4. Results

4.1. High glucose induces oxidant stress in Jurkat T-cells

First, I examined whether high glucose, similar to hyperglycemia, can induce reactive oxygen species (ROS) generation in lymphocytes. Jurkat T-cells grown in normal glucose (5.5mM/L) or high glucose (20mM/L) for 24h were stained for 15 min with DCF-DA and green fluorescence formed due to ROS was determined by fluorescent microscope and spectrofluorometry. Result in Fig-2 shows that high glucose treatment induced a significant 3-4 fold increase in intracellular ROS generation in Jurkat T-cells. Consistent with spectrum of reports, high glucose induced ROS formation in lymphocytes too.

4.2. High glucose induces the expression of pro-inflammatory cytokine and chemokine genes in Jurkat T-cells

Next I sought after to profile high glucose induced expression of pro-inflammatory cytokine and chemokine in Jurkat T-cells by using RT²-PCR profiler arrays. Results from two independent experiments shown in Figure-3 and Table-2 shown that high glucose treatment induced the expression of surface antigens like CD247, CD3G, CD4, CD40L and CD8; Th17 cell specific cytokines like IL-17A and IL-17F; Th2 cell specific cytokines IL-17E and IL-10; Chemokines like IL-8, CCL-1, CCL-7, CCL-12, CXCL-1, CXCL-2, CXCL-5 and CXCL-12. In addition, cytokines like IL-6, TGF-β and IL-21 which are master regulators of Th17 cell differentiation, and key transcription factors specific for Th17 cells like ROR-γ and NF-κB1 were also up-regulated by high glucose. These results suggested that high glucose could induce Th17 immune response via transcriptional activation of Th17 lineage specific transcription factors and Th17 regulatory cytokines.
Figure-2: High glucose induces ROS formation in Jurkat T-Cells

Figure-2. High glucose induces ROS formation in Jurkat T-cells. (A) Fluorescence photomicrograph from normal glucose (NG) or high glucose (HG) grown Jurkat T-cells stained with DCF-DA. (B) Bar graph showing a significant 3-4 fold increase in ROS formation upon high glucose treatment compared with NG (***(p<0.001 vs. NG, n=3).
Figure-3: High glucose induces Th17 immune response related genes expression in Jurkat T-Cells

Figure-3: High glucose induces Th17 immune response genes expression in Jurkat T-cells. cDNA synthesized from Jurkat T-cells cultured with either normal glucose or high glucose were used for the RT²-PCR profiling assay. Cytokines showing increased expression are shown in the bar graph. High glucose treatment resulted in increased expression of genes associated with Th17 immune response. All values are the average of two independent experiments.
### Table-2: High glucose-induced changes in Th17 immunity related genes

<table>
<thead>
<tr>
<th>S.No</th>
<th>Gene</th>
<th>Description</th>
<th>Fold change</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CD-247</td>
<td>Cluster of Differentiation 247</td>
<td>↑2.5</td>
<td>Surface Antigen</td>
</tr>
<tr>
<td>2.</td>
<td>CD3G</td>
<td>Cluster of Differentiation 3</td>
<td>↑1.6</td>
<td>Surface Antigen</td>
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<tr>
<td>3.</td>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
<td>↑1.8</td>
<td>Surface Antigen</td>
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<tr>
<td>4.</td>
<td>CD40LG</td>
<td>Cluster of Differentiation 40 ligand</td>
<td>↑1.6</td>
<td>Surface Antigen</td>
</tr>
<tr>
<td>5.</td>
<td>CD8A</td>
<td>Cluster of Differentiation 8A</td>
<td>↑1.6</td>
<td>Surface Antigen</td>
</tr>
<tr>
<td>6.</td>
<td>IL-2</td>
<td>Interleukin-2</td>
<td>↑1.6</td>
<td>Cytokine</td>
</tr>
<tr>
<td>7.</td>
<td>IL-22</td>
<td>Interleukin-22</td>
<td>↑2.0</td>
<td>Cytokine</td>
</tr>
<tr>
<td>8.</td>
<td>IL-4</td>
<td>Interleukin-4</td>
<td>↑2.6</td>
<td>Cytokine</td>
</tr>
<tr>
<td>9.</td>
<td>IL-6</td>
<td>Interleukin-6</td>
<td>↑2.0</td>
<td>Cytokine</td>
</tr>
<tr>
<td>10.</td>
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<tr>
<td>11.</td>
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<tr>
<td>12.</td>
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<td>13.</td>
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<td>Cytokine</td>
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<tr>
<td>14.</td>
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<td>Interleukin-17E</td>
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<td>Cytokine</td>
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<tr>
<td>15.</td>
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<td>16.</td>
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<tr>
<td>17.</td>
<td>CCL-1</td>
<td>Chemokine ligand-1</td>
<td>↑1.8</td>
<td>Chemokine</td>
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<td>18.</td>
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<td>Chemokine XC Ligand 1</td>
<td>↑2.1</td>
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<td>Chemokine</td>
</tr>
<tr>
<td>20.</td>
<td>CXCL-2</td>
<td>Chemokine XC Ligand 2</td>
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<td>Chemokine</td>
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<tr>
<td>21.</td>
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<td>Chemokine</td>
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<tr>
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<td>IL-17RA</td>
<td>Interleukin-17RA</td>
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<td>Cytokine receptor</td>
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<tr>
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<td>IL-17RC</td>
<td>Interleukin-17RC</td>
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<td>Cytokine receptor</td>
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<tr>
<td>24.</td>
<td>CLEC-7A</td>
<td>C-type lectin</td>
<td>↑2.3</td>
<td>Growth factor</td>
</tr>
<tr>
<td>25.</td>
<td>CSF-3</td>
<td>Colony Stimulating Factor</td>
<td>↑2.5</td>
<td>Growth factor</td>
</tr>
<tr>
<td>26.</td>
<td>NF-xB1</td>
<td>Nuclear Factor-xB1</td>
<td>↑1.8</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>27.</td>
<td>ROR-C</td>
<td>Retinoic acid Receptor gamma</td>
<td>↑1.9</td>
<td>Transcription factor</td>
</tr>
</tbody>
</table>

↑ - Upregulation, fold of induction is given next to arrow.
4.3. Characterization of high glucose induced IL-6, IL-17E and IL-17F mRNA expression in Jurkat T-cells

Next to validate array results by candidate gene approach, 6 interleukin genes IL-6, IL-17A, IL-17B, IL-17C, IL-17E, and IL-17F expressions were analyzed by RT-PCR. To find out the optimal time interval, Jurkat T-cells were treated with normal glucose or high glucose for different time intervals from 24 – 72h. Results in Fig-4A-F, bar graphs in upper panels show that high glucose induced expression of IL-6 and IL-17A peaked at 48h (***p < 0.001) and then gradually decreased to 72h; IL-17E expression attained its maximum at 24h (***p < 0.001) and then decreased to 72h; IL-17C (*p < 0.05) and IL-17F (***p < 0.001) expressions increased from 24h and reached their peak at 72h. IL-17B expression was not significantly affected by high glucose at any point of time. These results are consistent with the array results in which IL-6, IL-17A, IL-17E and IL-17F were up-regulated by high glucose.

4.4. High glucose induced IL-6 and IL-17 family cytokine mRNA expression in Jurkat T-cells is specific to high glucose.

Next, to find out the dose dependant effect of high glucose, Jurkat T-cells were treated with various concentrations of glucose (5.5, 11, 15 and 20mmol) for 48h. Results in Fig.5A-D show that optimal concentration for IL-6, IL-17C, IL-17E and IL-17F expression was 15-20mmol/L. Furthermore, Jurkat T-cells with were treated high glucose and equimolar concentrations of osmolality control mannitol (5.5mmol/L glucose + 14.5mmol/L Mannitol).
Figure-4: Validation of array results by candidate gene approach

Validation of high glucose induced IL6 and IL-17 family cytokines expression. Relative multiplex RT-PCRs were performed with total RNA isolated from Jurkat T-cells treated with normal glucose or high glucose for 24 to 72h using gene-specific primers and 18S or GAPDH internal controls. (A-F) shows the time course of mRNA expression of IL-6, IL-17A, IL-17B, IL-17C, IL-17E and IL-17F respectively. Lower panel: ethidium bromide-stained agarose gels of RT-PCR products. Sign ‘+’, or ‘-’, indicate with or without high glucose treatment. Upper panel: bar graph showing significant induction of IL-6, IL-17A, IL-17C, IL-17E and IL-17F mRNA by high glucose. There was no significant increase seen in IL-17B mRNA by high glucose. Values shown are mean ± SEM of 3 independent experiments. (*p<0.05, **p<0.01, ***p<0.001 vs. NG).
Figure-5: High glucose induced increase in IL-6 and IL-17 family mRNA expressions are dose dependent in Jurkat T-Cells

Dose response effects of glucose on IL-6 and IL-17 family cytokine expression. (A-D) shows dose-response effects of glucose on IL-6, IL-17C, IL-17E and IL-17F mRNA expression in Jurkat T-cells. Total RNA isolated from the Jurkat T-cells treated with 5.5, 11, 15 and 20 mmol/L glucose for 48h was used for RT-PCR using gene-specific primers and 18S primer. Bottom panel shows representative ethidium bromide-stained agarose gels of RT-PCR products. Bar graphs in upper panel show dose dependent increase of IL-6, IL-17C, IL-17E and IL-17F mRNA expression by high glucose (*p<0.05, **p<0.01, ***p<0.001 vs. NG).
Figure-6. High glucose induced IL-6 and IL-17 family cytokine expression is specific to high glucose. Jurkat T-cells were treated with normal glucose (NG-5.5mmol/L) or mannitol (MN-5.5mmol/L glucose + 14.5 mmol/L mannitol) or high glucose (HG-20 mmol/L) for 48h. Total RNA isolated from these cells was used for RT-PCR using gene-specific primers and internal control primer. Bottom panel shows representative ethidium bromide-stained agarose gels of RT-PCR products. Bar graphs in upper panel show that high glucose alone induced IL-6 and IL-17 family cytokine expressions, whereas, osmolality control mannitol did not produce any effect on these cytokines expression (**p<0.01, ***p<0.001 vs. NG).
Results in Fig.6A-D show that IL-6, IL-17C, IL-17E and IL-17F mRNA expressions were significantly increased (***p < 0.001) by high glucose alone and not by mannitol. These results confirmed that high glucose induced IL-6, IL-17C, IL-17E, and IL-17F mRNA expressions are specific to high glucose.

4.5. Functional annotation of high glucose regulated genes

Functional annotation of high glucose induced genes was analyzed by DAVID, a web based software. Results of functional clustering analysis shown in Table-3 suggested that high glucose can regulate several enriched cellular functions of lymphocytes including inflammatory response, chemotaxis, cellular activation, proliferation, differentiation, cell to cell signaling, cell adhesion, regulation of effector immune response and transcriptional regulation and regulation of cytokine productions. As shown in Table-4, top scoring diseases associated with high glucose induced genes are immunological diseases, type-1 diabetes, renal and cardiovascular diseases. Further, key signaling events altered by high glucose are cytokine receptor signaling, JAK-STAT signaling, IL-17 signaling, Nucleotide binding Oligomerization Domain receptor-like signaling and chemokine signaling (Table-5). High glucose induced the expression of transcription factors like NF-κB1 and ROR-γ, hence it is plausible that it can transcriptionally regulate the expression of cytokines and related molecules. Thus, overall annotation results suggested that high glucose could transcriptionally induce the expression of Th17 cytokines in lymphocytes which could in-turn lead to activation of cytokine signaling resulting in lymphocyte activation, differentiation, adhesion and progressive diabetic complications.
Table-3: Functional annotation of high glucose induced Th17 immune response related genes identified in array

<table>
<thead>
<tr>
<th>S.No</th>
<th>Function</th>
<th>Enrichment Score</th>
<th>No. of Genes</th>
<th>Candidate Genes</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inflammatory Response</td>
<td>19.90</td>
<td>16</td>
<td>CLEC7A, CD40L, CCL22, CCL7, CXCL1, CXCL2, IL-10, IL-13, IL-17A, IL-17F, IL-22, IL-23, IL-25, IL-6, IL-8, NF-κB1</td>
<td>4.4e-16</td>
</tr>
<tr>
<td>2</td>
<td>Chemotaxis</td>
<td>18.08</td>
<td>11</td>
<td>CCL1, CCL7, CCL22, CXCL1, CXCL12, CXCL2, CXCL5, IL-10, IL-4, IL-6, IL-8</td>
<td>4.1E-12</td>
</tr>
<tr>
<td>3</td>
<td>Cell activation</td>
<td>6.21</td>
<td>14</td>
<td>CLEC7A, CD3G, CD4, CD40LG, CD8A, IL8, IL7R, CXCL12, HPRT1, IL-10, IL-2, IL-23A, IL-4, IL-6, IL-8</td>
<td>8.4E-14</td>
</tr>
<tr>
<td>4</td>
<td>Lymphocyte Proliferation</td>
<td>6.21</td>
<td>7</td>
<td>CD40LG, CXCL12, HPRT1, IL-10, IL-23A, IL-7R</td>
<td>1.1e-12</td>
</tr>
<tr>
<td>5</td>
<td>Lymphocyte differentiation</td>
<td>6.21</td>
<td>7</td>
<td>CD4, CD40L, CD8A, IL-10, IL-2, IL-4, IL-7R</td>
<td>1.9e-7</td>
</tr>
<tr>
<td>6</td>
<td>Regulation of effector Immune response</td>
<td>3.79</td>
<td>8</td>
<td>CD40L, TBX21, IL-10, IL-13, IL-2, IL-4, IL-6, IL-7R</td>
<td>4.8e-9</td>
</tr>
<tr>
<td>7</td>
<td>Cell-cell signaling</td>
<td>3.79</td>
<td>10</td>
<td>CCL122, CCL7, CXCL5, IL-10, IL-13, IL-17A, IL-8, IL-2, IL-22, IL-6</td>
<td>1.2e-5</td>
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<tr>
<td>8</td>
<td>Cell adhesion</td>
<td>3.79</td>
<td>5</td>
<td>CLEC7A, CD4, CD40L, CXCL12, IL2</td>
<td>9.7e-2</td>
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<tr>
<td>9</td>
<td>Transcriptional regulation</td>
<td>3.79</td>
<td>11</td>
<td>ROR-C, TBX21, IL-10, IL-17A, IL-17F, IL-22, IL-4, IL-6, NF-κB1, STAT4</td>
<td>1.0e-1</td>
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<tr>
<td>10</td>
<td>Regulation of cytokine Production</td>
<td>3.68</td>
<td>8</td>
<td>CLEC7A, CD4, CD40L, IL-10, IL-17F, IL-18, IL-6, NF-κB1</td>
<td>2.7e-7</td>
</tr>
<tr>
<td>S. No</td>
<td>Disease</td>
<td>No. of Genes</td>
<td>% of genes</td>
<td>Candidate Genes</td>
<td>p-value</td>
</tr>
<tr>
<td>-------</td>
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<td>--------------</td>
<td>------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>1.</td>
<td>Immunological Diseases</td>
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<td>61.1</td>
<td>CD4, CD40L, TBX21, CCL1, CCL7, CXCL12, CXCL5, IL-10, IL-13, IL-17F, IL-18, IL-2, IL-23A, IL-4, IL-6, IL-17R, IL-8, MMP-13, MMP-9, NF-κB1, SOCS3</td>
<td>8.1e-9</td>
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<td>Diabetes, Type-1</td>
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<td>27.8</td>
<td>CD4, CXCL12, IL-10, IL-13, IL-17A, IL-2, IL-4, IL-6, NF-κB1, SOCS3</td>
<td>2.0e-5</td>
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<tr>
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<td>Renal diseases</td>
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<tr>
<td>4.</td>
<td>Cardiovascular Diseases</td>
<td>11</td>
<td>30.6</td>
<td>CD40L, CXCL12, CSF3, IL-10, IL-4, IL-2, IL-6, MMP-13, MMP-9, NF-κB1</td>
<td>4.6e-2</td>
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<td>5.</td>
<td>Cancer</td>
<td>13</td>
<td>36.1</td>
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<td>1.7e-2</td>
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### Table-5: Top 5 signaling pathways associated with high glucose induced Th17 immune response related genes identified in array

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Pathway</th>
<th>No. of Genes</th>
<th>% of genes</th>
<th>Candidate Genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cytokine-Cytokine receptor interaction</td>
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<td>58.3</td>
<td>CD40L, CCL1, CCL22, CCL7,CXCL1, CXCL12, CXCL2, CXCL5,CSF3, IL-18, IL-2, IL-22, IL-23A, IL-25, IL-4, IL-6, IL-7R, IL-17RC, IL-8, IL-17A,</td>
<td>5.5e-20</td>
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<td>2.</td>
<td>JAK-STAT Signaling</td>
<td>10</td>
<td>30.6</td>
<td>CSF3, IL-10, IL-13, IL-2, IL-22, IL-23A, IL-4, IL-6, IL-7R, SOCS3</td>
<td>6.3e-9</td>
</tr>
<tr>
<td>3.</td>
<td>IL-17 signaling</td>
<td>8</td>
<td>22.2</td>
<td>CD247, CD3G, CD4, CD8A, CSF3, IL-7R, IL-8, IL-17A, IL-17F</td>
<td>1.9e-10</td>
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<td>4.</td>
<td>NOD-like receptor signaling</td>
<td>7</td>
<td>20.0</td>
<td>CCL7, CXCL1, CXCL2, IL-18, IL-6, IL-8, NF-κB1</td>
<td>9.8e-7</td>
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<tr>
<td>5.</td>
<td>Chemokine signaling</td>
<td>9</td>
<td>25.0</td>
<td>CCL1, CCL22, CCL7, CXCL1, CXCL12, CXCL2, CXCL5, IL-8, NF-κB1</td>
<td>6.7e-6</td>
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</tbody>
</table>
4.6. High glucose induces IL-6 and IL-17 family genes mRNA expression in PBLC

Although Jurkat T-cells represent typical human lymphocytes, their phenotype may not be identical with that of PBLC. Hence, I checked whether high glucose can produce similar response in PBLC. PBLC were isolated from healthy volunteers and treated with normal glucose or high glucose for 48h. RNA isolated from the PBLC was reverse transcribed, IL-6, IL-17A, IL-17E and IL-17F family gene expressions were analyzed by RT-PCR (Fig-7A-D). Result in Figure-8A-B shows that high glucose caused a dose dependant increase in IL-6 and IL-17E mRNA expression (**p < 0.01 and *p <0.05) PBLC too. Result in Figure-8C-D shows that high glucose induced IL-6 and IL-17E mRNA expression in PBLC was also specific to glucose and osmolality control mannitol did not induce cytokine mRNA expression in PBLC (**p < 0.01 and *p <0.05). I also analyzed high glucose induced IL-6, IL-17E and IL-17F mRNA expression by real time PCR. RT-qPCR results shown in Fig.9A-C show that high glucose significantly induced (**p < 0.01 and *p <0.05) the expression of IL-6, IL-17E and IL-17F compared with normal glucose in PBLC also.

4.7. Elevated IL-6, IL-17E and IL-17F mRNA expression in diabetes patient’s PBLC

To check the **in vivo** relevance of these in vitro results, I examined whether PBLC of diabetes patients had any elevated IL-6, IL-17E, and IL-17F mRNA levels compared with non-diabetes healthy subjects. PBLC isolated from normal and diabetes patients were directly processed for RNA isolation and mRNA expression of IL-6, IL-17E and IL-17F by RT-PCR. Figure-10A-B show 2-3 fold (*p < 0.05) increased IL-6 and IL-17E mRNA levels seen in both T1D and T2D subjects compared with control subjects.
Figure-7: High glucose induces IL-6 and IL-17 family mRNA expression in PBLC

Total RNA isolated from (2 x 10⁵ cells) PBLC of normal healthy non-diabetes adult donors stimulated with high glucose (20 mmol/L) was analyzed for IL-6, IL-17A, IL-17E and IL-17F mRNA expression by RT-qPCR. (A-D) Ethidium bromide-stained agarose gels of RT-PCR products of IL-6, IL-17A, IL-17E and IL-17F mRNA expression in normal glucose and high glucose treated PBLC.
Figure-8: High glucose induced increase in IL-6 and IL-17E mRNA expression in PBLC is dose dependant and specific to high glucose.

(A-B) shows dose-response effects of glucose on IL-6 and IL-17E mRNA expression in PBLC. (C-D) shows that high glucose induced IL-6 and IL-17E mRNA expression in PBLC is specific to glucose and osmolality control mannitol did not induce IL-6 and IL-17E mRNA expression in PBLC (**p<0.01, ***p<0.001 vs. NG).
Figure-9. Real time quantitative PCR analysis of high glucose induced IL-6 and IL-17 family genes expression

(A-C) Total RNA isolated from PBLC of normal healthy non-diabetes adult donors treated with normal glucose and high glucose was analyzed for IL-6, IL-17E and IL-17F mRNA expression by RT-qPCR. Bar graph show the fold induction over normal glucose treated lymphocytes, calculated by comparative Ct method after normalization with GAPDH internal controls (*p<0.05, **p<0.01).
Figure-10C shows that IL-17F mRNA was significantly up-regulated (*p < 0.05) in T1D subjects but not in T2D patients. Thus, these results represent the *in vivo* relevance of my *in vitro* results.

4.8. **Increased IL-17E protein levels in high glucose treated Jurkat T-cells and diabetes patient’s serum.**

Next, I checked the effect of high glucose on IL-17E protein secretion in Jurkat T-cells. Secreted IL-17E protein levels were analyzed in culture supernatants of Jurkat T-cells treated with high glucose by ELISA. Bar graph in Fig-11A shows significantly increased IL-17E protein level (1.5-2 fold, *p<0.05) by high glucose. Then, I examined the IL-17E protein levels in diabetes patient’s serum by ELISA and found 2 fold (**p <0.01) elevated IL-17E protein levels in both T1D and T2D patient’s serum compared with control volunteers as shown in Fig.11B. These results suggest that elevated blood glucose levels seen in diabetes patients could activate the lymphocytes and increase the mRNA and protein expression of inflammatory cytokines.
Figure-10: Elevated IL-6, IL-17E and IL-17F mRNA expression in diabetes patient’s PBLC
Lymphocytes from diabetes patients have elevated IL-6, IL-17E and IL-17F mRNA expression. PBLC isolated from four T1D patients, five T2D patients or nine healthy non-diabetes control subjects (Ctrl) were directly processed for RNA isolation and RT-PCR. Data quantified from diabetes subjects were pooled and expressed as fold over control subjects. (A & B) Significantly elevated IL-6 and IL-17E mRNA expressions were seen in T1D and T2D patient’s PBLCs; (C) IL-17F mRNA expression was elevated in T1D patients only, but not in T2D patients (*p< 0.05, **p< 0.01 vs. control subjects, N=9, T1D=4, T2D=5).
Figure-11: Increased IL-17E protein levels in high glucose treated Jurkat T-cells and diabetes patient’s serum

(A) Conditioned cell culture supernatants of Jurkat-T cells treated with normal glucose and high glucose were assayed for IL-17E protein levels by ELISA. Results shown are mean ± SEM from three experiments ran in triplicates.

(B) Serum from indicated control and diabetes subjects were assayed for IL-17E protein by ELISA. Results in bar graphs show significantly elevated IL-17E protein in diabetes patient’s serum compared with control volunteers (*p< 0.05 Vs Ctrl, Ctrl=9, T1D=4, T2D=5).
4.9. High glucose induced IL-6 and IL-17E mRNA expressions are due to increased transcription

After conforming that high glucose significantly induced IL-6, IL-17C and IL-17E mRNA expression in lymphocytes, next I investigated the molecular mechanism involved in this high glucose induced cytokines expression. High glucose induced IL-6, IL-17C and IL-17E mRNA expressions may be due to either increased transcription or decreased mRNA degradation. To evaluate whether high glucose induced expression of IL-6, IL-17C and IL-17E is due to increased transcription, Jurkat T-cells were pre-treated with actinomycin-D, a transcription inhibitor, for 1h and stimulated cells with high glucose. The effect of transcription inhibitor on high glucose induced IL-6, IL-17C and IL-17E mRNA was analyzed by RT-PCR. Results in Figure-12A-C show that actinomycin-D treatment significantly (** p < 0.01) blocked the high glucose induced IL-6, IL-17C and IL-17E mRNA expression. Thus, high glucose induced IL-6, IL-17C and IL-17E mRNA expression could be due to increased transcription.

4.10. Transcription Factor (TF) binding sites of human IL-17C minimal promoter region

Since high glucose induced IL-6, IL-17C and IL-17E expressions were due to increased transcription, next I decided to check whether high glucose stimulates the promoters of these genes. In order to perform a luciferase reporter gene assay human IL-17C promoter (-1278/+112) region was cloned into pGL3 luciferase expression vector in which expression of luciferase gene was under the control of IL-17C promoter. Human IL-17C gene is present in the forward
strand of chromosome 16 spanning the region 88,704,999-88,706,881bp and it is 1882bp in length. This gene has two transcript variant forms; Transcript variant-1 consists of 3 exons. Transcript variant-2 consists of 2 exons. Human IL-17C promoter sequence was located using MapViewer tool of NCBI and retrieved the sequence spanning from 88705504 to 88706994.

Human IL-17C minimal promoter sequence spanning from -1278 to +112 was chosen. The chosen regions were analyzed for transcription factor binding sites using web based TF search tool. The tab delimited files were uploaded and threshold level was set as 90% or above. TF search results revealed that human Human IL-17C promoter sequence consists of binding sites of AP-1 (-1237/-1229), SRY(-862/-855), delta-E (-768/-757), USF (-475/-464), and NF-κB (-73/-64) as shown in Fig.13.

4.11. PCR amplification and cloning of IL-17C promoter fragment

Cloning strategy of human IL-17C into pGL3 vector has been depicted in Fig.13. Human IL-17C promoter fragment (-1287/+112) was PCR amplified using primers described earlier and analyzed by agarose gel electrophoresis. PCR result showed amplification of 1.4 kb DNA fragment (Fig.14A). This amplified DNA fragment was forced cloned in desired orientation in pGL3 luciferase reporter plasmid and clone was confirmed by restriction digestion (Fig.14B) and DNA sequencing as shown in Fig.15.
Figure-12. High glucose induces transcription of IL-6, IL-17E and IL-17C mRNA in Jurkat T-cells. Jurkat-T cells were pre-treated with Actinomycin-D (0.1ug/ml) and then stimulated with high glucose. (A-C) RT-PCR analysis shows that high glucose induced IL-6, IL-17E and IL-17C mRNA expressions were significantly inhibited by Act-D respectively; signs “-” and “+” above the lanes indicate without or with Act-D or high glucose.
**Human IL-17C Promoter - Transcription Factor Binding Site Map**

```plaintext
1  ACCTCACACC TCACCTCATCA CACCTCCAAC TCAGACTTTT TAGCTTAGAC TTTTTTCTCA ATAAAACCAC AGAAGCCCAAG GCCGGGTGCA
   AP1 (94.3)
91  GTGACTCATG CCTGTGATCC CAGCACTTTG CGAGGCCGAG GTGGGCAGAT CACGAGGTCA GGAGATCGAG ACCATCCTGG CTAACATGGT
   AML-1a (100)
181 GAAACCCGGT CTCTACCTAA AATACAAAAA AATTAGCCAG GTGTGGTGGC GGGCGCCTGT AGTCCCAGCT ACCCGGCAGG CTGAGGCAGG
   SRY (96.4)
361 TCAAAA [AAAC AAC] [AAAAA AAAAACAAACAA AAAAACCCAA AAGAGACCTG AAGACGTGCC TGCCTCCCC ATCTCCGGGA CTCCTCCCAC
   USF (92.8)  delta E (94.4)
451 CTGGTGAAG [GGGCCAGCTG AACACTCCT CAGATTTGAC ATCTCAGCTA AATACAAAAA AAAAAAGTTT GATAATGTCA GACCAACAC CACCCTGTGT
   GATA1/2 (91.0)
721 ATCACTTGAG GTCCAGGCCG TCAGCCACC C [GGGCGATA TGG] [AAAAAC CTGTCTGTAC TAAAAATACA AAAATTAGCC AGGCATGGTG
   NF-κB (96.0)
811 GGGGGTGCCGCT TAACTCGCCG TACCTCCCCC GGACGCTCCG GGAATCCTGC GTGTTCTCGT GTCTCTCTCG GGGGAGGGCC GTAACAGGAC AGCGCCTCCC
   TATA Box
901 ACCATTTCGGGC ACCACAGGCGA GACCGCTCTC CAAAAAATA AAAAAGTTT GATAATGTC AAGCAAGGCA CACCCCTTCTGT
991 AGGTGTCAAG GTCAGGGCTC TCTCTCGTGT GGGAGGCGG GAAAGCTCCT CTGATGCTCT TGGTCTGATG TGGTCTGATG
1081 CACAGCCAGG GCCGGGTGGGA GGGACCGGCA AGGGAGGCCA GCAGTGTGAGC GATGTTGAAC TAATTTTTCT GTACCAGCG
   GAGTTGAAGG CCCAGTCTGG GCATGCTTCCT GTCCTCGGCT TCACTCCTC
1171 AGGTGTCAAG GTCAGGGCTC TCTCTCGTGT GGGAGGCGG GAAAGCTCCT CTGATGCTCT TGGTCTGATG TGGTCTGATG
   TATA Box
1261 GCCCACGCTG
```

**Figure-13:** Transcription factors binding sites were identified by TF search. Human IL-17C promoter sequence was submitted in the TF search at threshold level of 90%. The transcriptions factors related to human were selected respectively. Boxed sequences are human specific transcription factor binding sites. Percentages of homology are shown within brackets.
Figure-14: Cloning strategy of human IL-17C promoter into pGL3 reporter plasmid

Schematic representation shows cloning strategy used for cloning of the human IL-17C promoter into luciferase reporter plasmid pGL3.
Figure-15: Cloning of human IL-17C promoter into pGL3 Vector

A: Agarose gel picture PCR-amplified of DNA fragment

B: Confirmation of clone by restriction digestion

Figure-15: Cloning of human IL-17C promoter fragment (-1278/+112). A: Lanes 1-4 show PCR amplified DNA fragment using genomic DNA template. B: Lane 5 shows DNA molecular weight marker. B: Lane 1 shows DNA molecular weight marker. Lane 2 shows cloned PCR fragment in pGL3-Luc basic vector (pGL3IL-17C). Lane 3 shows HindIII and KpnI digested pGL3-IL-17C plasmid (-1278/+112). Arrow shows the released 1.4 kb DNA fragment from pGL3-IL-17C plasmid.
Figure-16: Confirmation of clone by sequencing

Figure-16A: Chromatogram picture of sequence of pGL3-IL-17C by using sense primer using the ABI –XL-3730 DNA sequencer.
Figure-16B: Chromatogram picture of sequence of pGL3-IL-17C by using anti-sense primer using the ABI – XL-3730 DNA sequencer.
Deduced nucleotide sequence from plasmid pGL3-IL-17C after DNA sequencing

1  ACCTCACACCTCACCTCACCTCACCTCACCTCAACTCTTTTAGCTTTTGAACCTTTTTCTCA
61  ATAAAAACACAGAAGGCCAGGCTCCGCTTCGCTATGCTGTCGATCTCCCTCGGACTCTCCCTCTCTGT
121  CGAGGCCGAGGTGGGAGAAGTCAAGGAGGAGGTTCAGGAGATCGAGACCATCCTGGCTAACATGGT
181  GAAACCCCGTCTCTCTTTGACTACCCACCCAGGCTCCAGGCTGGCGGCGCCTGT
241  AGTCCCAGCTACCCGGCAGGCTGAGGCAGGAGAATGGCGTGAACCCAGGAAGCTGAGCTTT
301  GTAGTGAGCCGAGATCAGACCATCCTGTACTCCCCGCCTGGGGCGACAGAGTCAGACTGCCGCC
361  TCAAAAAACAACAAAAAACAAAAAACAACAAAAAACAACAAAAACCTATGCTATGATTTAC
421  TGCCCTCCCCCTCTCCGGGACTGCTCTCCTGCACCTGGTGAAGGGGGACGTGAGCCTCACCTC
481  GATCTTGACCATCTCAGCTAAACACTGAAACCCACCTTTCGACACGCACAGCGACAGACGG
541  AGAAGAAGCTGTATAGGTTCAACCAGGGCCGGCCCAACATCAGGCTGAGACCCCAATGAAAAGCTAC
601  AAGTGAATCAGCAAGGTGTAGCTGACCGATACGCAAGCACAACACCTTCTTTATAGGAGTTCA
661  AGTGGGCGGAGATGAGGCTGAGGTGGCGGCGGCGGCGGCGGCAAGGCGGCCAGCGAGAG
721  ATCACTTGAGGTCAGGAGCCTCAGACCAGCCTGGCCGATATGGCAAAACCCTGTCTGTAC
781  TAAAAATACAAATATAGGTTCAACCAGGGCCGGCCCAACATCAGGCTGAGACCCCAATGAAAAGCTAC
841  GCCCTAGGGCAGAGTGCTAGTTGCCACCTGCATATGCAAGCCAGAGAGCTGAGGGACGC
901  ACCCACTCCCCCTCAGTCTCCGGGACTGAGACCCAGCCACTCTGGTCTCTCAAAAAAAAAAAAAAGTTT
961  GATAATGTCAGACAGCGGACACCCACCCCTCTGCTAGGTGTGTCAGGTGTTGGCCGAGCTCTCCTCG
1021  GGGGAGGGGGGTCAACAGGACAGGCCGCTCCGGGCTCCCTGGCTATGCTGTCGCTGCTGATG
1081  CACACGAGCGGGCTGGGCTGGGACCACAGAGGAAGGAAAGGCGGGCCACACCTGGCCACTCC
1141  CGAGGAGGAGTGTGGTGGCGGCAATTTTCCCCCAGATGGTGGCCCTCAGGTATAAAGACGCCGGCTG
1201  TGCCAGGGCGATGCGGCGGCAGTCCACTCTGTTGGGATTGCCGCCAGGTGTGCAGGCCCTCCAA
1261  GCCAAGGGCCTG
Homologous analysis of cloned human IL-17C promoter sequence with mouse IL-17C promoter

 Clone  ...ACCTCAC ACCTCACTCC TCACACCTCC AACTCAGACT TTTTAGCTTG AACTTTTTTC TCAATAAAAC CACAGAAGCC
 Human  ...ACCTCAC ACCTCACTCC TCACACCTCC AACTCAGACT TTTTAGCTTG AACTTTTTTC TCAATAAAAC CACAGAAGCC
 Mouse  AGGACAGCCA GGCTACACA GAGAAACCTT GTCTCGGAAA AAATAAAT.. AAATAAACA ACAAACAAAC CT.GTTGGAG

 Clone  CAGGCCGGGT GCAGTGACTCATGCCTGTGA TCCCAGCACT TTGCGAGGCC GAGGTGGGCA GATCACGAGG TCAGGAGATC
 Human  CAGGCCGGGT GCAGTGACTCATGCCTGTGA TCCCAGCACT TTGCGAGGCC GAGGTGGGCA GATCACGAGG TCAGGAGATC
 Mouse  AGGGTCTGGA GAGAGGATTCAGA....GGT TAAAAGCACT ..GGGTGTTC TTACAGCCAT GATTCCAGTT CCAGGAGATC

 Clone  ...GAGACCA .TCCTGGCTA ACATGGTGAA ACCCGTCTC TACTAAAAAT ACAAAAAAAT TAGCCAGGTG TGGTG....
 Human  ...GAGACCA .TCCTGGCTA ACATGGTGAA ACCCGTCTC TACTAAAAAT ACAAAAAAAT TAGCCAGGTG TGGTG....
 Mouse  AAAGGGTCCA CTCTTGGCCC TGCTGGGGGT ...CCAGACA ATACGTGCAG ACAAAACACT  CATCCACGGT GTTTGTCTTT

 Clone  .GCGGGCGCC TGTAGTCCCA GCTACCCGGC AGGCTGAGGC AGGAGAA..T GGCGTGAACC CAGGAAGCTG AGCTTGTA.G
 Human  .GCGGGCGCC TGTAGTCCCA GCTACCCGGC AGGCTGAGGC AGGAGAA..T GGCGTGAACC CAGGAAGCTG AGCTTGTA.G
 Mouse  TGGGGTGGTT TATTGTTT..G TTTGTTTTGT TTGTTATTTG TGTGTTATTT ATGAGACAGC GGGTTTCTCTA

 Clone  TG.AGCCGAG ATCGCACCAC TGTACTCCCC GCCTGGGCGA CAGAGTCAGA CTCCGCCTCA AAAAAACAAC AAAAAAACAAC AAAA
 Human  TG.AGCCGAG ATCGCACCAC TGTACTCCCC GCCTGGGCGA CAGAGTCAGA CTCCGCCTCA AAAAAACAAC AAAAAAACAAC AAAA
 Mouse  TGAGCCCTGG TGGTGCCTCA ..GAACACTG ...GGTACCA CAGAGCTGGC CTCAAACTCA CAAAGGATCTG CCTGCTCTGC

 Clone  ACAACAAAGA AACCCAACAG AGACCTGAAG ACGTGCCTGC CTCCCCCATC TCCGGGACCT CTCCCACCTG GGTGAAGGGG
 Human  ACAACAAAGA AACCCAACAG AGACCTGAAG ACGTGCCTGC CTCCCCCATC TCCGGGACCT CTCCCACCTG GGTGAAGGGG
 Mouse  CCTCCTGAGT GCTCAGATTA AAGGCAAGTG CCAA.CATAC CTAGCTCACA CAAAAGGCTT ATTATTATTA TTATAAAACTT
Figure-18: Multiple sequence alignment analysis showed nearly 74% homologous with mouse IL-17C promoter sequence.
The resulting plasmid has expression of luciferase gene under the control of human IL-17C (-1287/+119) promoter sequence. The sequence and orientations were confirmed by DNA sequencing in both orientations using sense and anti-sense primers as shown in Fig.16(A-B). DNA sequence deduced from sequencing is given in Figure-17 (Submitted in NCBI, GenBank Accession ID. KF147854). Comparative analyses of cloned human IL-17C promoter region with human and mouse species showed nearly 99% and 74 % homology respectively (Figure-18).

4.12. High glucose stimulates IL-6, IL-17C and IL-17E promoter activation

To confirm whether high glucose can activate IL-6, and IL-17E promoters, luciferase reporter gene assay was performed using luciferase reporter plasmids hIL-17C- Luc (-1278/+112), hIL-17E-Luc (1587/+57), hIL-6-Luc (596/+14), and its mutants in which luciferase gene expression is under the control of IL-17C, IL-17E, IL-6 and its mutated promoter fragments. Human IL-6 promoter region contains transcription factors binding sites like NF-κB (-73/-64), NF-IL-6 (-83/-75, -153/-145), CREBP(-174/-164) and AP-1(-283/-276); IL-17C promoter contains binding sites for AP-1(-1237/-1229), SRY (-862/-855), Delta-E (-785/-775), USF (-768/-757), GATA-1 (-475/-464), and NF-κB (-73/-64)IL-17E promoter contains transcription factors binding sites like three MZF1 sites (-288/-280, -633/-625, -972/-964), STATx (-723/-714), p300 (-489/-475), GATA-1 (-451/-441) and NF-κB (-205/-195) binding elements. Jurkat T-cells were transfected with hIL-17C-Luc, hIL6-Luc and hIL17E-Luc plasmids and treated with either normal glucose or high glucose. Results of luciferase reporter assays (Fig.19) suggested that high glucose stimulate IL-6 and IL-17E promoters significantly (***p < 0.001 and * p < 0.05 respectively),
but not IL-17C promoter. These results also underscored the importance of key transcription factors associated with these promoters.

4.13. Role NF-κB and AP-1 in high glucose-induced IL-6 expression

Next, I used mutated IL-6 promoter plasmids to explore the key transcriptional, high glucose specific, regulatory element involved in high glucose induced IL-6 promoter activation. Jurkat T-cells were transfected with mutated IL-6 promoter plasmids in which NF-κB, NF-IL-6, CREBP and AP-1 binding sites are mutated (Fig.20) and stimulated with high glucose. Luciferase reporter assay result in Fig.20 shows that high glucose induced IL-6 promoter activation was inhibited in cells transfected with NF-κB and AP-1 sites mutated plasmids (mAP-1 and mNF-κB), but, not with CREBP and NF-IL-6 site mutated plasmids. These results confirm the role of oxidative stress sensitive transcription factors like NF-κB and AP-1 in high glucose induced IL-6 expression.

4.14. Signaling mechanisms involved in high glucose induced IL-6, IL-17E and IL-17F mRNA expression

Since my earlier results showed that high glucose induced ROS production in lymphocytes, I investigated whether high glucose induced ROS signaling is associated with increased IL-6 and IL-17E mRNA expression. Towards this Jurkat T-cells were pre-treated with N-Acetyl Cysteine (NAC), an antioxidant, for 1h and treated with normal glucose and high glucose for 48 h (Fig.21A-B). Similar experiment was performed with PBLC also (Fig.21C-D). Results shown in Fig.17 indicate that high glucose induced IL-6 and IL-17E mRNA expressions were significantly blocked by NAC in both Jurkat T-cells and PBLC. This implies that high glucose induced ROS signaling is involved in IL-6 and IL-17E mRNA expression in lymphocytes.
Figure-19. High glucose stimulates IL-6 and IL-17E promoter activation but not IL-17C. Jurkat T-cells were transfected with either control plasmid pGL3, or phIL-17C-Luc(-1278/+112), phIL6-Luc (-596/+14) or phIL17E-Luc (-1587/+57) plasmids and treated with normal glucose or high glucose. High glucose induced luciferase activity was determined as Relative Light Units (RLUs). RLUs were normalized to 100 µg total protein and expressed as fold over normal glucose. High glucose significantly induced the activation of IL-6 and IL-17E promoters, but not IL-17C promoter.
Figure-20: Involvement of transcription factors NF-κB and AP-1 in high glucose induced IL-6 expression

Jurkat T-cells were transfected with various mutant IL-6 promoter plasmids (mutation at NF-κB, AP-1, and CREBP, proximal and distal NF-IL6 binding sites (Filled in shapes indicate mutated elements). Luciferase activity was determined and results were expressed as fold over normal glucose as mentioned above (*p<0.05, ***p<0.001 vs. NG).
Figure-21: Effect of N-acetyl cysteine on high glucose induced IL-6 and IL-17E mRNA Expression in Jurkat T-cells and PBLC

(A) Jurkat T-cells were pretreated with or without the N-acetyl cysteine (1mM/L) (NAC) for 1h and treated for normal glucose and high glucose for 48h. Total RNA was isolated and RT-PCR performed using internal control and genes specific primers. (C &D)PBLC were treated with or without NAC and high glucose as mentioned above. Results in A & C show that high glucose induced IL-6 mRNA expression significantly blocked by NAC in both Jurkat T-cells and PBLC respectively. B & D show that high glucose induced IL-17E mRNA expression significantly blocked by NAC in both Jurkat T-cells and PBLC respectively (+, - ) sign shows with or without NAC or high glucose respectively.

Figure-21. Effect of anti-oxidant NAC on high glucose induced IL-6 and IL-17E mRNA expression. (A-B) Jurkat T-cells were pretreated with or without the N-acetyl cysteine (1mM/L) (NAC) for 1h and treated for normal glucose and high glucose for 48h. Total RNA was isolated and RT-PCR performed using internal control and genes specific primers. (C &D)PBLC were treated with or without NAC and high glucose as mentioned above. Results in A & C show that high glucose induced IL-6 mRNA expression significantly blocked by NAC in both Jurkat T-cells and PBLC respectively. B & D show that high glucose induced IL-17E mRNA expression significantly blocked by NAC in both Jurkat T-cells and PBLC respectively (+, - ) sign shows with or without NAC or high glucose respectively.
Figure-22. Effect of NF-κB inhibitor (Bay-11) on high glucose induced IL-6, IL-17E and IL-17F mRNA expression. Jurkat T-cells were pretreated with or without the NF-κB inhibitor (Bay-11) for 1h and treated with normal glucose and high glucose for 48h. Total RNA was isolated and RT-PCR performed using internal control and genes specific primers. (A-C) shows that BAY11 significantly blocked the high glucose induced IL-6, IL-17E and IL-17F mRNA expression. (+) and (-) signs show with or without Bay-11 or high glucose respectively.
Figure-23: Effect of p38 MAPK inhibitor (SB-2090) on high glucose induced IL-6, IL-17E and IL-17F mRNA expression

(A) (B) (C)

Figure-23. Effect of p38 MAPK inhibitor (SB-2090) on high glucose induced IL-6, IL-17E and IL-17F mRNA expression. Jurkat T-cells were pretreated with or without the p38 MAPK inhibitor (SB-2090) for 1h and treated with normal glucose and high glucose for 48h. Total RNA was isolated and RT-PCR performed using internal control and genes specific primers. (A-C) shows that SB-2090 significantly blocked the high glucose induced IL-6, IL-17E and IL-17F mRNA expression. (+) and (-) signs show with or without SB-2090 or high glucose respectively.
Figure-24: Effect of PKC inhibitor (GFX) on high glucose induced IL-6, IL-17E and IL-17F mRNA expression

(A)  
(B)  
(C)  

Figure-24.  Effect of PKC inhibitor (GFX) on high glucose induced IL-6, IL-17E and IL-17F mRNA expression. Jurkat T-cells were pretreated with or without the PKC inhibitor (GFX) for 1h and treated with normal glucose and high glucose for 48h. Total RNA was isolated and RT-PCR performed using internal control and genes specific primers. (A-C) shows that GFX significantly blocked the high glucose induced IL-6 and IL-17E mRNA expression, but not IL-17F mRNA expression. (+) and (-) signs show with or without GFX or high glucose respectively.
Figure-25: Effect of JAK-STAT inhibitor (AG-490) on high glucose induced IL-6, IL-17E and IL-17F mRNA expression

(A)  
(B)  
(C)  

Figure-25. Effect of JAK-STAT inhibitor (AG-490) on high glucose induced IL-6, IL-17E and IL-17F mRNA expression. Jurkat T-cells were pretreated with or without the JAK-STAT inhibitor (AG-490) for 1h and treated with normal glucose and high glucose for 48h. Total RNA was isolated and RT-PCR performed using internal control and genes specific primers. (A-C) shows that AG-490 significantly blocked the high glucose induced IL-17E and IL-17F mRNA expression, but not IL-6 mRNA expression. (+) and (-) signs show with or without AG-490 or high glucose respectively.
Figure-26. Effect of MEK inhibitor (PD9859) on high glucose induced IL-6, IL-17E, and IL-17F mRNA expression. Jurkat T-cells were pretreated with or without the MEK inhibitor (PD-9859) for 1h and treated with normal glucose and high glucose for 48h. Total RNA was isolated and RT-PCR performed using internal control and genes specific primers. (A-C) shows that PD-9859 significantly blocked the high glucose induced IL-17E and IL-17F mRNA expression, but not IL-6 mRNA expression. (+) and (-) signs show with or without PD-9859 or high glucose respectively.
Figure-27: High glucose treatment skews T-lymphocytes to Th17 Subset

The stained pattern of IFN-γ, IL-4 and IL-17A of CD4+ T-lymphocytes in normal glucose and high glucose grown PBLC for are shown (A-F). Dual-parameter fluorescence histograms generated from lymphocyte gated region, showing the fluorescent distribution of cells stained with surface CD4, intracytoplasmic IFN-γ, IL-4, and IL-17A in PBLCs grown in normal glucose (upper panel) and high glucose (lower panel). The percentage of CD4+ and CD4− IFN-γ, IL-4 and IL-17A T-cells were calculated from the stained population and indicated in quadrants. These results show that high glucose treatment of lymphocytes resulted in a marked increase in CD4+ Th17 subset compared with CD4+ Th1 and Th2 subsets.
Furthermore, to explore the key signal transduction pathways involved in high glucose induced IL-6, IL-17E and IL-17F mRNA expressions, Jurkat T-cells were pre-treated with inhibitors of various cell-signaling pathways like NF-κB pathway, p38MAPK pathway, PKC-C pathway, Janus Activated Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway and MAPK Kinase (MEK) pathway for 1h and then treated with high glucose. RT-PCR results in Fig. 22-26 show that high glucose induced IL-6 mRNA expression was significantly blocked by inhibitors of NF-κB, p38 MAPK and PKC pathways in a dose dependent manner, but not by JAK-STAT and MEK inhibitors; Fig. 22-26 show that IL-17E mRNA expression was significantly blocked by inhibitors of NF-κB, p38 MAPK, PKC and JAK-STAT pathways, but not by MEK pathway; Fig. 22-26 show that IL-17F mRNA expression was blocked by inhibitors of NF-κB, p38MAPK, MEK and JAK-STAT pathways, but not by PKC pathway. These results suggest that high glucose regulates IL-6, IL-17E and IL-17F expression via several cell signaling kinases. Intriguingly, all these inflammatory gene expressions were significantly blocked by NF-κB inhibitor suggesting it to be indispensible for the expression all these cytokines.

4.15. High glucose treatment skews T-lymphocytes into CD4+ Th17 subset.

Inflammatory interleukins are key regulators of not only inflammation, but also priming T-cell subset. My array results showed elevated expression of Th2 signature cytokine IL-4 and Th17 cytokine IL-17, but not Th1 cytokine IFN-γ. Thus, I hypothesized that prolonged exposure of lymphocytes to high glucose can influence lymphocytes differentiation. Therefore, I analyzed Th1/Th2/Th17 phenotypic changes in PBLC by flow cytometry to check
whether high glucose can induce T-cells skewing to particular subset. PBLC
grown either in normal glucose or high glucose were stained with antibody
cocktail containing antibody for surface marker CD4+ tagged with fluorescent
PerCP-CY5.5 and intracellular staining markers for IFN-γ, IL-4 and IL-17A
tagged with fluorescent Fluorescein isothiocyanate (FITC), Allo-phycocyanin
(APC) & Phycoerythrin (PE) respectively. Lymphocytes were gated from PBLC
population further to ensure the purity of lymphocyte population and CD4+ and
CD4– IFN-γ secreting Th1 cells, IL-4 secreting Th2 cells and IL-17A secreting
Th17 cells were counted from respective quadrants of dot plots and histograms.
FACS analyses of PBLC grown in high glucose (Fig-27) showed increased
IL-17A positive CD4+ cells compared to normal glucose grown cells. However,
IL-4 and IFN-γ positive CD4+ cells showed no considerable change during high
glucose treatment (Fig-27). These results suggest that high glucose can skew
T-cell population into Th17 subset; this increased subset may further exacerbate
diabetes and its complications.