Chapter 8:

Results and Discussion 5

Studying the peripheral effects of mutant huntingtin (mHTT) expression in cell line models of HD

This part of the study aims to investigate the peripheral effects of mutant huntingtin (mHTT) expression and secretome induced non-cell autonomous features of HD in healthy cells. Huntington's Disease primarily affects medium spiny striatal and cortical neurons due to the polyQ expansion in huntingtin, leading to neuronal dysfunction, involuntary movements, psychiatric symptoms, and cognitive decline [1-3]. While research has focused on neuronal mHTT toxicity, both normal and mutant HTT are expressed throughout the body, causing non-neuronal issues like gastrointestinal dysfunction, testicular atrophy, osteoporosis, muscle atrophy, cardiac failure, and weight loss [4]. Recent studies reveal that high mHTT-expressing cells, secrete factors causing pathological changes in low mHTT-expressing cells, such as immune cells, which can lead to elevated pro-inflammatory cytokines and chemokines years before classical HD symptoms [5-7]. Research also suggests that soluble factors from mHTT-expressing neurons can activate peripheral immune cells to secrete pro-inflammatory cytokines, potentially crossing the blood-brain barrier [6]. A landmark study showed that injecting HD patient-derived fibroblasts or iPSCs into wild-type mice caused HD-like motor and cognitive impairments, demonstrating the non-cell autonomous effects of mHTT and the role of secretome in converting normal cells to HD cells [8]. HD is associated with inflammatory responses that contribute to disease progression, with chronic inflammation being a hallmark of HD pathology that exacerbates neuronal damage and accelerates the disease, marked by upregulated key inflammatory markers like IL-6, TNF-α, and IL-1β. [9-14]. This study assessed the impact of Kinetin, BMS 345541, and Bay 11-7082 on the expression of these inflammatory markers in in-house developed HD Lymphoblastoid cell lines.

8.1 Establishment and Characterization of Lymphoblastoid Cell Lines (LCLs) from Healthy Controls and Huntington's Disease Patients

8.1.1 Epstein Barr Virus (EBV) Production

The marmoset cell line B95-8, known for its high permissiveness to Epstein-Barr virus (EBV) replication, was successfully employed to generate infectious viral particles. Culturing B95-8 cells in medium with reduced serum concentration induced the lytic cycle, resulting in a substantial production of infectious viral particles. Under normal culture conditions, only a small fraction of B95-8 cells enter the lytic cycle. However, by transitioning to medium with low serum concentration (2% FBS), a larger proportion of cells were induced into the lytic phase, leading to enhanced virus production.

B95-8 cells were initially cultured in complete RPMI medium supplemented with 10% heat-inactivated FBS and 1% Pen-Strep. Once the cells reached the logarithmic growth phase, they were transferred to a medium containing 2% FBS and maintained for two weeks (**Fig. 8.1.1**). During this period, the culture supernatant was periodically collected, centrifuged, and filtered to remove cells and debris (**Fig. 8.1.2**). The resulting virus-containing supernatant exhibited a high virus titer, suitable for direct use in generating continuous B lymphoblastoid cell lines.

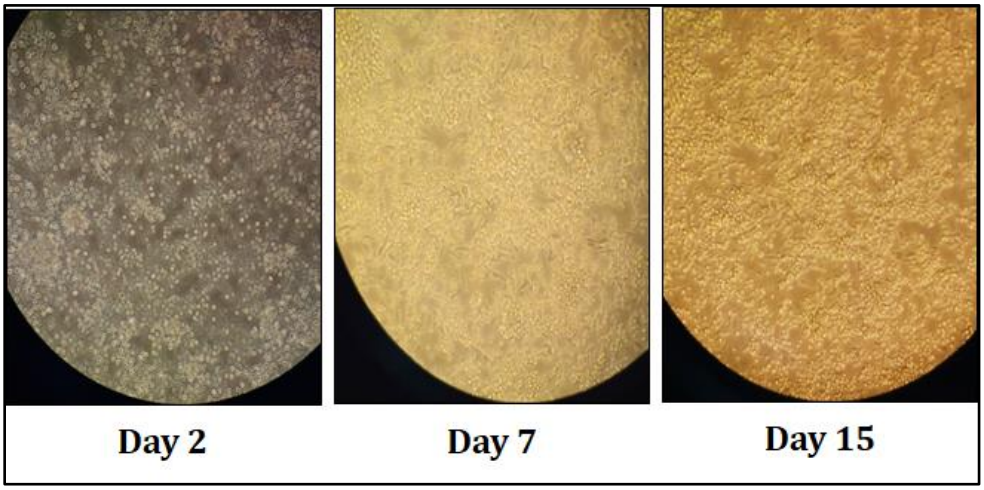


Figure 8.1.1: Representative microscopy images showing the growth of B95-8 cells. Images were captured at 10X magnification.

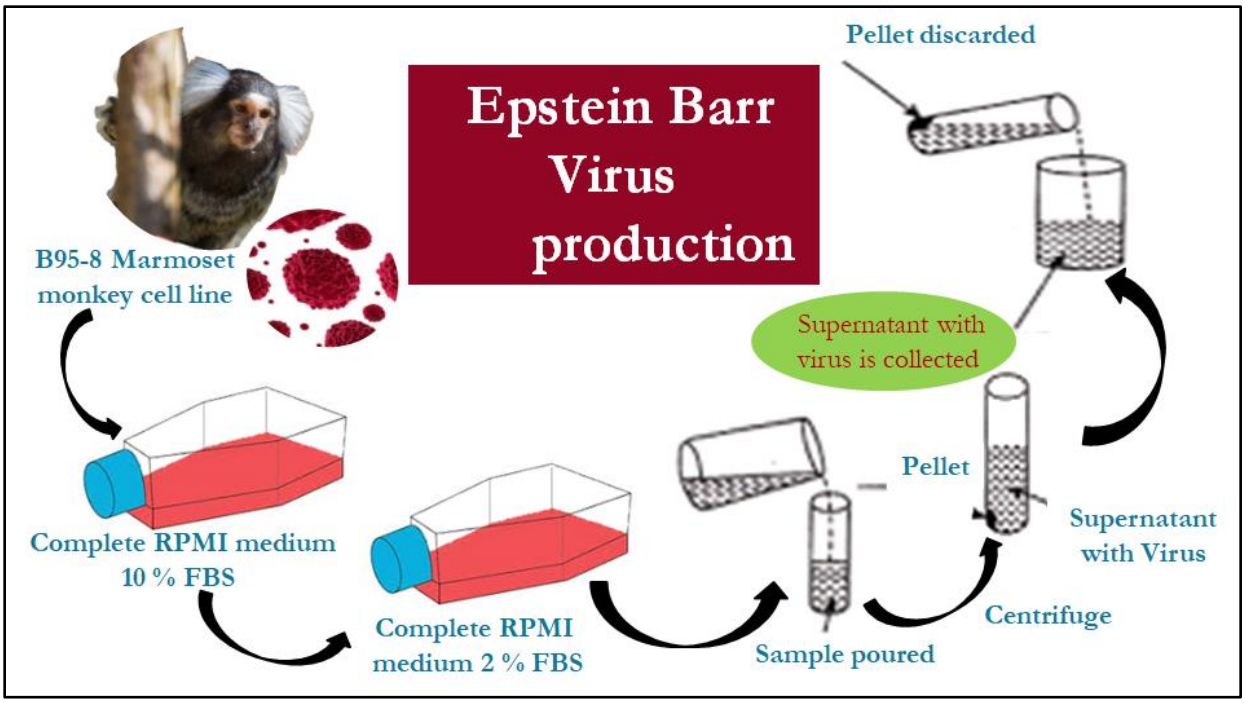


Figure 8.1.2: A schematic representation illustrating Epstein Barr Virus production.

8.1.2 Generation of Lymphoblastoid Cell Lines (LCLs)

8.1.2.1 Isolation of Mononuclear Cells from Blood

Mononuclear cells were isolated from blood samples using a standard Ficoll Paque density gradient centrifugation method. Blood was diluted with an equal volume of PBS and carefully layered onto Ficoll Paque to avoid mixing. Following centrifugation at 400 ×g for 20 minutes at 20°C with the centrifuge brake set to zero, the mononuclear cell layer formed at the interface was carefully collected. These cells were washed twice with PBS, resuspended in complete RPMI medium, and counted, resulting in a highly purified suspension of lymphocytes and monocytes. This suspension was either used immediately for experiments or cryopreserved for future use.

8.1.2.2 *In-vitro* Infection with EBV

The purified mononuclear cells were infected with EBV by exposure to virus-containing supernatant from B95-8 cells as depicted in **Fig. 8.1.3**. Approximately 3-5 × 10^6 cells were pelleted, resuspended in 3-5 mL of the supernatant, and incubated at 37°C for 2 hours. Post-incubation, the cells were pelleted again, resuspended in complete RPMI medium supplemented with 0.4 µg/mL cyclosporine A (CsA), and distributed into T25 flasks at a density of 2.8-3 × 10^6 cells per flask. The cultures were maintained in a humidified incubator at 37°C with 5% CO_2, with weekly medium changes to sustain cell growth.

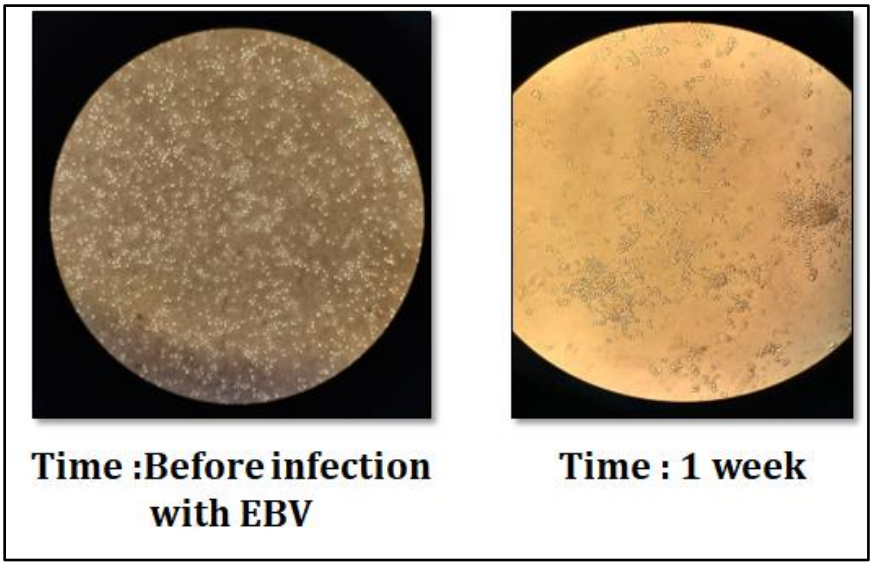


Figure 8.1.3: A diagram depicting the *in-vitro* infection process of purified mononuclear cells with Epstein-Barr virus. Images were captured at 10X magnification.

8.1.3 Characterization of Lymphoblastoid Cell Lines (LCLs)

Upon successful generation of LCLs, comprehensive characterization was performed to confirm their identity and functionality.

8.1.3.1 Genotypic Characterization

Genotypic analysis involved marker gene expression studies to confirm the presence of B lymphocytes. PCR analysis demonstrated the expression of CD-19, a specific surface marker for B lymphocytes, confirming the B lymphocyte origin of the generated LCLs. Additionally, the presence of Beta-actin (a housekeeping gene), CD3 (a marker for T lymphocytes), and NCAM (a marker for Natural Killer cells) was assessed to evaluate the purity and specificity of the LCL population (**Fig. 8.1.4**).

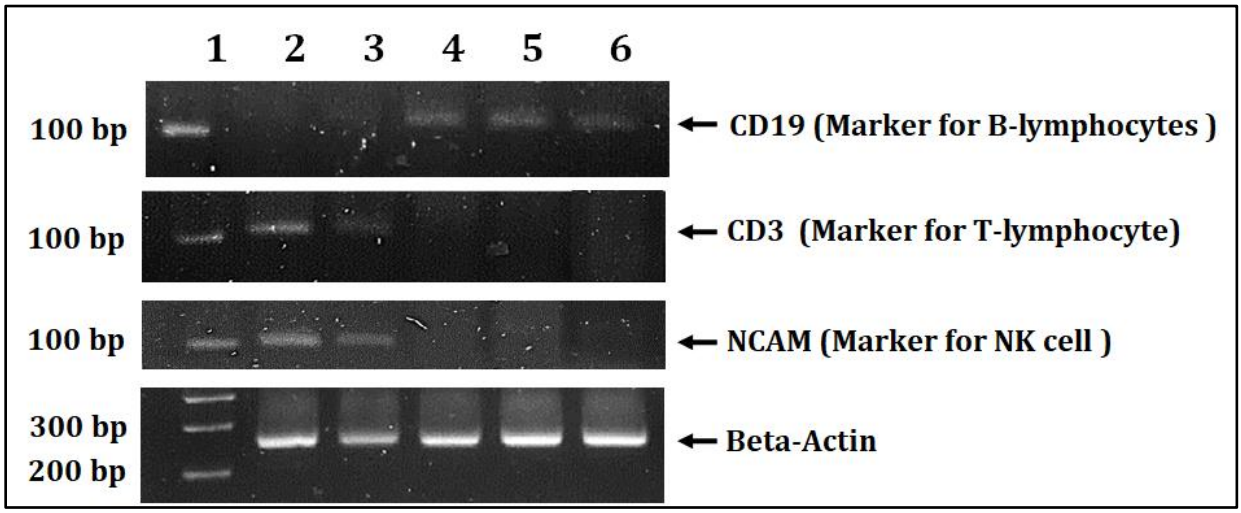


Figure 8.1.4: 2% Agarose gel showing PCR confirmation of marker genes expression of healthy control and HD patient LCLs. (1- DNA ladder, 2- PBMC 1, 3- PBMC 2, 4- Control LCL 1, 5- Control LCL 2, 6- HD LCL).

8.1.3.2 Phenotypic Characterization

Phenotypic characterization involved assessing cell morphology and growth characteristics. Microscopic examination of the LCLs revealed typical lymphoblastoid morphology, characterized by large, round cells with a high nuclear-to-cytoplasmic ratio. These observations indicated active proliferation. Further microscopic analysis demonstrated robust cell growth over time, indicating the viability and health of the LCL cultures (**Fig. 8.1.5**).

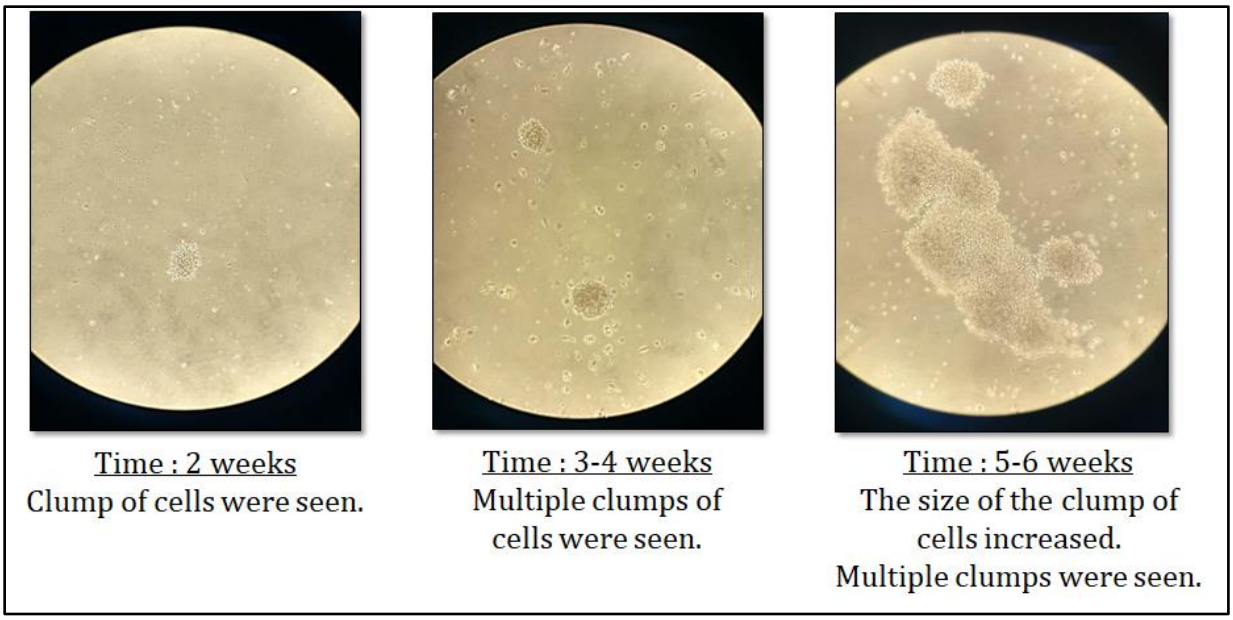


Figure 8.1.5: Representative microscopy images depicting cell growth post-infection with Epstein-Barr Virus (EBV). Images were captured at 10X magnification.

8.1.3.3 Conservation of PolyQ Repeats

To ensure the genetic fidelity of the LCLs, PCR (**Fig. 8.1.6**) and western blotting (**Fig. 8.1.7**) were used to confirm the presence of polyQ repeats, previously identified in fresh B lymphocyte samples. The polyQ repeats were successfully conserved in the generated LCLs, validating their suitability for research on Huntington's disease.

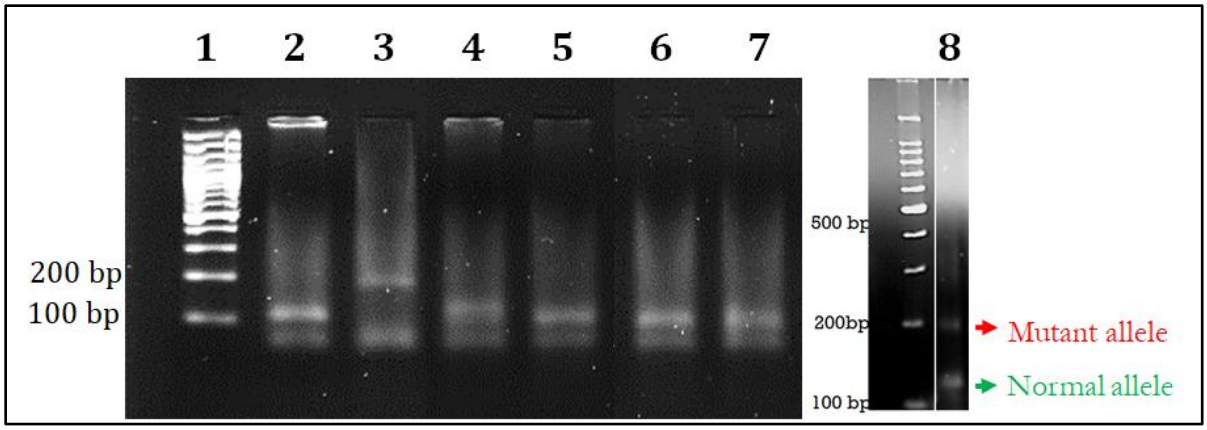


Figure 8.1.6: 2% Agarose gel showing PCR confirmation for *HTT* gene using *HTT* specific primers. (1- DNA ladder, 2- HttQ23 plasmid as a positive control, 3- HttQ46 plasmid as a positive control, 4- PBMC 1, 5- PBMC 2, 6- Control LCL 1, 7- Control LCL 2 and 8- HD LCL).

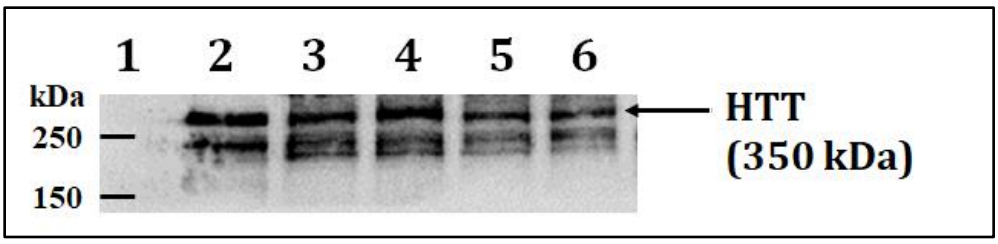
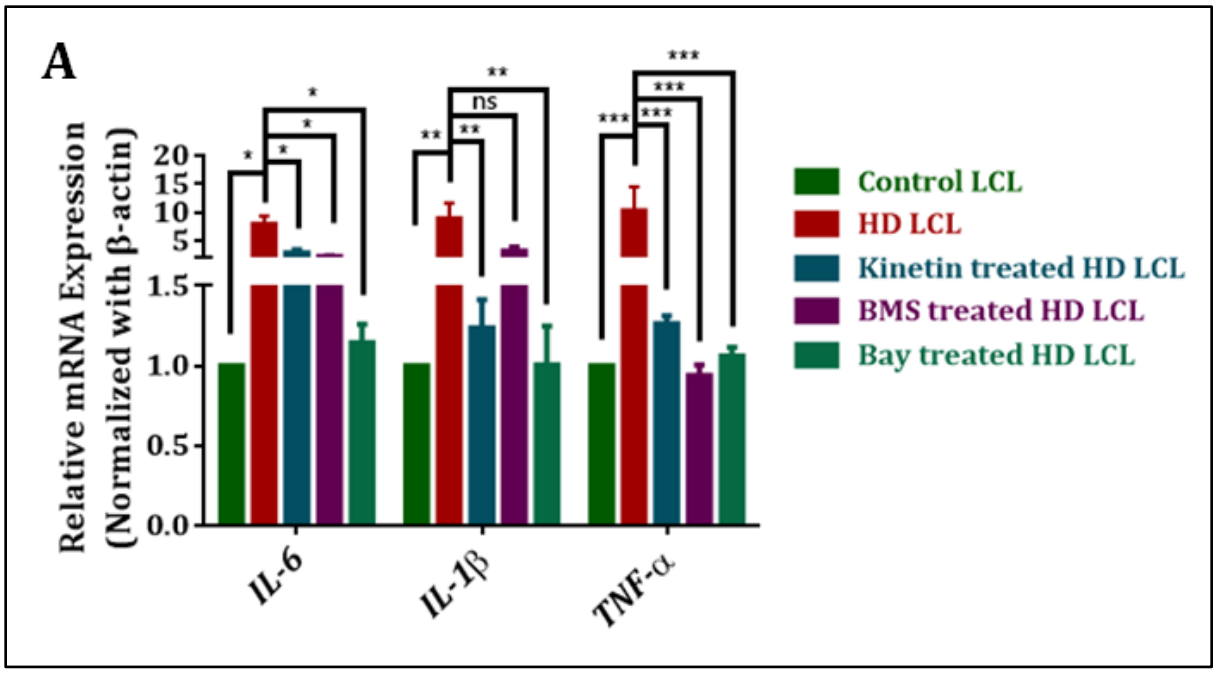


Figure 8.1.7: Western Blot image showing huntingtin expression from control and HD LCLs. (1- protein markers, 2- PBMC 1, 3- PBMC 2, 4- Control LCL 1, 5- Control LCL 2, 6- HD LCL).

In summary, the LCLs established from healthy controls and Huntington's disease patients were thoroughly characterized. They were confirmed to be of B lymphocyte origin, exhibited robust growth, and retained essential genetic features, including polyQ repeats, necessary for this study.

8.2 Effect of Kinetin, BMS 345541, and Bay 11-7082 on Inflammatory Marker Gene Expression in HD LCL Cells

To investigate the impact of Kinetin, BMS 345541, and Bay 11-7082 on inflammatory marker gene expression in Huntington's Disease derived Lymphoblastoid Cell Lines, cells were treated with these compounds for 24 hours. Quantitative Real-Time PCR analysis revealed significantly elevated expressions of both anti-inflammatory (*TGF-β1* and *IL-10*) and pro-inflammatory (*IL-1β*, *IL-6*, and *TNF-α*) marker genes in untreated HD LCL cells compared to control LCL cells (**Fig. 8.2.1A** and **B**).



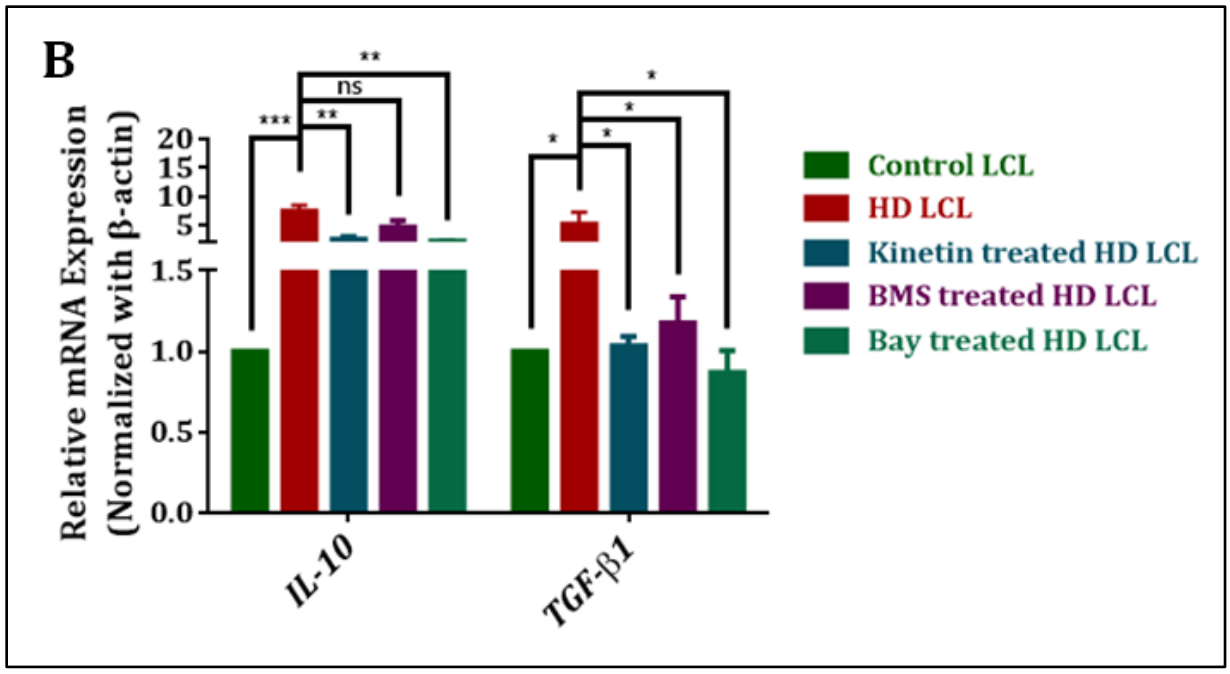


Figure 8.2.1: Gene expression profiles of key (A) pro-inflammatory and (B) anti-inflammatory genes in Kinetin, BMS 345541, and Bay 11-7082 treated LCLs, as determined by RT-qPCR (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)).

Upon treatment with Kinetin (1 µM), BMS 345541 (300 nM), and Bay 11-7082 (1 µM), the expression levels of inflammatory markers were significantly reduced to basal levels, comparable to those observed in control LCL cells (**Fig. 8.2.1A** and **B**). Specifically, Kinetin treatment led to a marked decrease in the expression of all five inflammatory genes compared to untreated HD LCLs. Similarly, BMS 345541 treatment resulted in a significant reduction in the expression of *IL-6*, *TNF-α*, and *TGF-β1*. Bay 11-7082 treatment also significantly decreased the expression of all five inflammatory markers compared to untreated HD LCLs.

These findings highlight the therapeutic potential of Kinetin, BMS 345541, and Bay 11-7082 in mitigating inflammatory responses associated with Huntington's Disease. The observed reductions in inflammatory marker gene expression underscore the role of these molecules in modulating peripheral effects of mutant huntingtin, suggesting novel therapeutic avenues for managing inflammation in HD patients. The ability of these compounds to restore inflammatory markers to basal levels indicates their potential efficacy in reducing the neuroinflammatory burden in HD, which is a critical aspect of disease pathology and progression.

8.3 Impact of HD150Q Cell Secretome on Immune Cells Expressing Normal Huntingtin

To investigate the effects of secretome derived from HD150Q cells on immune cells expressing normal huntingtin, freshly isolated human peripheral blood mononuclear cells (PBMCs) from normal controls and the monocyte-derived THP-1 cell line were exposed to conditioned media from uninduced or Ponasterone A-induced HD150Q cells for varying durations.

8.3.1 Gene Expression Changes in PBMCs and THP-1 Cells

Upon incubation with HD150Q cell secretome, PBMCs displayed altered expression levels of key pro-inflammatory (*IL-1β*, *IL-6*, *TNF-α*) and anti-inflammatory (*TGF-β1*, *IL-10*) marker genes, as determined by real-time PCR (**Fig. 8.3.1**). Similarly, THP-1 cells showed changes in the expression levels of crucial pro-inflammatory (*IL-1β*, *TNF-α*) and anti-inflammatory (*TGF-β1*) marker genes when incubated with HD150Q cell secretome, as assessed by real-time PCR (**Fig. 8.3.1**). Compared to untreated controls, HD150Q secretome induced significantly elevated expression of these markers in PBMCs and THP-1 cells, indicative of an inflammatory response mediated by mutant huntingtin.

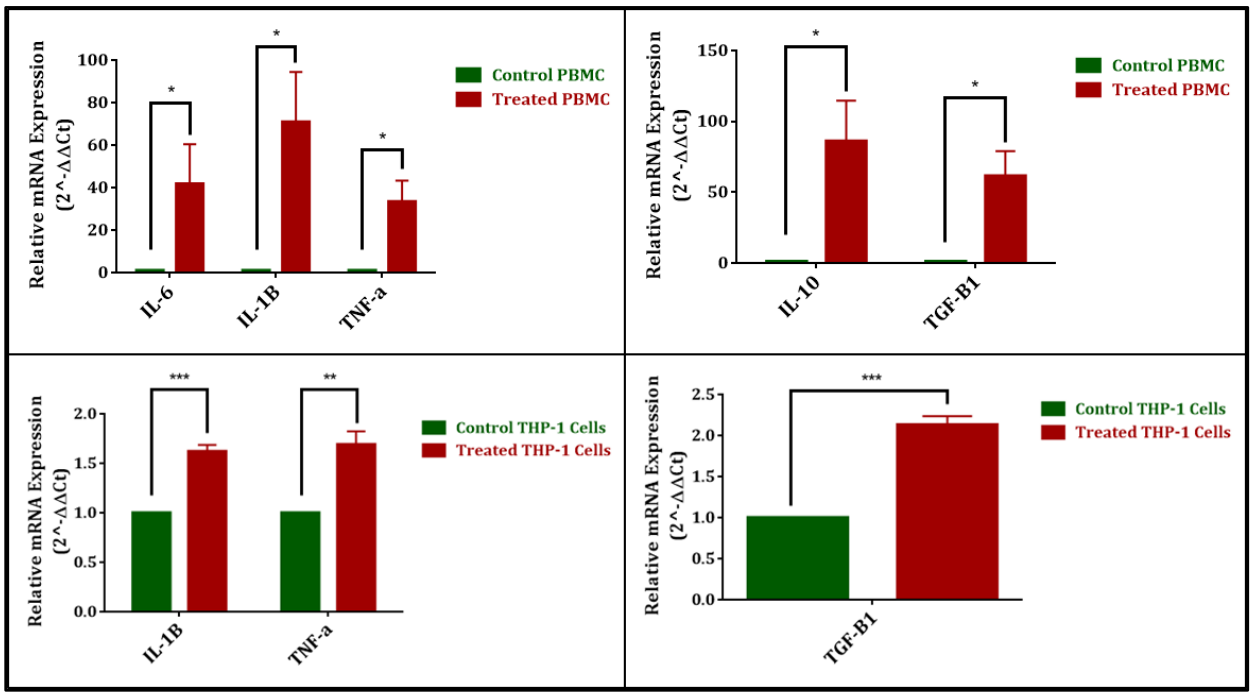


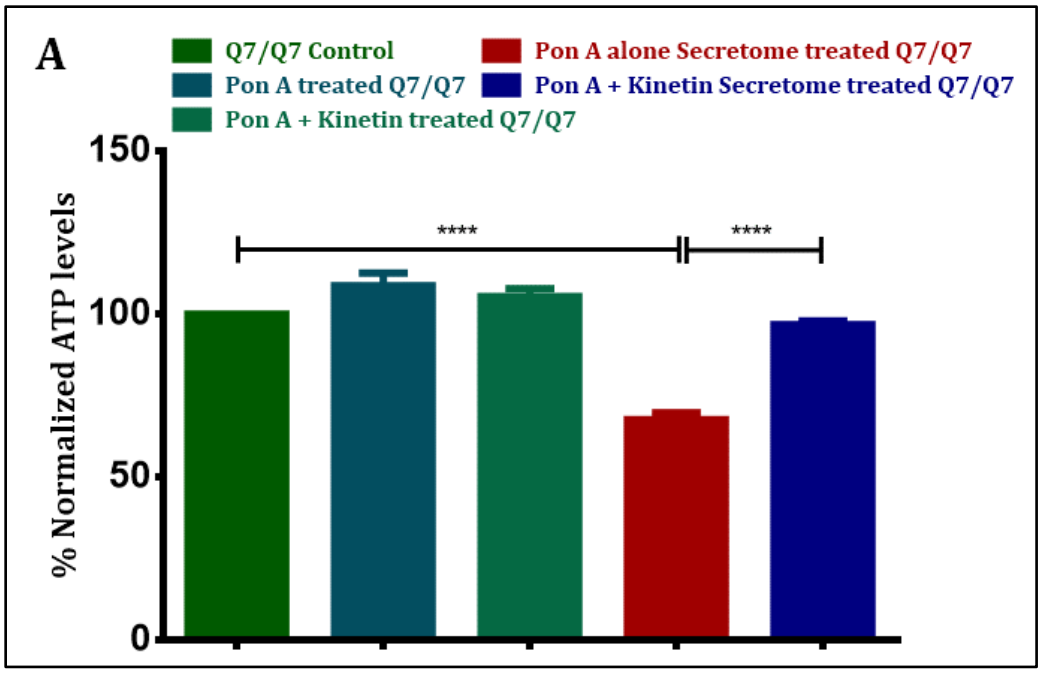
Figure 8.3.1: Expression profile of key pro-inflammatory and anti-inflammatory genes in PBMCs and THP-1 cells, following treatment with HD150Q cell secretome, as determined by RT-qPCR. (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

8.3.2 ATP Levels in *STHdh*$^{Q7/Q7}$ Cells

STHdh$^{Q7/Q7}$ cells treated with HD150Q secretome showed altered ATP levels compared to untreated controls. Specifically, cells treated with conditioned media from Ponasterone A-induced HD150Q cells exhibited variations in ATP levels after 24 and 48 hours of exposure (**Fig. 8.3.2A** and **B**). ATP measurements, normalized to protein content, revealed significant changes in cellular energy metabolism upon exposure to HD150Q secretome, highlighting potential metabolic impacts of mutant huntingtin on wild type huntingtin expressing cells.

8.3.3 Effect of Ponasterone A and Kinetin-treated HD150Q Secretome on ATP Levels

Further investigations involved treating *STHdh*$^{Q7/Q7}$ cells with secretome derived from HD150Q cells that had been incubated with Ponasterone A and Kinetin for 48 hours. Following 24- and 48-hours treatment with this conditioned media, the *STHdh*$^{Q7/Q7}$ cells exhibited a significant increase in ATP levels as shown in **Fig. 8.3.2A** and **B**. The ATP measurement was conducted using a luminescence-based assay, with normalization to protein content determined by the Bradford assay. These results indicate that the Pon A and Kinetin-treated secretome effectively rescued ATP levels in the *STHdh*$^{Q7/Q7}$ cells, suggesting a role in restoring cellular energy homeostasis affected by mutant huntingtin. These findings underscore the complex interplay between mutant huntingtin and immune cell function, as well as potential therapeutic strategies involving secretome manipulation to mitigate peripheral effects associated with Huntington's disease.



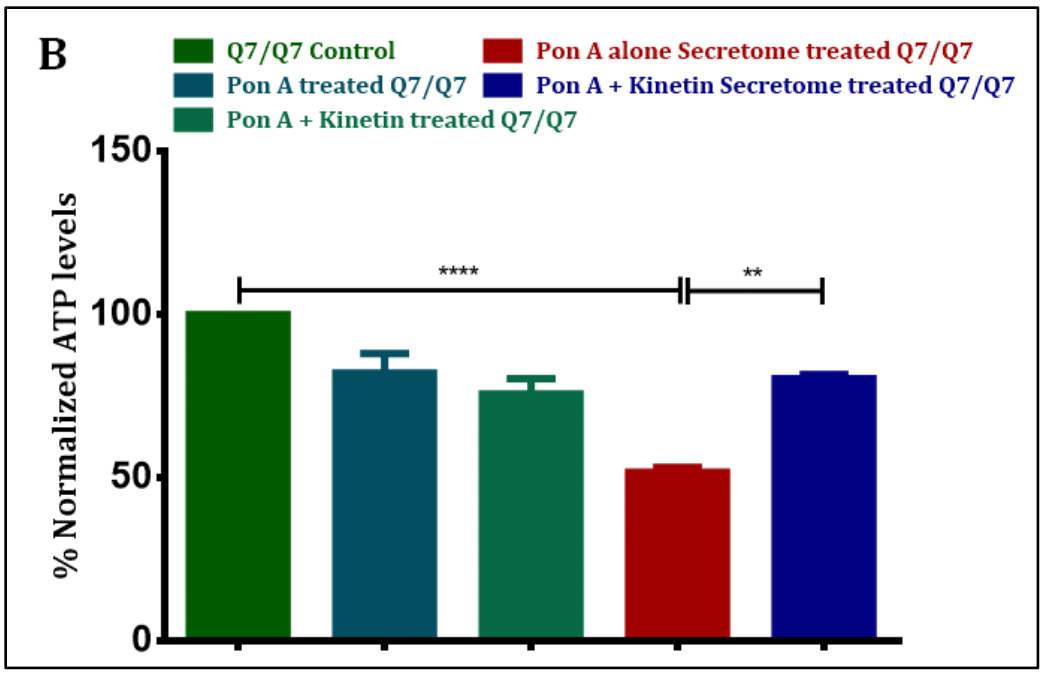


Figure 8.3.2: Graphical representation of normalized ATP levels in *STHdh*$^{Q7/Q7}$ cells after different treatments of HD150Q secretome for (A) 24 hour and (B) 48 hours obtained through ATP determination kit (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

8.4 Discussion

In this part of the study, we explored the impact of Kinetin, BMS 345541, and Bay 11-7082 treatments on the expression levels of key pro-inflammatory (*IL-1β*, *IL-6*, *TNF-α*) and anti-inflammatory (*TGF-β1*, *IL-10*) marker genes in HD and control lymphoblastoid cell lines. Inflammation is a key component of HD pathology, and its modulation could ameliorate disease symptoms and progression [15]. Our results demonstrate significantly elevated expression levels of pro-inflammatory and anti-inflammatory markers in HD LCLs compared to controls. However, treatment with Kinetin, BMS 345541, and Bay 11-7082 for 24 hours reduced these elevated levels to basal, indicating their potential role in modulating inflammatory responses in HD. This aligns with previous studies indicating that inflammation is a critical component of HD pathology [6,16-18,15] and suggests these compounds could mitigate peripheral immune dysfunction in HD.

The significant upregulation of pro-inflammatory markers *IL-1β*, *IL-6*, and *TNF-α* in HD LCLs is consistent with the known inflammatory pathology associated with HD. These cytokines are key mediators of the inflammatory response and have been implicated in neuronal damage in HD

models [6,18]. Our findings suggest that the inflammatory environment in HD LCLs could contribute to the peripheral immune dysregulation observed in HD patients. Similarly, the increased expression of anti-inflammatory markers *TGF-β1* and *IL-10* in HD LCLs highlights a compensatory mechanism to counteract the heightened pro-inflammatory response. *TGF-β1* and *IL-10* are crucial for resolving inflammation and maintaining immune homeostasis [19-22,10]. The upregulation of these markers suggests that while there is an active inflammatory response, there is also an effort by the cells to mitigate damage and restore balance.

Treatment with Kinetin, BMS 345541, and Bay 11-7082 effectively reduced the elevated expression levels of both pro- and anti-inflammatory markers to basal levels in HD LCLs. Kinetin, a cytokinin known for its role in plant growth, has been shown to possess anti-inflammatory properties in mammalian cells [23,24]. BMS 345541 is a selective inhibitor of IKK, which plays a pivotal role in NF-κB-mediated inflammatory responses [25-27]. Bay 11-7082 inhibits NF-κB activation by targeting IκBα phosphorylation [28-30]. The normalization of inflammatory markers upon treatment suggests that these compounds can effectively modulate the inflammatory response, potentially providing therapeutic benefits for managing inflammation in HD. Future studies should focus on *in vivo* validation of these findings and the development of these compounds for clinical use.

Hyperactivation of the immune system is an early hallmark of HD and is closely linked to disease progression [13]. Given that chronic inflammation can result in neurodegeneration [9-14], early intervention to mitigate this phenomenon holds significant therapeutic potential. Interestingly, the findings from this study demonstrate that conditioned media containing the secretome from a neuronal cell line expressing the Exon 1 fragment of mutant huntingtin (mHTT) can induce a profound immune response in isolated peripheral blood mononuclear cells (PBMCs) and in the monocyte cell line THP-1, which expresses normal huntingtin (HTT). In PBMCs, the expression of pro-inflammatory cytokines (*IL-1β*, *IL-6*, and *TNF-α*) exhibited a 20-60-fold increase compared to the control. Additionally, there was a compensatory rise in anti-inflammatory cytokines *IL-10* and *TGF-β1*, similar to observations in the serum of HD patients [6]. These findings highlight the role of secreted factors in conditioned media in eliciting an inflammatory response in immune cells expressing normal HTT.

Moreover, treating a transgenic mouse striatal cell line expressing normal HTT (*STHdh*$^{Q7/Q7}$) with HD secretome resulted in a significant reduction in ATP levels. Based on these results, it can be concluded that the secretome of cells expressing mHTT has the potential to induce key hallmarks of HD pathogenesis, including immune hyperactivation and mitochondrial dysfunction, even in cells expressing normal HTT, in a non-cell-autonomous manner. Identifying and characterizing these factors in the secretome may provide novel therapeutic avenues for the treatment of HD.

8.5 References

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