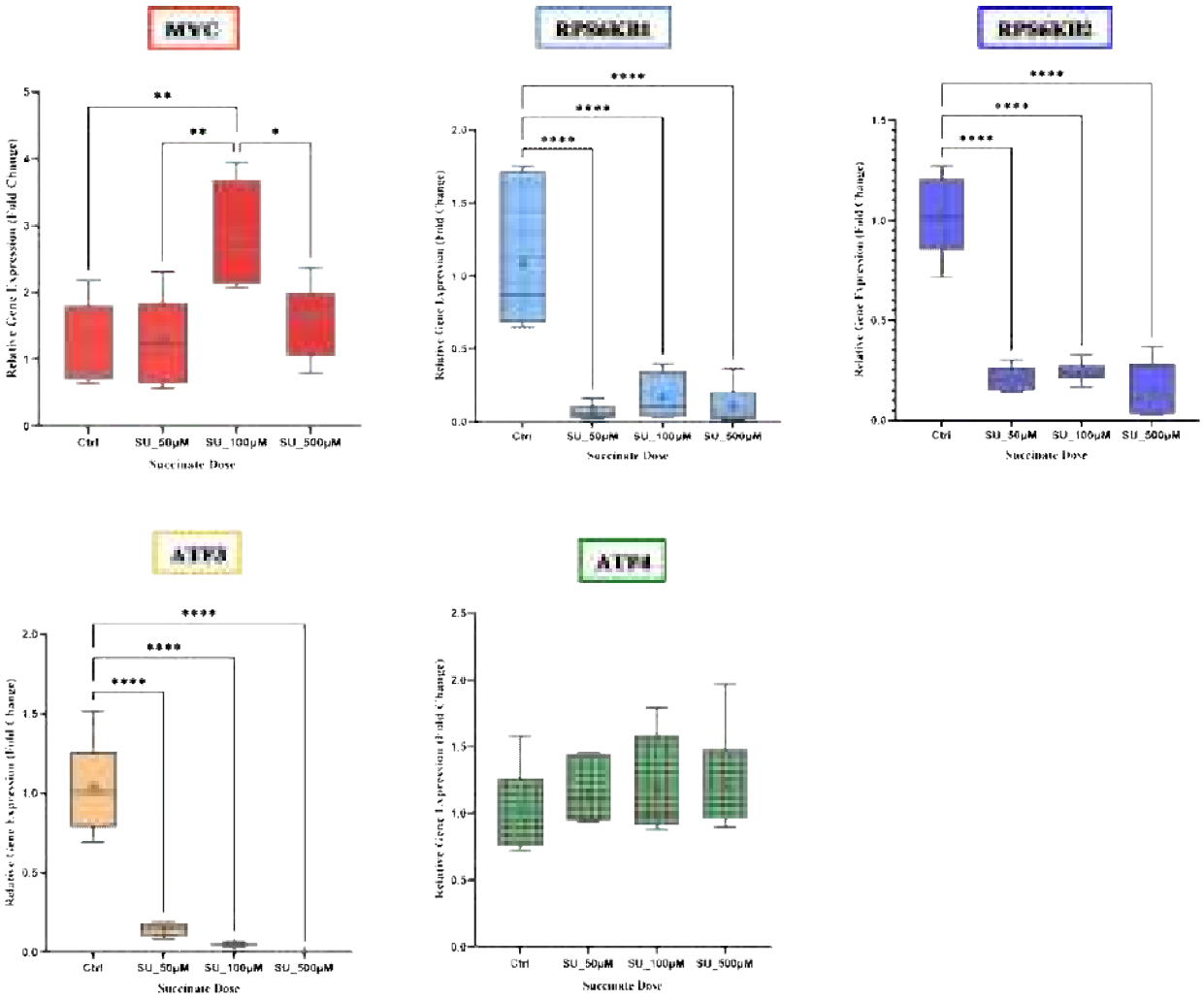

Figure (28): Box plots showing the effect of succinate treatments after 72 hrs on the relative gene expression of mTOR pathway-related genes including *MYC*, *RPS6KB1*, *RPS6KB2*, *ATF3* and *ATF4*.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ****$p < 0.0001$.

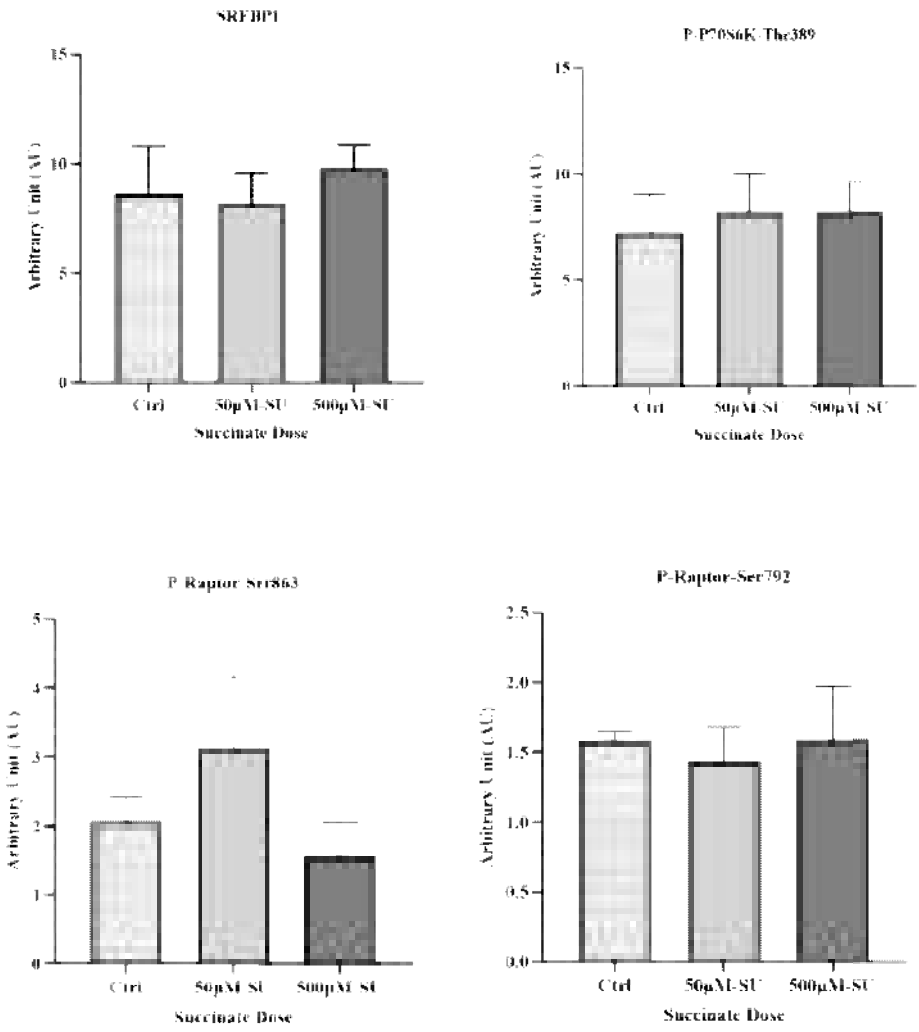

Figure (29): Bar plots showing the effect of succinate treatments on the protein levels (represented by the arbitrary units; AU) of SREBP1, and the phosphorylated proteins including p-P70S6K-Thr389, p-Raptor-Ser863, and p-Raptor-Ser792 in mono-cultured NK92 cells after 72hrs. Data presented as mean ± SEM (n = 6). Statistical analysis was conducted by one-way ANOVA test.

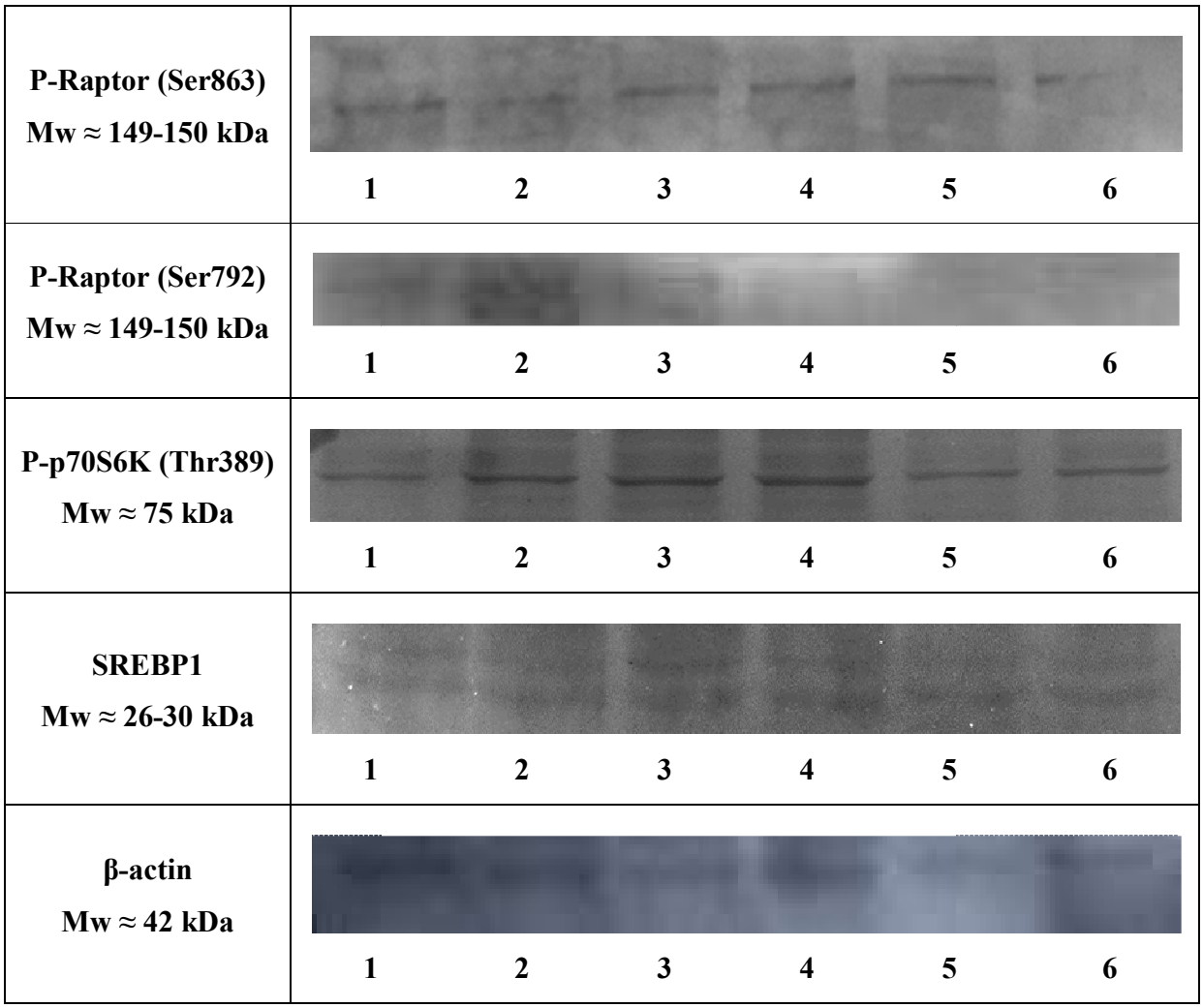

Figure (30): Representative Western Blot analysis of SREBP1 and the phosphorylated proteins in the mTOR pathway, including phosphorylated Raptor at the Ser863 and Ser 792 sites, and phosphorylated P70S6K at the Thr389 site in mono-cultured NK92 cells under different experimental treatments of folate and succinate. The cells were treated for 72 hrs with the treatments included: **1).** 0.02 mM FA (ctrl), **2).** 0.2 mM FA, **3).** 0.2 mM 5-MTHFA, **4).** NoF (no folate added), **5).** 50 µM succinate, **6).** 500 µM succinate.

The Western blotting image shows the approximate molecular weights (Mw) for the target proteins in kDa depicted on the left side of the images. β-actin was used as a loading control. The Western blotting images shows a single band for each protein target, except for SREBP1, which exhibited two distinct bands at approximately Mw of 26 kDa and 30 kDa.

4.3. Effect of folate treatments on NK92 cell activation status and cytokines/ chemokines profile in co-culture conditions with MDAMB231 cancer cell line:

4.3.1. Effect of folate status on the co-cultured NK92 cell viability:

NK92 cells were co-cultured with MDAMB231 breast cancer cell line for 72hrs in media with different folate conditions (NoF; no folate added, 0.2 mM of either FA or 5-MTHFA, and 0.02mM of FA as a control group).

The viability of co-cultured NK92 cells was significantly reduced in the presence of high dose of FA and in the folate-deficient condition (Figure 31).

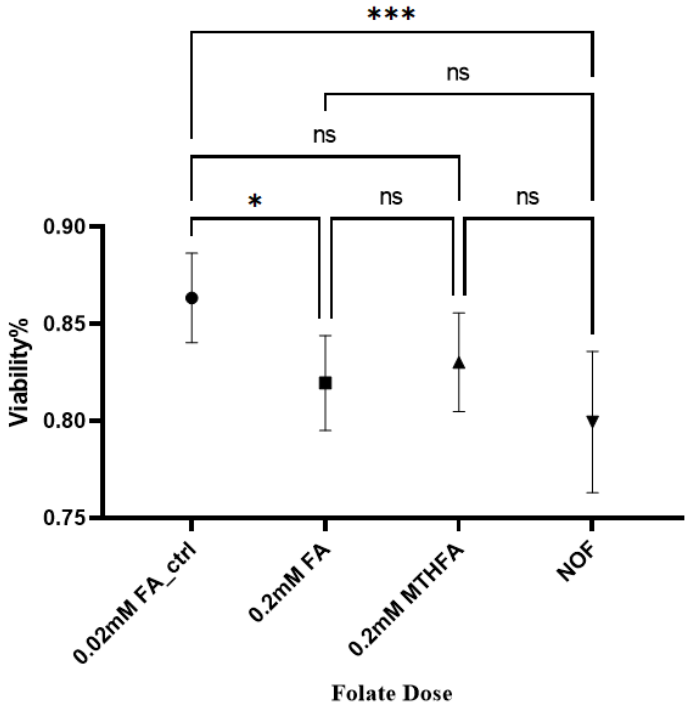

Figure (31): Symbol graph showing the effect of folate treatments on the viability of co-cultured NK92 cells with MDA-MB-231 after 72hrs.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test, Significance levels: *$p < 0.05$; ***$p < 0.001$; ^{ns} not significant.

4.3.2. Effect of folate treatments on the co-cultured NK92 cell activation status:

After 72 hrs, a significant increase in the MFI values for CD56 in FA over-supplemented group (0.2mM) compared to the control group. In contrast, folate-deficient group exhibited lower MFI values for CD56 than all the other groups (Figure 32A). However, the percentage of CD56+ NK92 cells in excess doses of both folate forms and deficient folate condition remained higher than the control group (Figure 32B). The MFI values for IFNγ in co-cultured NK92 cells and the percentage of IFNγ+ cells decreased significantly in FA over-supplemented and folate-deficient groups (Figure 32). However, a notable increase in the percentage of IFNγ+ cells was observed in the 0.2 mM of 5-MTHFA form (Figure 32).

4.3.3. Effect of folate treatments on HIF1α in the co-cultured NK92 cells:

The flow cytometry analysis revealed a significant decrease in the MFI values of HIF1α in co-cultured NK92 cells in folate over-supplemented groups (mainly; in 5-MTHFA form) and in folate-deficient condition compared to the co-cultured NK92 cells in the control FA treatment (Figure 33).

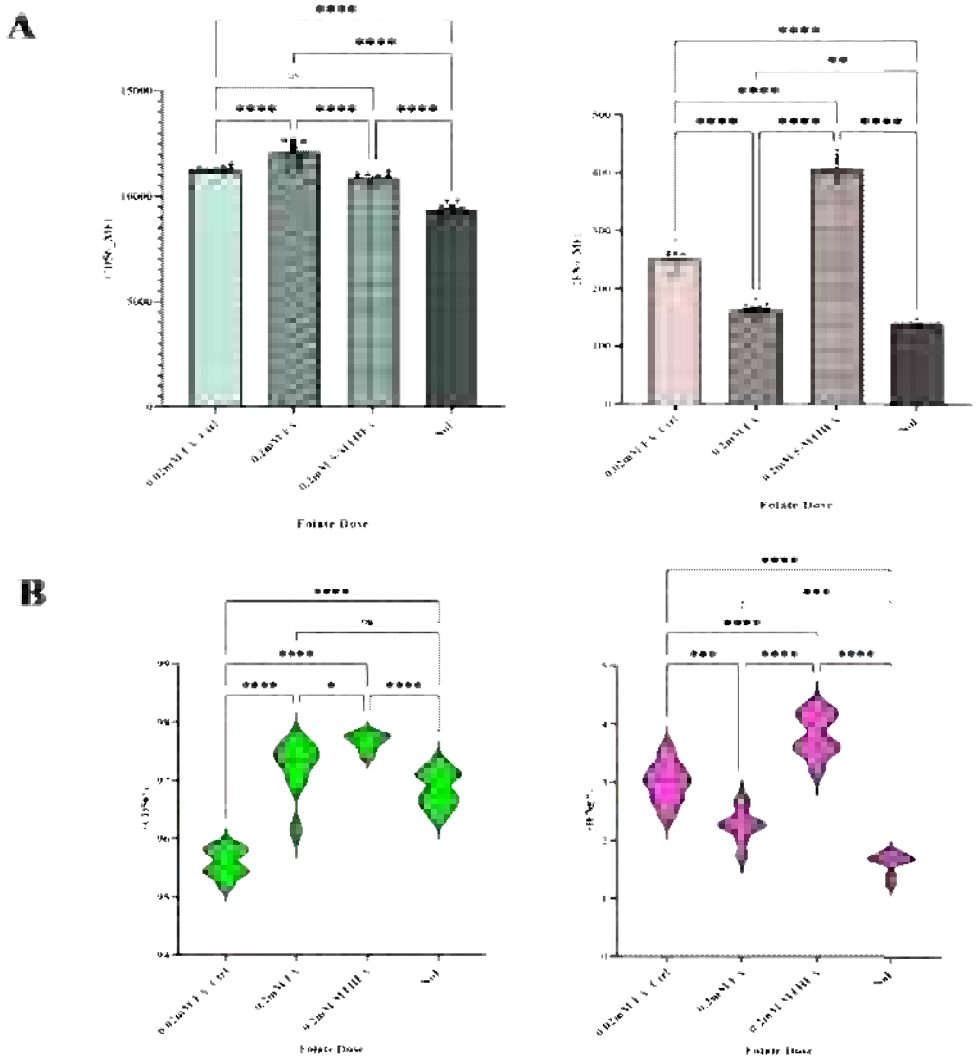

Figure (32): The effect of folate treatments on the activation status of co-cultured NK92 cells after 72 hrs. A). Column graphs representing the effect of folate treatments on the expression levels (represented by MFI) of CD56 and IFNγ in co-cultured NK92 cells. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Violin plots depicting the effect of folate treatments on the percentage (%) of positive cells expressing CD56 and IFNγ in co-cultured NK92 cells. The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ***$p < 0.001$; ****$p < 0.0001$; ^{ns} not significant.

A). **B).**

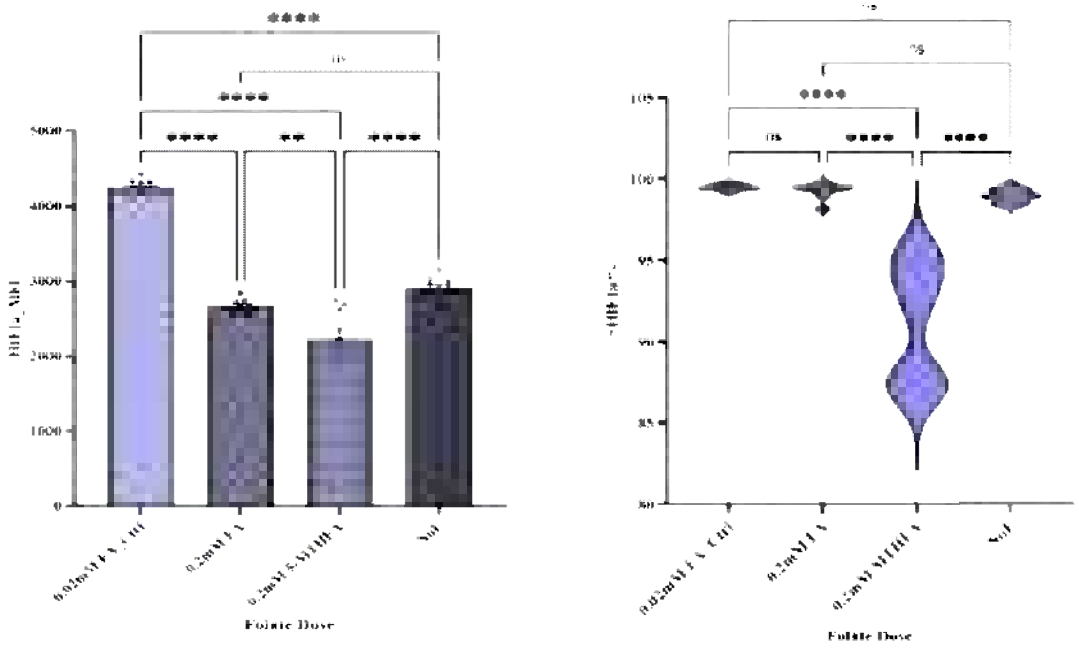

Figure (33): The effect of folate treatments on HIF1α in the co-cultured NK92 cells with MDAMB231 breast cancer cells after 72hrs.

A). Column graphs representing the effect of folate treatments on the expression levels of HIF1α (represented by MFI). Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Violin plot representing the effect of folate treatments on the percentage (%) of positive NK92 cells for HIF1α. The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: $^{**}p \leq 0.005$; $^{****}p < 0.0001$; ^{ns} not significant.

4.3.4. Effect of folate treatments on the profile of secreted cytokines and chemokines in the co-culture system of NK92 cells with MDAMB231 cancer cells:

The concentration of IL10 significantly increased in cultures supplemented with an excess dose of FA ($p = 0.036$), on the other hand, IL8 showed a significant decrease in both folate over-supplemented and folate-deficient cultures compared to the control group ($p < 0.0001$). However, the concentration of IL6 remained comparable across all folate conditions (Figure 34).

The concentrations of chemokines were also affected by the excess and deficient folate conditions. CCL2 (MCP1) concentration increased in these treatments ($p < 0.001$). In contrast, CXCL10 (IP10) concentration was comparable among all folate treatments ($p > 0.05$) (Figure 35). CCL5 (RANTES), a key chemokine involved in immune cell recruitment, showed a significant increase in cultures with over FA supplementation and folate-deficient condition ($p < 0.05$).

Interestingly, the concentration of CXCL9 (MIG), a chemotaxis chemokine, significantly decreased in cultured with high doses of folate and folate deficient conditions ($p < 0.0001$) (Figure 35).

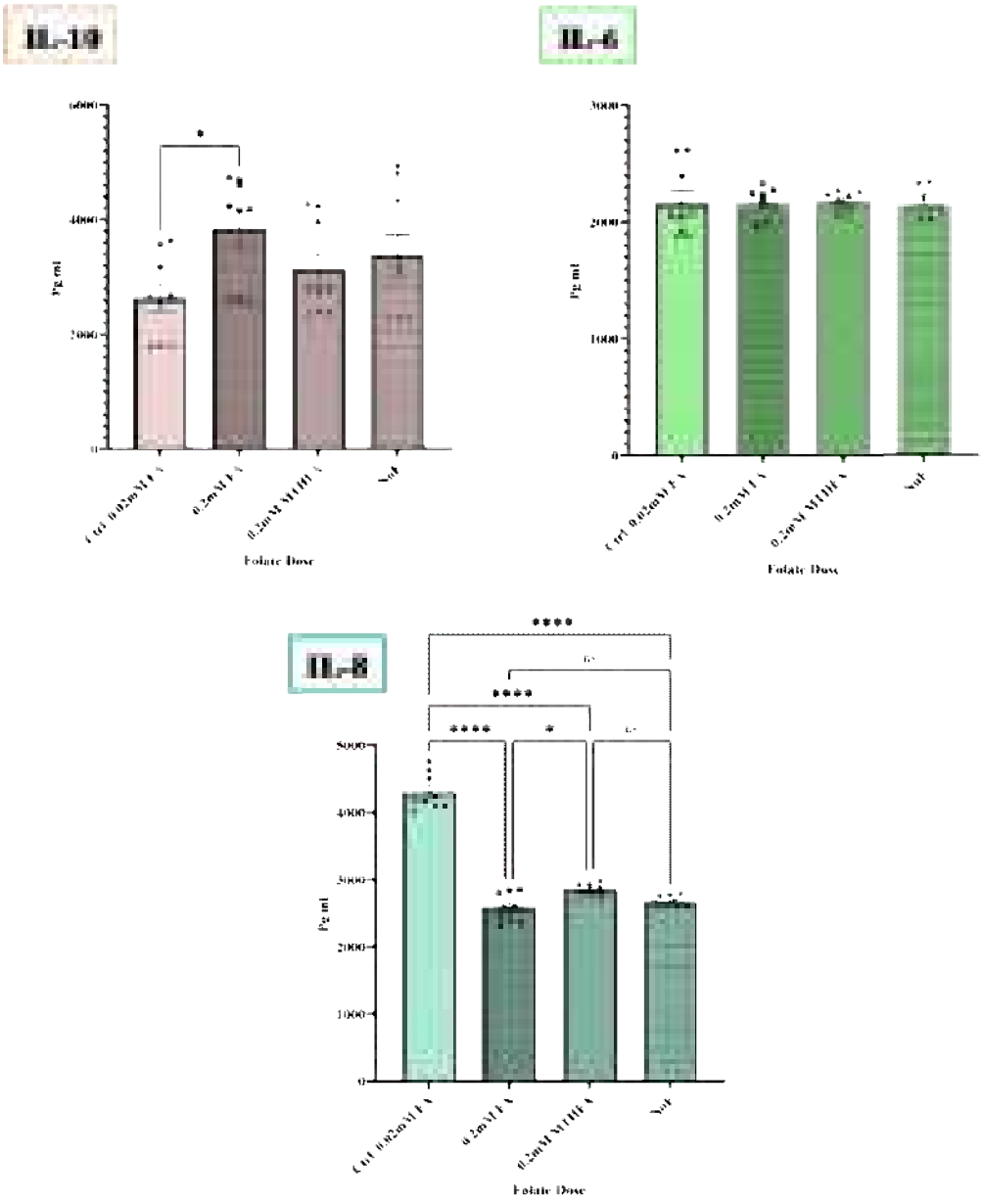

Figure (34): Column graphs representing the effect of folate treatments on the production of cytokines in the co-cultured system of NK92 cells with MDAMB231 cancer cells.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test. The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: **p* < 0.05; *****p* < 0.0001; [ns] not significant.

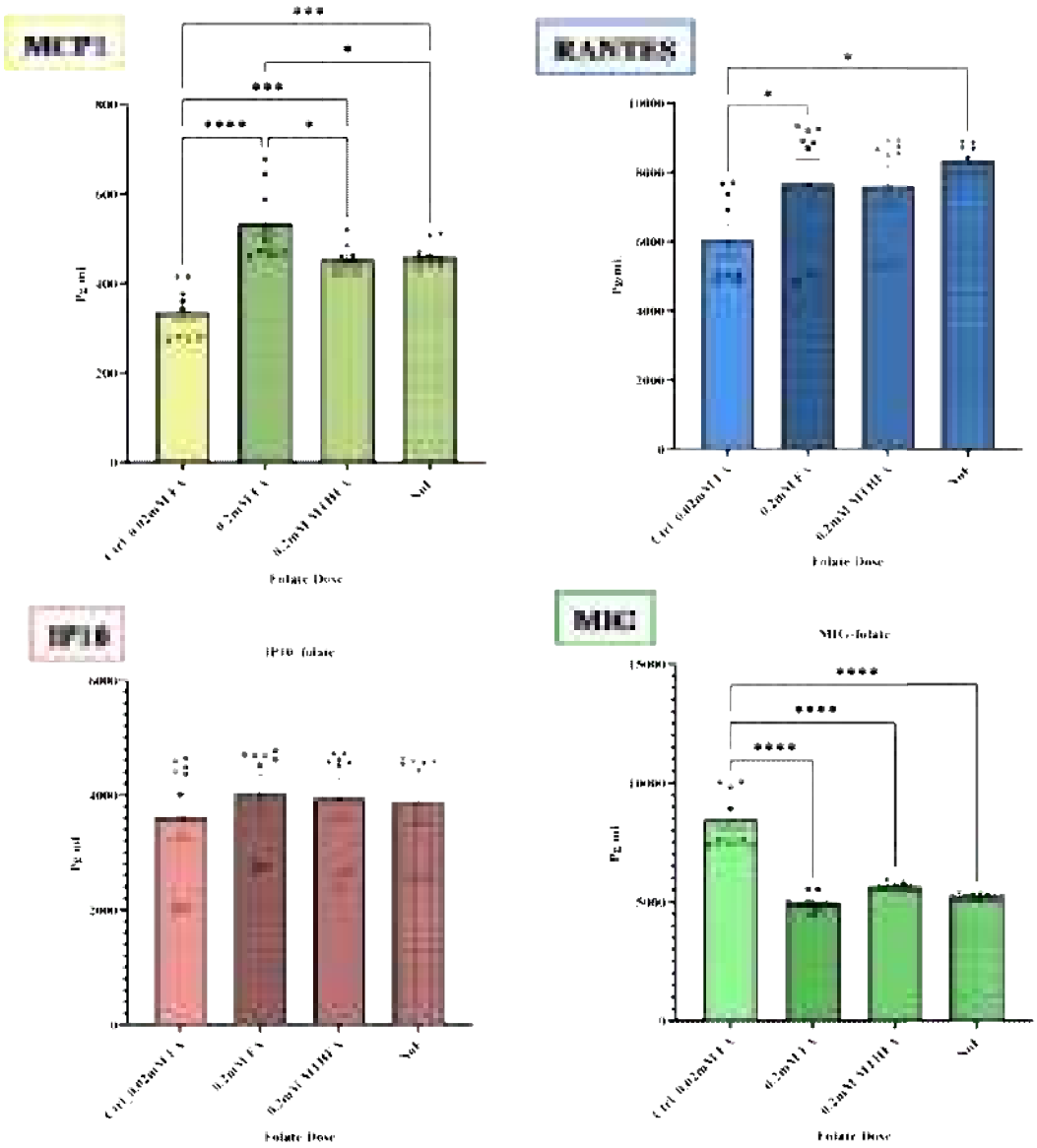

Figure (35): Column graphs representing the effect of folate treatments on the production of chemokines in the co-cultured system of NK92 cells with MDAMB231 cancer cells.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test.

The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

4.4. Effect of succinate treatments on NK92 cell activation status and cytokines / chemokines profile in co-culture conditions:

4.4.1. Effect of succinate treatments on the co-cultured NK92 cell viability:

NK92 cells were co-cultured with MDAMB231 breast cancer cell line for 72hrs in media supplemented with different succinate concentrations (50 µM and 500 µM of succinate, non-succinate media as a control group).

The viability of co-cultured NK92 cells was significantly reduced in the presence of high dose of succinate (500 µM) compared to the control group (Figure 36).

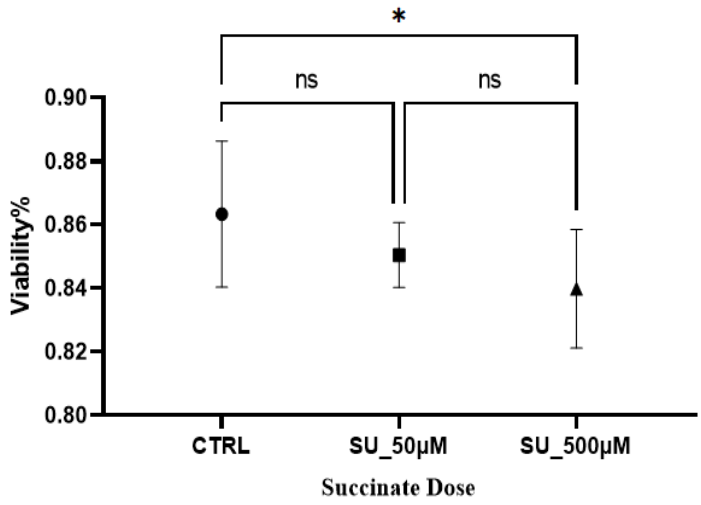

Figure (36): Symbol graph showing the effect of succinate treatments on the viability of co-cultured NK92 cells with MDAMB231 after 72hrs. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test, Significance levels: $*p < 0.05$; ^{ns} not significant.

4.4.2. Effect of succinate treatments on the co-cultured NK92 cell activation status:

The MFI values for CD56 and percentage of CD56+ cells after 72 hours were significantly increased in the succinate-treated groups (50 µM and 500 µM) compared to the control group without succinate supplementation ($p < 0.0001$). Additionally, the MFI values and the percentage of IFNγ+ cells were significantly elevated in the high-dose succinate group (500 µM) (Figure 37).

4.4.3. Effect of succinate treatments on HIF1α in the co-cultured NK92 cells:

The MFI- values for HIF1α and the percentage of HIF1α+ cells were significantly higher in the co-cultured NK92 cells treated with high-dose succinate (500 µM) compared to the control condition (Figure 38).

4.4.4. Effect of succinate treatments on the profile of secreted cytokines and chemokines in the co-culture system:

The concentration of IL10 did not show any significant changes in cultures supplemented with succinate. However, IL8 concentration exhibited a significant increase in succinate-treated cultures compared to the control culture ($p < 0.001$). Additionally, the concentration of IL6 was significantly elevated in cultures treated with 50 µM of succinate (Figure 39).

In terms of chemokines, succinate treatments did not appear to have a noticeable effect on the concentrations of CCL5 (RANTES), CXCL9 (MIG), and CXCL10 (IP10). However, CCL2 (MCP1) concentration showed a significant increase in cultures treated with 50 µM of succinate compared to the control group (Figure 40).

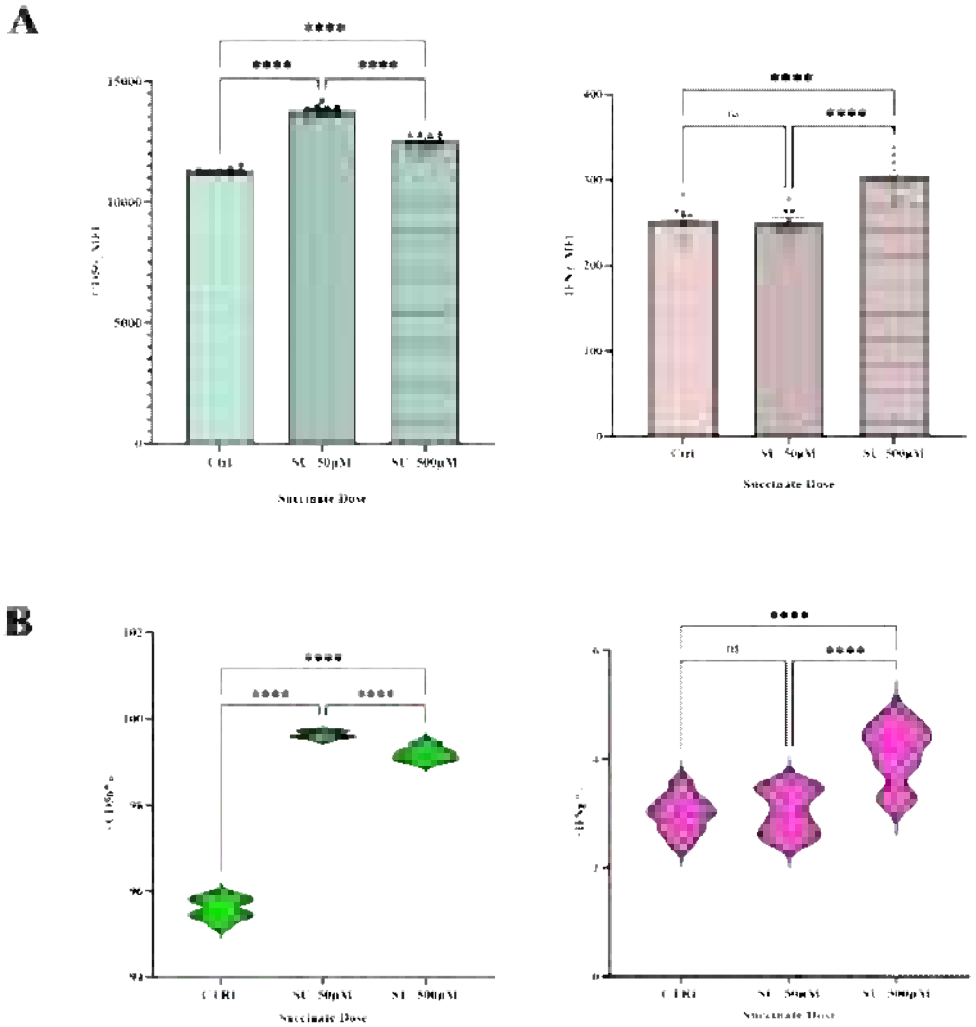

Figure (37): The effect of succinate treatments on the activation status of co-cultured NK92 cells after 72 hrs. A). Column graphs representing the effect of succinate treatments on the expression levels (represented by MFI) of CD56 and IFNγ in co-cultured NK92 cells. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Violin plots depicting the effect of succinate treatments on the percentage (%) of positive cells expressing CD56 and IFNγ in co-cultured NK92 cells. The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: ****$p < 0.0001$; ^{ns} not significant.

A). **B).**

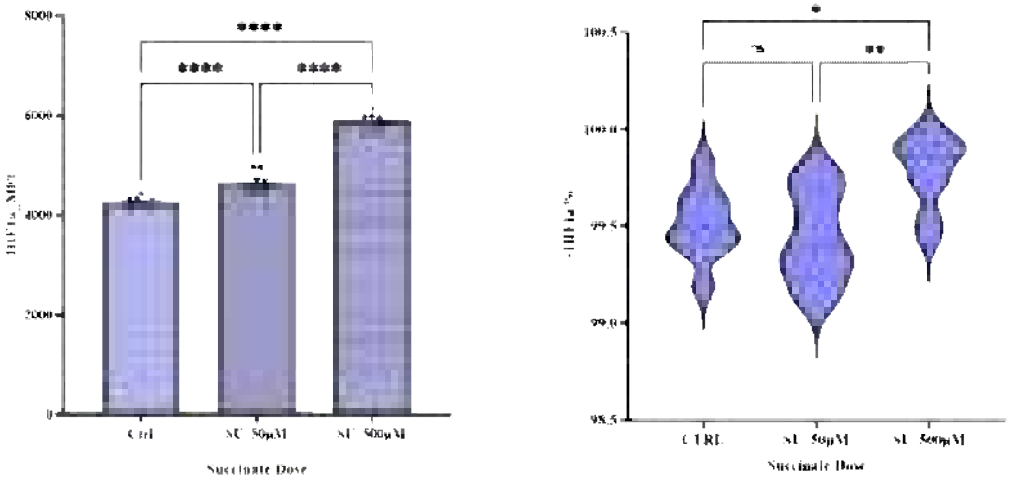

Figure (38): The effect of succinate treatments on HIF1α in the co-cultured NK92 cells with MDAMB231 breast cancer cells after 72hrs.

A). Column graphs representing the effect of succinate treatments on the expression levels of HIF1α (represented by MFI). Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Violin plot representing the effect of succinate treatments on the percentage (%) of positive NK92 cells for HIF1α. The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ****$p < 0.0001$; ^{ns} not significant.

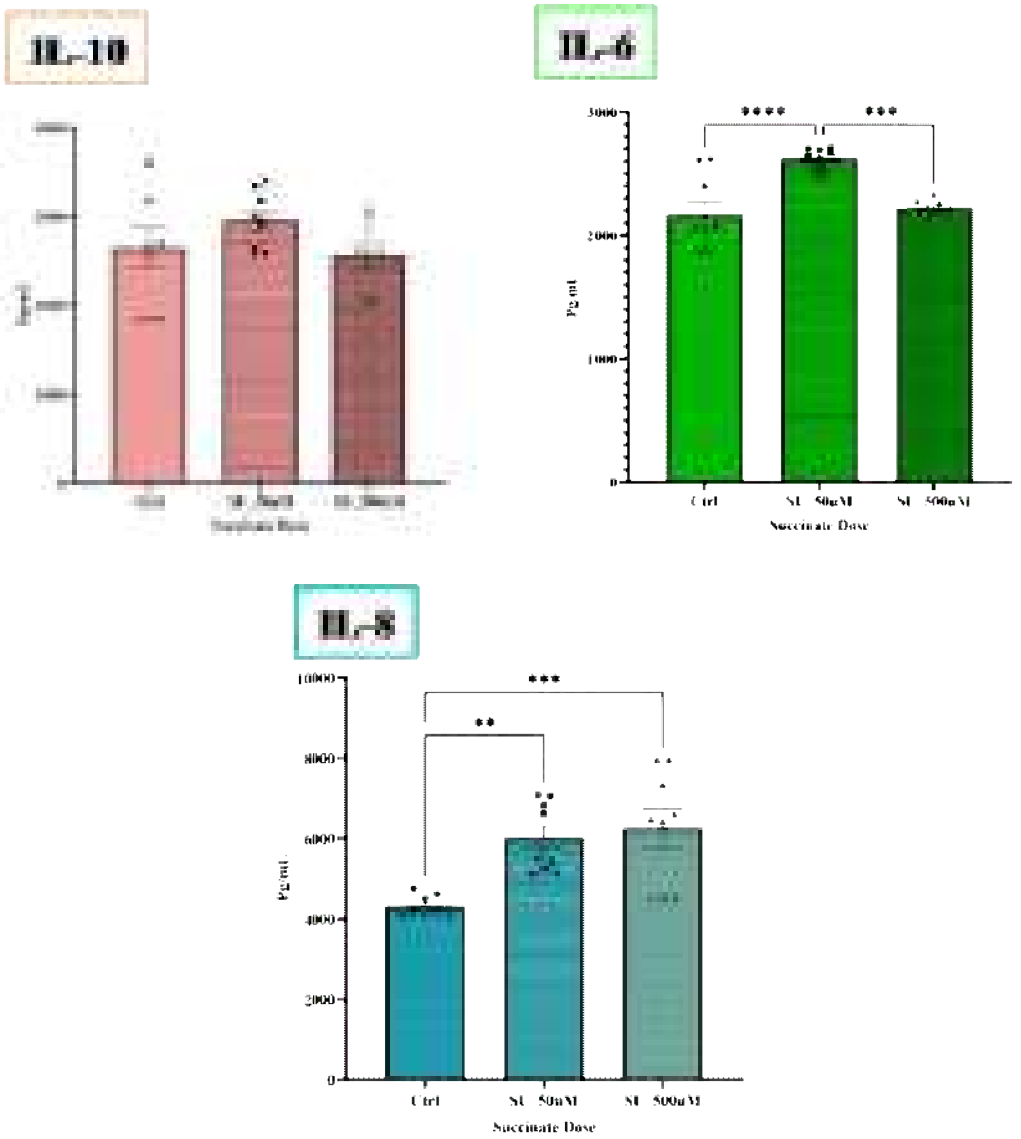

Figure (39): Column graphs representing the effect of succinate treatments on the production of cytokines in the co-cultured system of NK92 cells with MDAMB231 cancer cells.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test.

The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: $^{**}p \leq 0.005$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

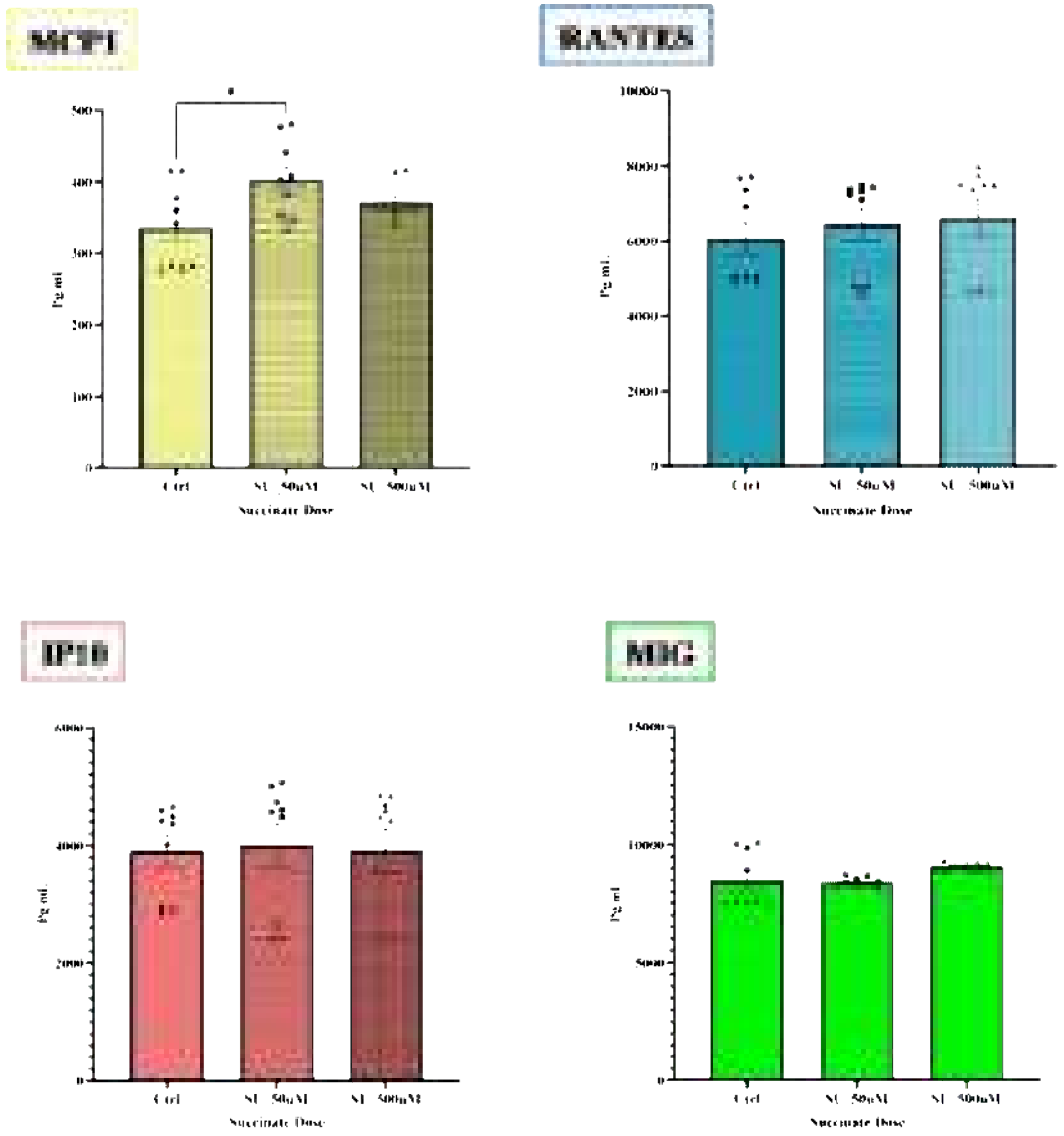

Figure (40): Column graphs representing the effect of succinate treatments on the production of chemokines in the co-cultured system of NK92 cells with MDAMB231 cancer cells.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test.

The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: $*p < 0.05$.

4.5. Effect of supplementation with 50µM succinate in different folate treatments on NK92 cell activation status and cytokines / chemokines profile in co-culture conditions:

4.5.1. Effect of folate treatments in the presence of 50µM succinate on the co-cultured NK92 cell viability:

NK92 cells were co-cultured with MDAMB231 breast cancer cell line for 72hrs in media with different folate conditions (NoF; no folate added, 0.2mM of either FA or 5-MTHFA and 0.02mM of FA as a control group) supplemented with 50 µM of succinate.

The viability of co-cultured NK92 cells was significantly reduced in the presence of high dose of folate, in 5-MTHFA less than FA, and in the folate deficient condition in the presence of succinate (Figure 41).

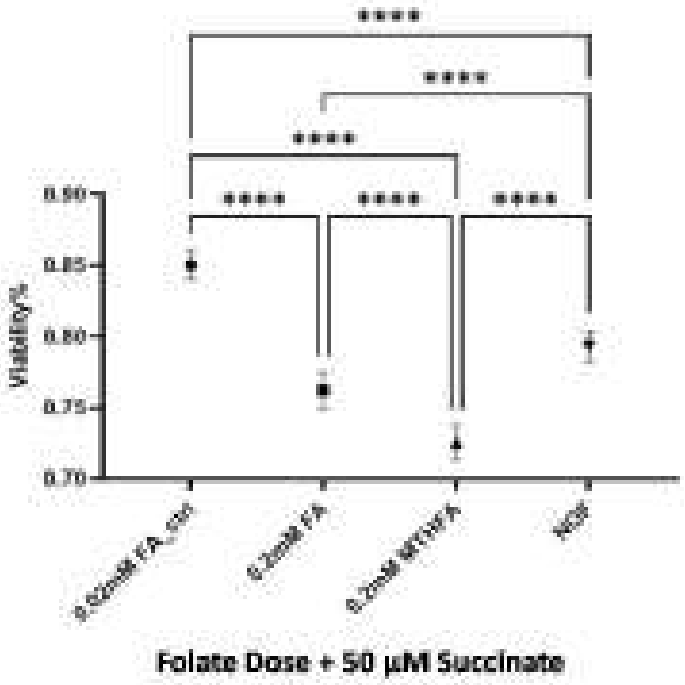

Figure (41): Symbol graph showing the effect of supplementation with 50µM succinate in different folate treatments on the viability of co-cultured NK92 cells with MDAMB231 after 72hrs.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test, Significance levels: ****$p < 0.0001$.

4.5.2. Effect of folate treatments in the presence of 50µM succinate on the co-cultured NK92 cell activation status:

The MFI values for CD56 and IFNγ exhibited a significant decrease in high folate treatments and folate-deficient conditions compared to the control group (0.02 mM of FA) (Figure 42A). Furthermore, the percentage of CD56+ NK92 cells in the excessive dose of FA displayed a notable decrease compared to both the control group and other folate conditions (Figure 42B). Similarly, the percentage of IFNγ+ cells significantly decreased in high folate treatments and folate-deficient condition compared to the control group (Figure 42B).

4.5.3. Effect of folate treatments in the presence of 50µM succinate on HIF1α in the co-cultured NK92 cell:

The MFI values of HIF1α showed a significant increase in all folate conditions compared to the control group (Figure 43A). However, there were no notable changes observed in the percentage of HIF1α+ cells among the different treatment groups (Figure 43B).

4.5.4. Effect of folate treatments in the presence of 50µM succinate on the profile of secreted cytokines and chemokines in the co-culture system:

The concentrations of cytokines, including IL10, IL6 and IL8 exhibited a significant increase in cultures with excessive and deficient folate conditions in the presence of 50µM of succinate ($p < 0.0001$) (Figure 44).

The concentration of chemokine CCL2 (MCP1) was also significantly increased in cultures under both excess and deficient folate conditions with 50 µM of succinate ($p < 0.0001$).
Interestingly, the concentration of CXCL9 (MIG) significantly increased in the high dose of FA compared to other folate conditions (Figure 45). However, there were no significant changes were observed in the concentrations of CXCL10 (IP10) and CCL5 (RANTES) compared to the control group (Figure 45).

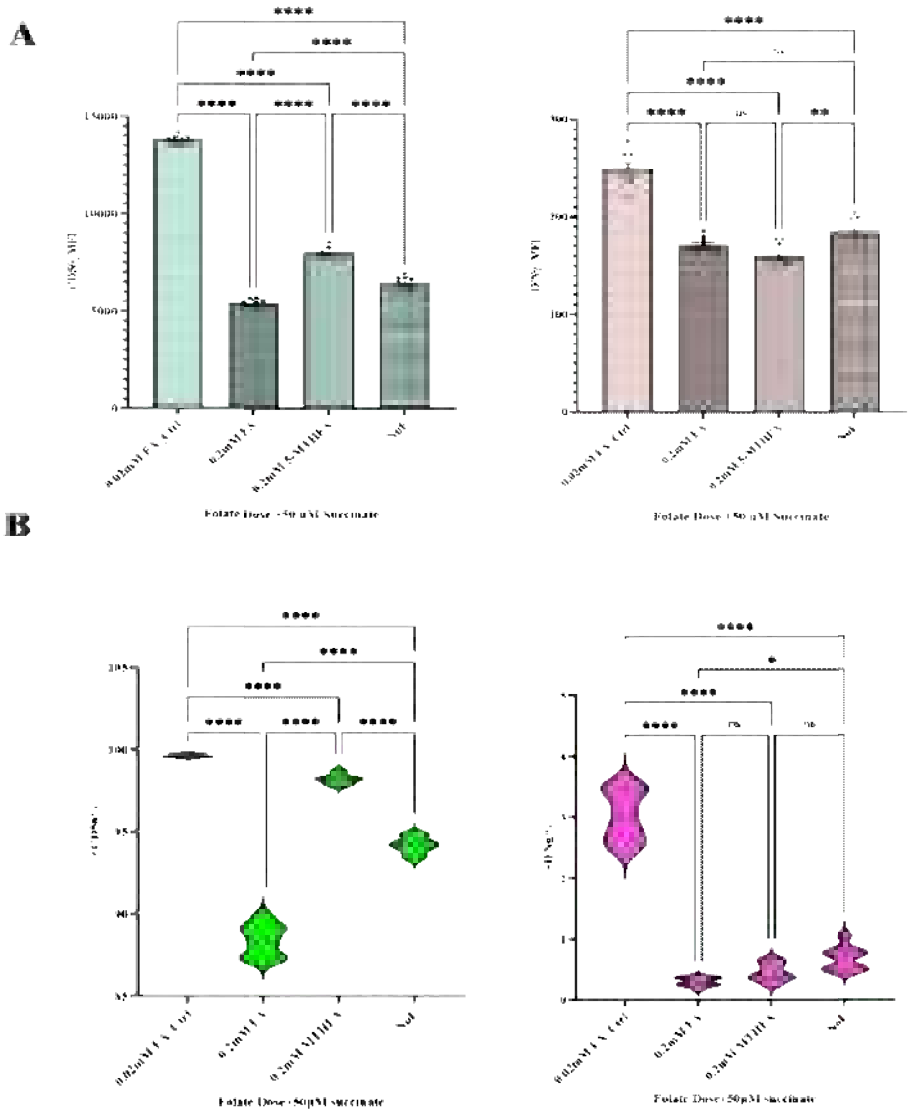

Figure (42): The effect of folate treatments in the presence of 50µM succinate on the activation status of co-cultured NK92 cells after 72 hrs. A). Column graphs representing the effect of folate treatments in the presence of 50µM succinate on the expression levels (represented by MFI) of CD56 and IFNγ in co-cultured NK92 cells Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). **B). Violin plots depicting the effect of folate treatments in the presence of 50µM succinate on the percentage (%) of positive cells expressing CD56 and IFNγ in co-cultured NK92 cells.** The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values. *P*-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: $^{**}p \leq 0.005$; $^{****}p < 0.0001$; ^{ns} not significant.

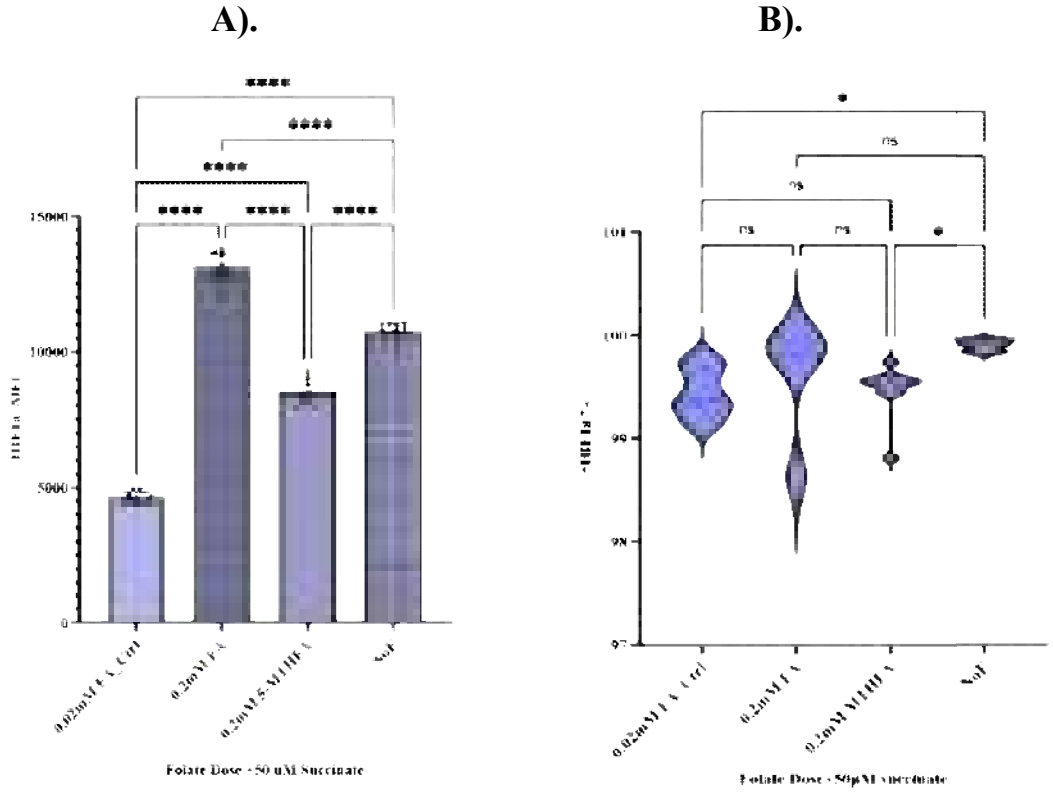

Figure (43): The effect of folate treatments in the presence of 50µM succinate HIF1α in the co-cultured NK92 cells with MDAMB231 breast cancer cells after 72hrs.

A). Column graphs representing the effect of folate treatments in the presence of 50µM succinate on the expression levels of HIF1α (represented by MFI). Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Violin plot representing the effect of folate treatments in the presence of 50µM succinate on the percentage (%) of positive NK92 cells for HIF1α. The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

P-values were obtained by one-way ANOVA test. The significant differences between the treatments are indicated as follows: *$p < 0.05$; **$p \leq 0.005$; ****$p < 0.0001$; ^{ns} not significant.

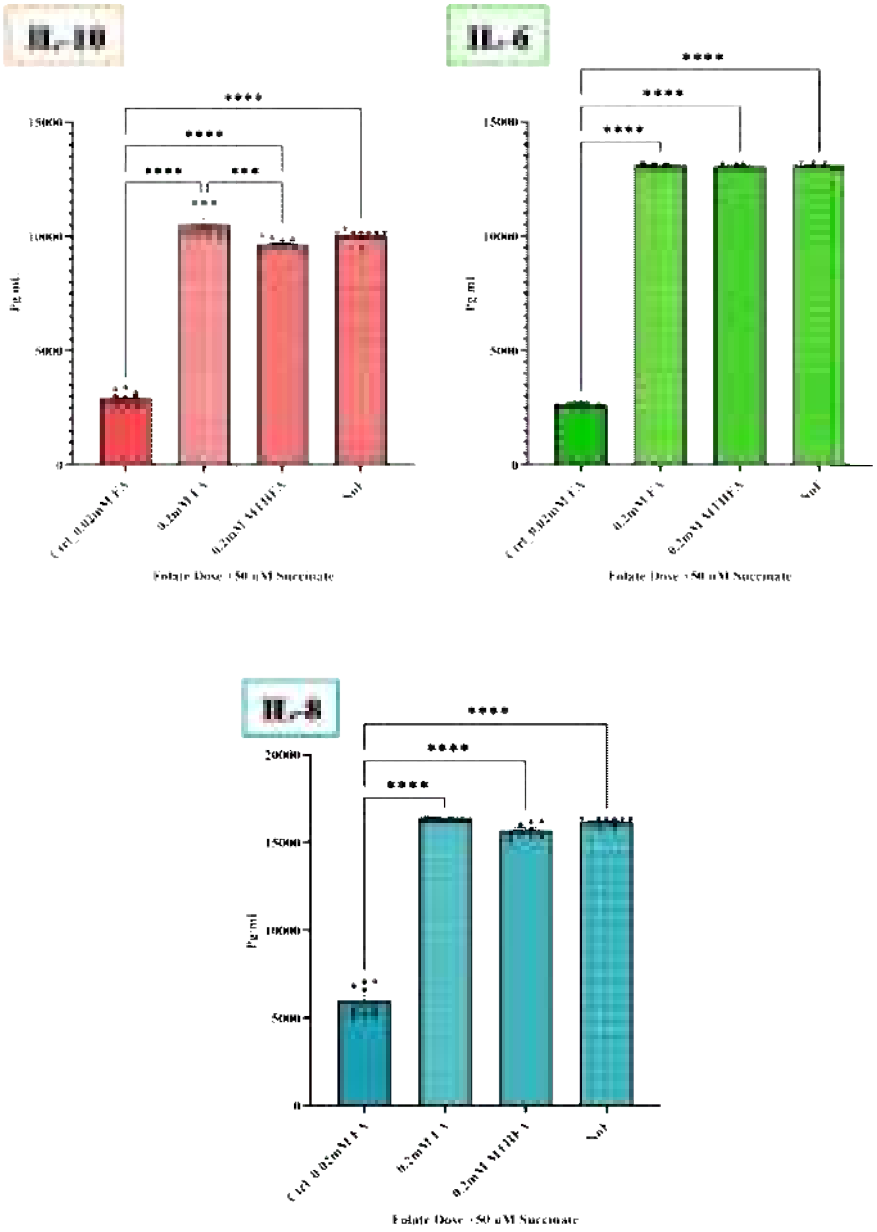

Figure (44): Column graphs representing the effect of folate treatments in the presence of 50µM succinate on the production of cytokines in the co-cultured system of NK92 cells with MDAMB231 cancer cells.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). *P*-values were obtained by one-way ANOVA test.

The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: ***$p < 0.001$; ****$p < 0.0001$.

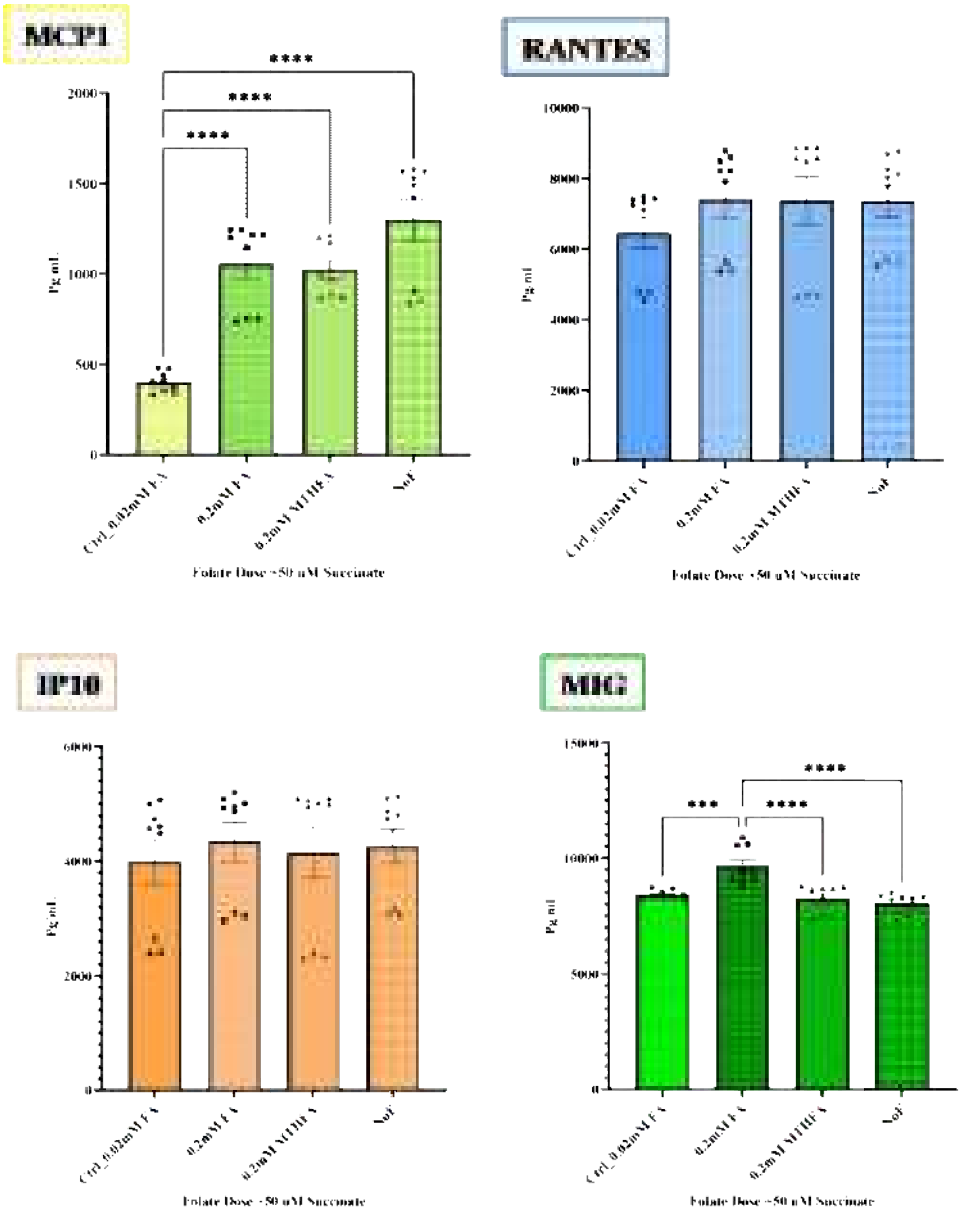

Figure (45): Column graphs representing the effect of folate treatments in the presence of 50µM succinate on the production of chemokines in the co-cultured system of NK92 cells with MDAMB231 cancer cells.

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by one-way ANOVA test.

The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: ***$p < 0.001$; ****$p < 0.0001$.

4.6. Differences in the effect of folate treatments on NK92 cells between mono-culture and co-culture systems:

4.6.1. Differences in the effect of folate treatments between mono-culture and co-culture systems on the activation status of NK92 cells:

NK92 cells in co-cultured conditions showed a general decrease in the MFI values of CD56 and percentage of CD56+ cells in all folate treatments compared to NK92 cells in the mono-culture conditions in the same treatments. However, a strong significant decrease in CD56-MFI values between mono-cultured and co-cultured cells was in 0.2mM of 5-MTHFA and NoF ($p < 0.0001$). Additionally, the percentage of CD56+ cells was notably higher in mono-culture in all folate treatments than in co-cultured conditions ($p < 0.0001$) (Figure 46).

Similarly, the IFNγ-MFI values were notably lower in co-cultured NK92 cells than in mono-cultured cells with strong significance in FA and NoF treatments ($p < 0.0001$) and with ($p = 0.003$) in 0.2mM of 5-MTHFA. The percentage of IFNγ+ cells increased in co-cultured NK92 cells in all treatments with strong significance in FA and 5-MTHFA treatments ($p<0.0001$) but low significance in the NoF condition ($p < 0.05$) (Figure 46).

4.6.2. Differences in the effect of folate treatments between mono-culture and co-culture systems on HIF1α in NK92 cells:

The HIF1α-MFI values were comparable between mono-and co-cultured NK92 cells in the control treatment (0.02mM of FA). However, the HIF1α-MFI values in high folate dose and folate-deficient conditions were notably lower in co-cultured NK92 cells ($p < 0.0001$) than in mono-cultured cells. There was no substantial change in the percentage of HIF1α+ cells between mono- and co-cultured NK92 cells (Figure 47).

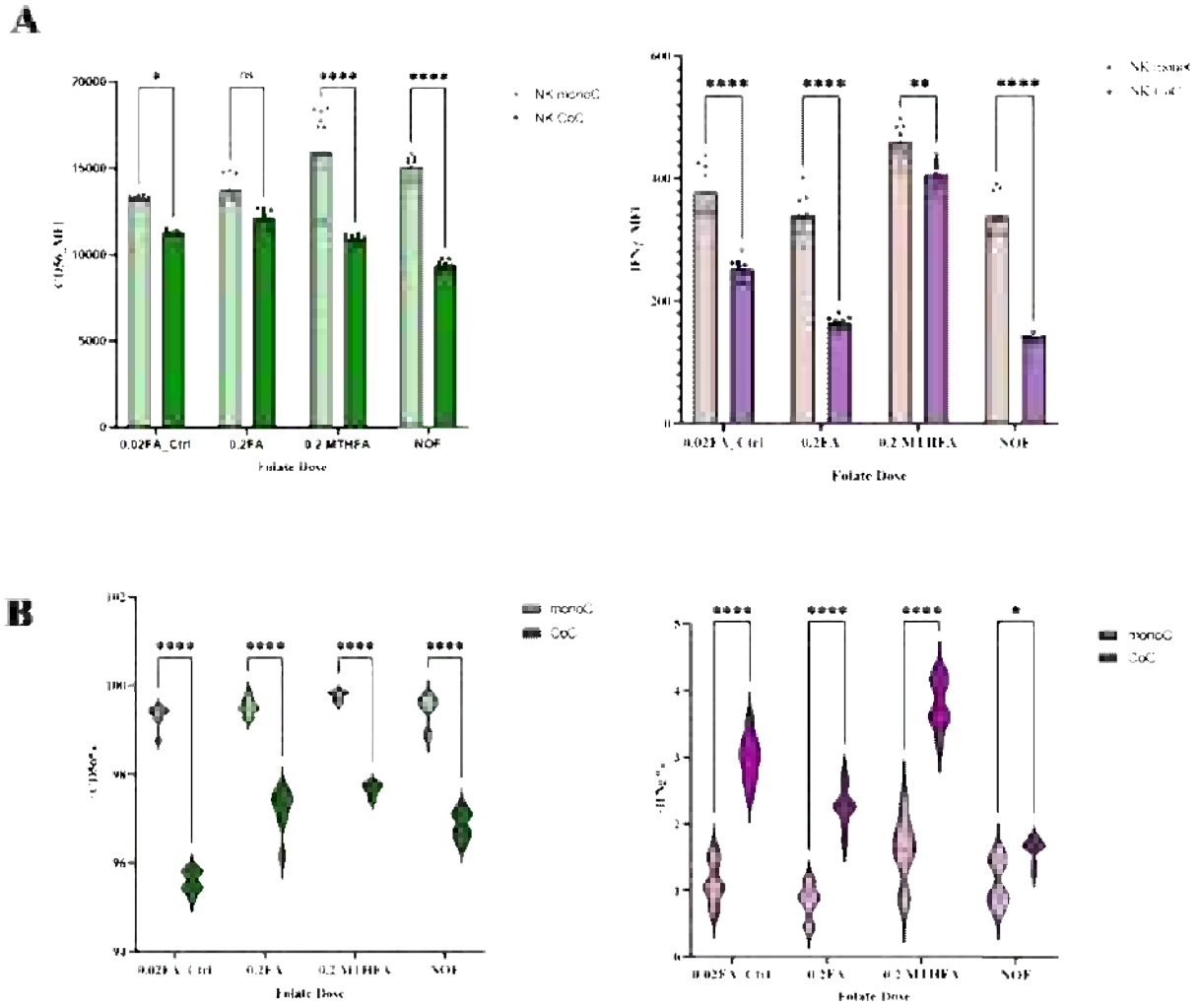

Figure (46): Differences in the effect of folate treatments on the activation status of NK92 cells between mono-culture and co-culture systems after 72 hrs.

A). Column graphs showing the differences in the effect of folate treatments on the expression levels (represented by MFI) of CD56 and IFNγ. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). **B). Violin plots depicting the differences in the effect of folate treatments on the percentage (%) of positive cells expressing CD56 and IFNγ.** The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values. monoC; mono-culture, CoC; co-culture. *P*-values were obtained by two-way ANOVA test. The significant differences between the culture systems are indicated as follows: $*p < 0.05$; $**p \leq 0.005$; $****p < 0.0001$; ^{ns} not significant.

A). **B).**

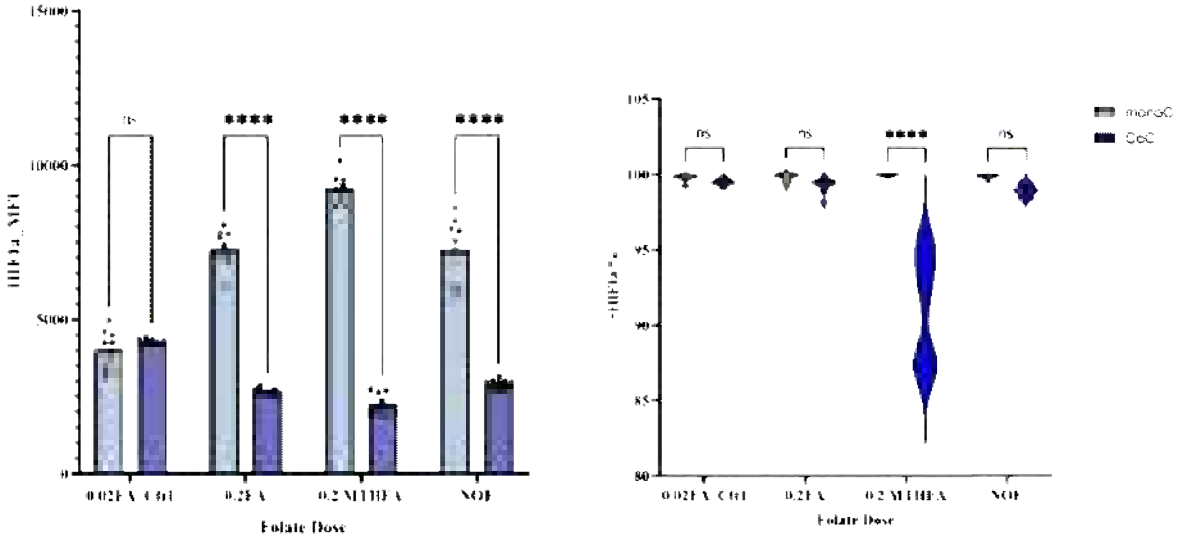

Figure (47): Differences in the effect of folate treatments on HIF1α between mono-culture and co-culture systems after 72hrs.

A). Column graph showing the differences in the effect of folate treatments on the expression levels of HIF1α (represented by MFI).

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Violin plot showing the differences in the effect of folate treatments on the percentage (%) of positive cells expressing HIF1α. The centre lines are median, the upper and lower lines are quartiles, and whiskers are minimum to maximum values.

monoC; mono-culture, CoC; co-culture.

P-values were obtained by two-way ANOVA test. The significant differences between the culture systems are indicated as follows: ****$p < 0.0001$; ^{ns} not significant.

4.7. Differences in the effect of succinate treatments on NK92 cells between mono-culture and co-culture systems:

4.7.1. Differences in the effect of succinate treatments between mono-culture and co-culture systems on the activation status of NK92 cells:

The CD56-MFI values and percentage of CD56+ cells in co-cultured NK92 cells were lower than in mono-cultured cells in the non-succinate control group. However, in succinate-treated cells, the CD56-MFI values notably increased in co-cultured NK92 cells than that in mono-cultured cells. The percentage of CD56+ NK92 cells in the co-culture condition in 500µM was comparable to that in the mono-culture condition (Figure 48).

The IFNγ-MFI values were lower in co-cultured NK92 cells than that in mono-cultured NK92 cells. However, the percentage of IFNγ+ cells was higher in all co-culture conditions (the control and succinate treated groups) compared to that in monoculture conditions (Figure 48).

4.7.2. Differences in the effect of succinate treatments between mono-culture and co-culture systems on HIF1α of NK92 cells:

The HIF1α-MFI values and the percentage of HIF1α+ cells increased notably in co-cultured cells in high concentration of succinate (500 µM) (Figure 49).

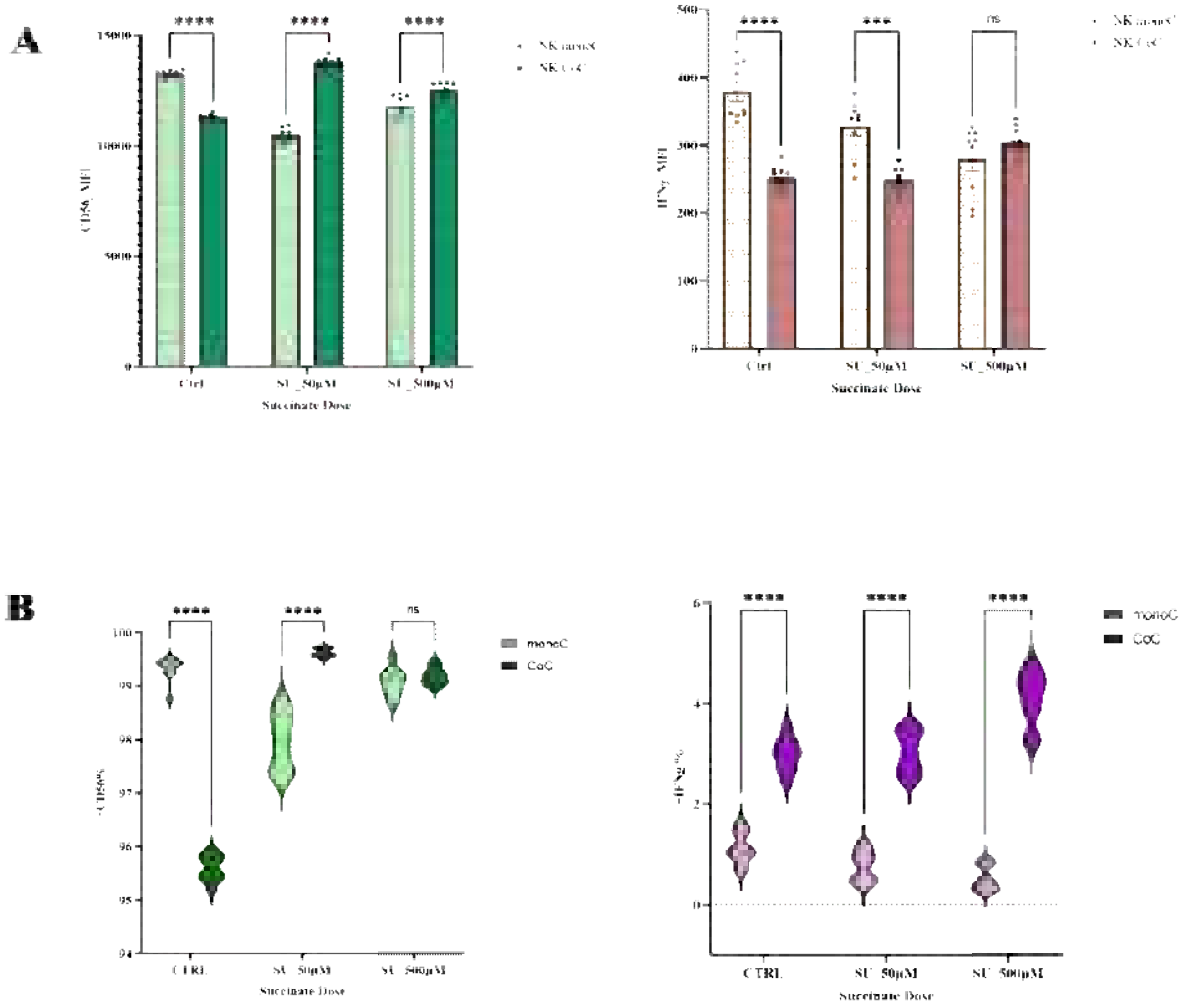

Figure (48): Differences in the effect of succinate treatments on the activation status of NK92 cells between mono-culture and co-culture systems after 72 hrs.

A). Column graphs showing the differences in the effect of succinate treatments on the expression levels (represented by MFI) of CD56 and IFNγ. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). **B). Violin plots depicting the differences in the effect of succinate treatments on the percentage (%) of positive cells expressing CD56 and IFNγ.** The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

"monoC"; mono-culture, "CoC"; co-culture. *P*-values were obtained by two-way ANOVA test. The significant differences between the culture systems are indicated as follows: **p* <0.05; ****p* <0.001; *****p* < 0.0001; ^{ns} not significant.

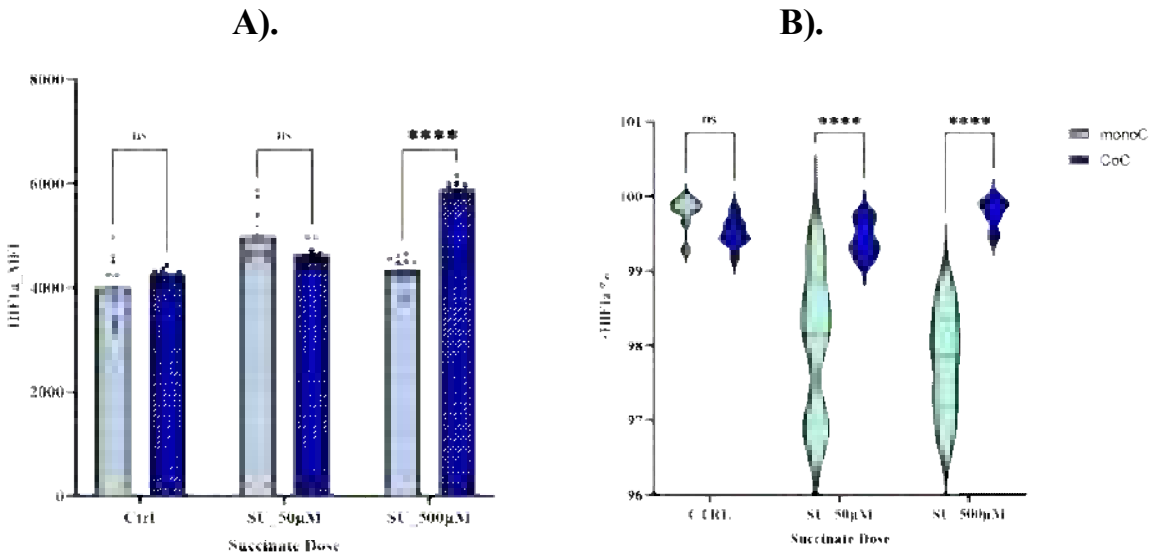

Figure (49): Differences in the effect of succinate treatments on HIF1α between mono-culture and co-culture systems after 72hrs.

A). Column graph showing the differences in the effect of succinate treatments on the expression levels of HIF1α (represented by MFI).

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Violin plot showing the differences in the effect of succinate treatments on the percentage (%) of positive cells expressing HIF1α. The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

"monoC"; mono-culture, "CoC"; co-culture.

P-values were obtained by two-way ANOVA test. The significant differences between the culture systems are indicated as follows: ****$p < 0.0001$; ^{ns} not significant.

4.8. Differences in the effect of folate treatments on NK92 cells between co-culture without succinate intervention and co-culture with 50µM succinate:

4.8.1. Differences in the effect of folate treatments on the activation status of NK92 cells in the co-culture system between with and without 50µM succinate intervention:

The CD56-MFI values of co-cultured NK92 cells increased in control folate media (0.02 mM of FA) supplemented with 50 µM of succinate more than that in the co-culture without succinate intervention (p <0.0001). However, adding succinate to the high and deficient folate treatments decreased the CD56-MFI levels of co-cultured NK92 cells (p < 0.0001) (Figure 50).

The IFNγ-MFI values of NK92 co-cultured cells with 50 µM of succinate supplementation maintained comparable values in FA treatment while increased in the folate-deficient condition, and decreased in 5-MTHFA treatment. However, the percentage of IFNγ+ cells decreased drastically in high doses and deficient conditions of folate after adding succinate to the co-culture setting (Figure 50).

4.8.2. Differences in the effect of folate treatments on HIF1α of NK92 cells in the co-culture system between with and without 50µM succinate intervention:

Succinate intervention in the co-culture with high doses and deficient conditions of folate increased substantially the HIF1α-MFI values in co-cultured NK92 cells (Figure 51).

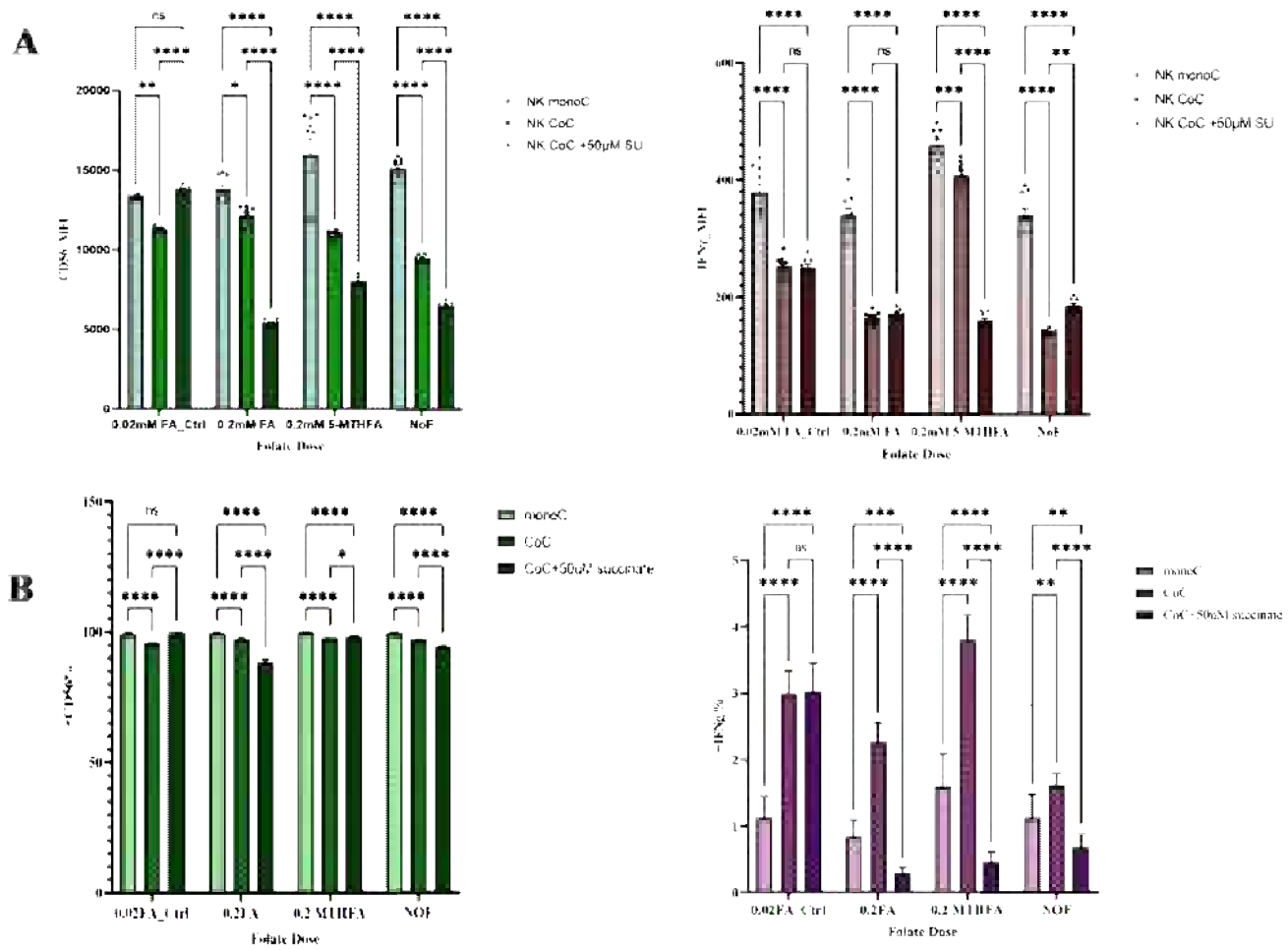

Figure (50): Differences in the effect of folate treatments on the activation status of NK92 cells between mono-culture and co-culture systems (with and without 50µM succinate intervention) after 72 hrs.

A). Column graphs showing the differences in the expression levels (represented by MFI) of CD56 and IFNγ between the culture systems. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM). **B). Column plots depicting the differences in the percentage (%) of positive cells expressing CD56 and IFNγ between the culture systems.** The centre lines are median, the upper and lower lines are quartiles, and whiskers are minimum to maximum values. "monoC"; mono-culture, "CoC"; co-culture, "CoC+50µM succinate"; co-culture system with 50µM succinate. *P*-values were obtained by two-way ANOVA test. The significant differences between the culture systems are indicated as follows: $*p < 0.05$; $**p \leq 0.005$; $***p < 0.001$; $****p < 0.0001$; ^{ns} not significant.

A). **B).**

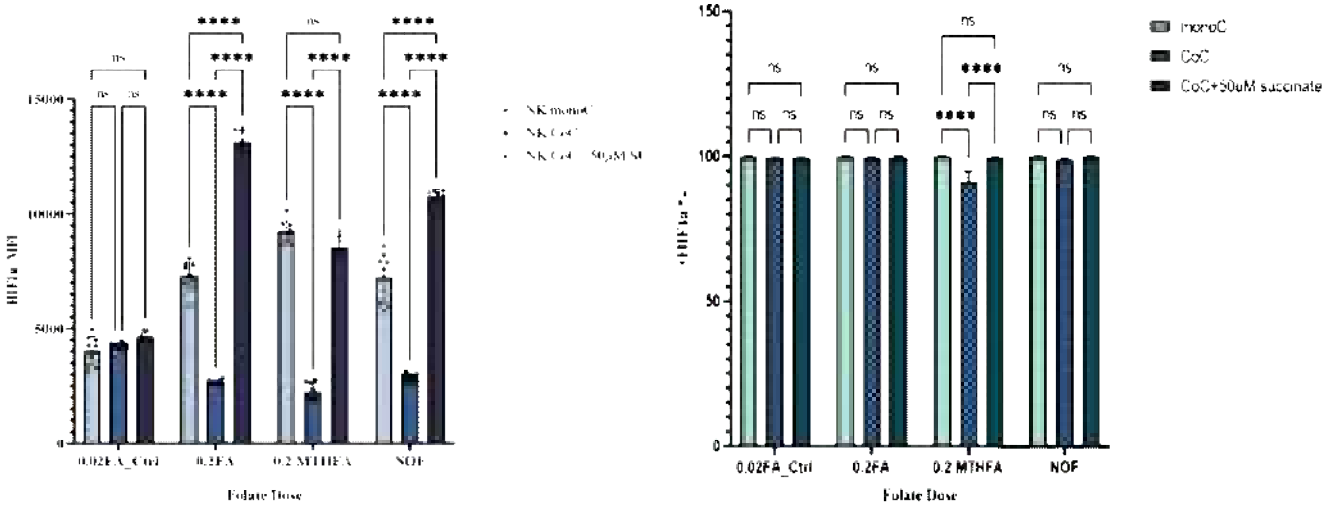

Figure (51): Differences in the effect of folate treatments on HIF1α between mono-culture and co-culture systems (with and without 50µM succinate intervention) after 72 hrs.

A). Column graph showing the differences in the effect of folate treatments on the expression levels of HIF1α (represented by MFI) between the culture systems. Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

B). Column plot showing the differences in the effect of folate treatments on the percentage (%) of positive cells expressing HIF1α between the culture systems. The centre lines are median, the upper and lower lines are quartiles, and the whiskers are minimum to maximum values.

"monoC"; mono-culture, "CoC"; co-culture, "CoC+50µM succinate"; co-culture system with 50µM succinate.

P-values were obtained by two-way ANOVA test. The significant differences between the treatments are indicated as follows: ****$p < 0.0001$; ^{ns} not significant.

4.9. Differences in the effect of folate treatments on cytokines / chemokines profile between co-culture systems without and with 50µM succinate intervention:

The addition of 50µM of succinate to co-culture conditions with both high folate and deficient folate conditions resulted in a significant increase in the levels of secreted cytokines including; IL6, IL8, and IL10 ($p < 0.0001$) (Figure 52).

The inclusion of 50µM of succinate in co-culture conditions with both high folate and deficient folate conditions led to a notable rise in the concentration of secreted chemokines CCL2 (MCP1) and CXCL9 (MIG) ($p < 0.0001$) (Figure 53). However, the chemokines CCL5 (RANTES) and CXCL10 (IP10) appeared unaffected under these conditions (Figure 53).

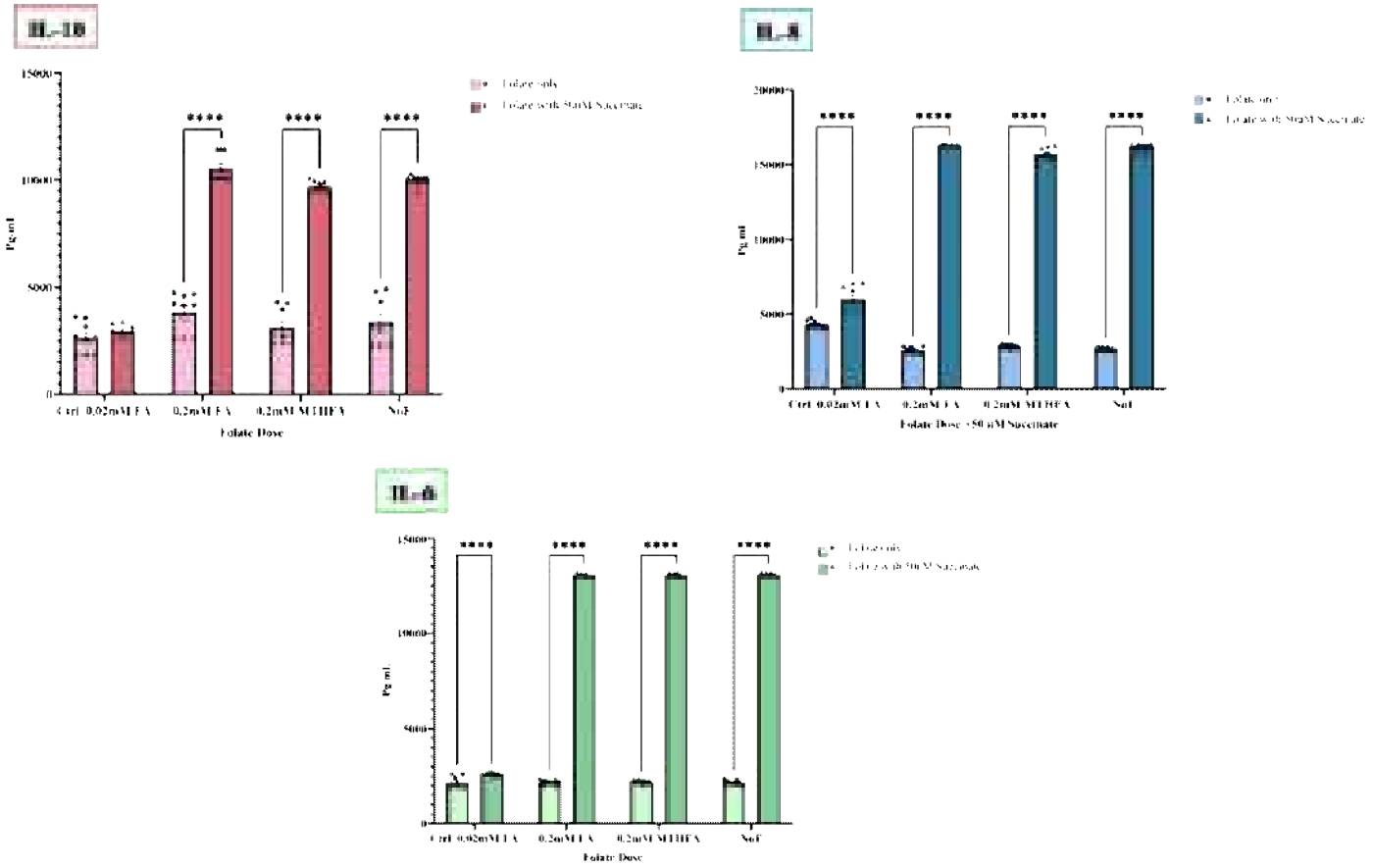

Figure (52): Column graphs representing the differences in the effect of folate treatments on the production of cytokines between co-culture systems without and with 50µM succinate intervention:

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by two-way ANOVA test.

The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: ****$p < 0.0001$.

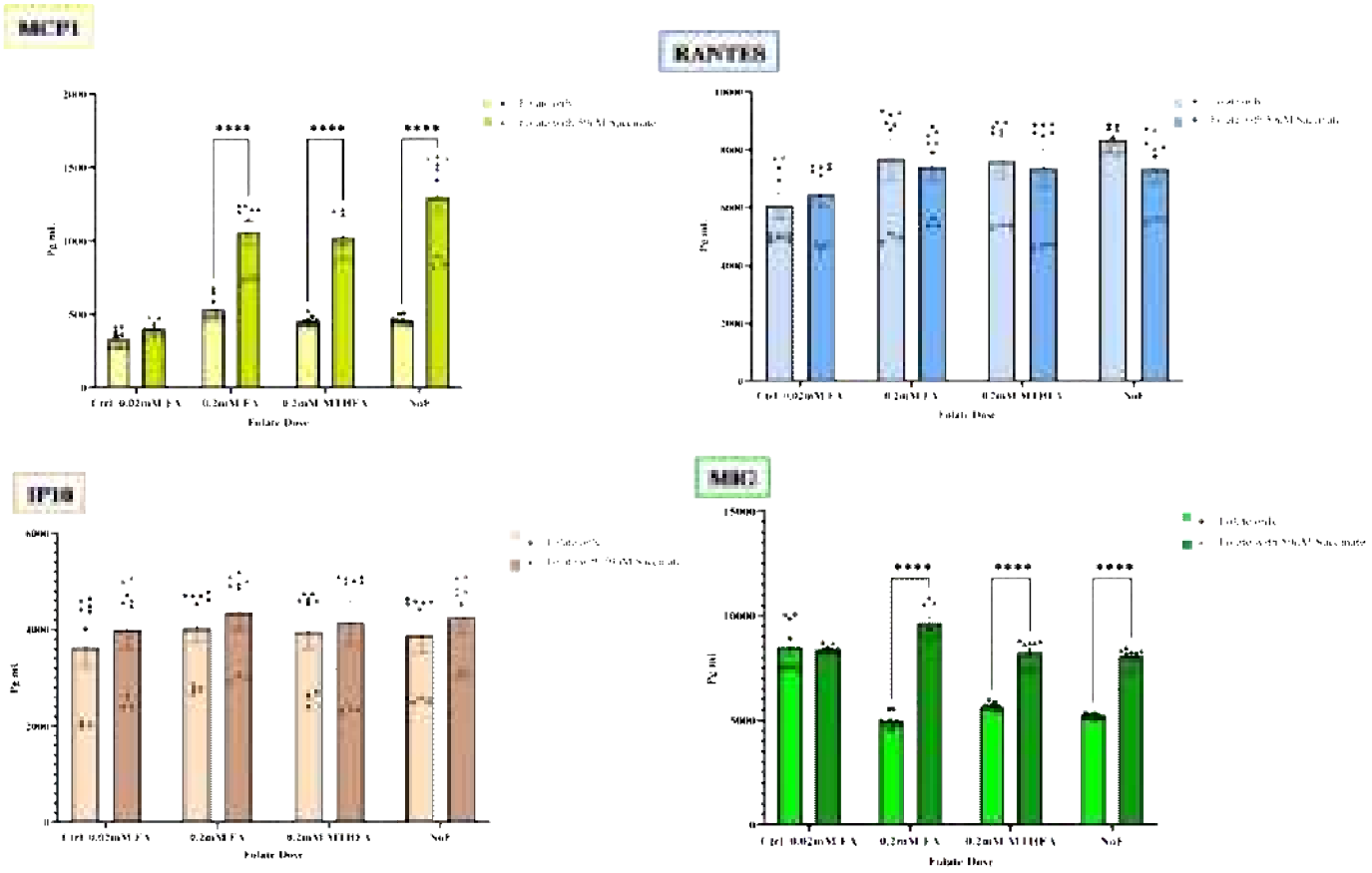

Figure (53): Column graphs representing the differences in the effect of folate treatments on the production of chemokines between co-culture systems without and with 50µM succinate intervention:

Values are means of (n = 9) and the error bars represent standard errors of the means (SEM).

P-values were obtained by two-way ANOVA test.

The significant differences in the concentration (Pg/mL) between the treatments are indicated as follows: ****$p < 0.0001$.