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Neurokinin-1 receptor signaling induces a pro-inflammatory transcriptomic profile in CD16+ monocytes

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Abstract

Neurokinin-1 receptor (NK1R) signaling can be immunomodulatory and it can lead to preferential transmigration of CD14+CD16+ monocytes across the blood brain barrier, potentially promoting the development of inflammatory neurological diseases, such as neuroHIV. To evaluate how NK1R signaling alters monocyte biology, RNA sequencing was used to define NK1R-mediated transcriptional changes in different monocyte subsets. The data show that NK1R activation induces a greater number of changes in CD14+CD16+ monocytes (152 differentially expressed genes), than in CD14+CD16- monocytes (36 genes), including increases in the expression of NF- κ B and components of the NLRP3 inflammasome pathway. These results suggest that NK1R may alter the inflammatory state of CD14+CD16+ monocytes, influencing the development of neuroinflammation.

Keywords

neurokinin-1 receptor; substance P; monocyte subsets; CD16; RNA sequencing

1. Introduction

Activation of the neurokinin-1 receptor (NK1R) by its primary ligand, the neuropeptide substance P (SP), has a broad spectrum of biologic effects including immunomodulation. In the CNS, SP promotes neurogenic inflammation and increased blood brain barrier (BBB) permeability (Martinez and Philipp, 2016). In the periphery, increased concentrations of plasma SP are observed in viral infections, such as HIV, and are associated with increased levels of inflammatory mediators (Spitsin et al., 2018).

In monocytes, NK1R activation can induce the production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- α . SP also enhances transmigration of CD14+CD16+

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monocytes in an *in vitro* model of the human BBB (Spitsin, Pappa, 2018, Spitsin et al., 2017), highlighting the potential impact of this neuropeptide on CNS inflammation. Although CD14+CD16+ monocytes comprise only 5–15% of the total circulating monocyte population (Patel et al., 2017, Williams et al., 2014), they are significantly expanded in HIV and they preferentially transmigrate across the BBB, thus becoming central to the establishment of CNS viral reservoirs and the development of HIV-associated neurocognitive disorders (HAND) (Williams et al., 2015, Williams, Veenstra, 2014).

Activation of NK1R in monocytes is poorly understood. It is thought to be connected to inflammatory disease progression (Ho et al., 1996) (Lai et al., 2001) through NK1R-mediated calcium mobilization or PKC δ and ERK phosphorylation, which increase NF- κ B activity (Lai et al., 2008, Spitsin, Pappa, 2018). To better define the inflammatory impact of NK1R activation in monocytes, we examined the quantitative and qualitative differences in inflammatory gene expression mediated by NK1R activation in both CD14+CD16– (CD16–) and CD14+CD16+ (CD16+) monocytes using RNA sequencing.

2. Materials and Methods

2.1. Monocytes

Fresh, de-identified monocytes (purity: 92.3 – 96.4 %, mean 95.0 % as confirmed by flow cytometry, see representative cell subsets on Fig. 1), isolated with negative selection (RosetteSep™ Human Monocyte Enrichment Cocktail, StemCell Technologies) from apheresis products from 8 healthy, HIV-negative donors (age 25 – 52, 3 male / 5 female) were purchased from the Human Immunology Core Facility of the University Of Pennsylvania School Of Medicine. Monocytes were maintained in RPMI 1640, 10% FBS, 1% Pen/Strep and 1% MEM Non-Essential Amino Acids solution. For SP treatment, monocytes were incubated in polypropylene tubes for 3 hours at 37°C with 10 μ M SP (Sigma-Aldrich) or 50 μ M acetic acid (vehicle, Sigma-Aldrich).

2.2. Cell sorting

SP-treated and vehicle-treated control monocytes were washed with RPMI 1640 without Phenol Red, containing 50% Accumax (Millipore), 1% FBS, 1% Pen/Strep, 10 mM HEPES and 0.5 mM EDTA. Sorted cells were stained with the antibodies in Table 1, with the FITC channel used for negative selection of contaminating cells. Monocytes were sorted into CD16– and CD16+ populations using the BD FACSJazz™ cell sorter in the Flow Cytometry Core at the Children’s Hospital of Philadelphia Research Institute (CHPRI) (Fig. 1) and collected in RPMI 1640, 20% FBS, 1% Pen/Strep, 10 mM HEPES, 1 U/ μ l Anti-RNase (Thermo Fisher Scientific).

2.3. RNA sequencing

The RNeasy Mini Kit (Qiagen) was used to purify RNA from sorted monocytes. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer in the Nucleic Acid and PCR Core at the CHPRI. Approximately 200 ng of RNA was submitted to the High Throughput Sequencing Core/BGI at the CHPRI (2 samples) and the BGI Americas Corporation (6 samples). Libraries were constructed using the Illumina TruSeq stranded

RNA sample preparation and Paired End 150 bp RNA sequencing was performed using the Illumina HiSeq 4000 Sequencing System (2 samples) and the NovaSeq 6000 (6 samples) with a coverage of 30 million reads. The Illumina HiSeq software was used to create RNA sequencing data files (GEO accession number: GSE109635, <https://www.ncbi.nlm.nih.gov/geo/>).

2.4. Statistical Analysis

For RNA sequencing analysis, STAR software (Dobin et al., 2013) was used to align RNA sequencing data to the Ensembl hg38 human reference genome. For differential gene expression, gene counts were estimated using the HTSeq Python package (Anders et al., 2015). The DESeq2 (Love et al., 2014) and DEXSeq (Anders et al., 2012) R packages were used to determine differentially expressed genes (DEGs) and for differential exon usage analysis. False discovery rate (FDR) adjusted p-values < 0.05 were considered statistically significant. The DAVID Functional Annotation tool (Huang da et al., 2009a, b) and the GPlot (Walter et al., 2015) R package were used for the Gene Ontology (GO) analysis.

3. Results

Human monocytes from 8 donors were treated with substance P (10 μ M, SP) for 3 hours, then sorted into mature (CD16+) and immature (CD16-) subsets and processed for mRNA, which was analyzed by RNA sequencing. Differential gene expression analysis of these data shows that SP treatment led to transcriptional changes in both monocyte subsets. A greater number of changes were seen in CD16+ monocytes (152 differentially expressed genes (DEGs), supplementary Table 1) than in CD16- monocytes (36 DEGs, supplementary Table 2). DEGs were then used for a Gene Ontology (GO) analysis, which showed upregulation of annotations that were associated with the inflammation, glycolysis, cell adhesion and negative regulation of apoptosis in CD16+ monocytes (Fig. 2) (Fisher's Exact test, $p < 0.05$). There were no significant GO annotations in the SP-treated CD16- subset (Fisher's Exact test, $p > 0.05$). These results suggest that exposure to SP could mediate increases in inflammation in CD16+ monocytes through transcriptional changes.

The first GO term associated with SP-treated CD16+ monocytes was the "Cellular response to lipopolysaccharide (GO:0071222)" (Fig. 2). Our analysis showed that SP-treated CD16+ monocytes displayed significant upregulation of transcripts for NFKB2 (\log_2 Fold Change (\log_2 FC): 0.128, FDR-adjusted $p = 0.049$) and NLRP3 (\log_2 FC: 0.207, FDR-adjusted $p = 0.017$) (supplementary Table 1). In addition, analysis of differential exon usage was performed on the RNA sequencing data to assess the effect of SP on alternative splicing and it showed that in SP-treated CD16+ monocytes there was differential exon usage in caspase-1 (transcript: ENST00000527979, \log_2 FC: 0.16, FDRadjusted $p = 0.004$) (Fig. 3) (supplementary Table 3). These results suggest that SP treatment of CD16+ monocytes may increase the priming or activation of the NLRP3 inflammasome. Activation of inflammasome-mediated inflammation is further supported by GO data indicating that SP treatment of CD16+ monocytes has additional inflammatory effects, showing upregulated transcripts within the GO term "Inflammatory response (GO:0006954)" (Fig. 2). Notably, SP treatment of CD16+ monocytes increased transcription of IRAK1 (\log_2 FC: 0.3, FDR-

adjusted $p = 0.006$), OSCAR (\log_2 FC: 0.189, FDR-adjusted $p = 0.026$) and CD300c (\log_2 FC: 0.153, FDR-adjusted $p = 0.0491$) (supplementary Table 1).

3. Discussion

These data indicate that in CD16+ monocytes, NK1R signaling has a pro-inflammatory transcriptional effect that may involve the NLRP3 inflammasome. The SP-treated CD16+ monocytes show upregulation of transcripts implicated in the cellular response to bacterial lipopolysaccharide (LPS) molecules, which activate toll-like receptor 4 (TLR4) on monocytes and macrophages. This further suggests that SP could predispose these cells to a more robust inflammatory response, as TLR4 activation provokes an immune response that includes activation of inflammasomes, which are multiprotein complexes that regulate the production of IL-1 cytokines (Swanson et al., 2019).

Activation of inflammasomes is usually a two-step process, involving an initial priming step followed by an activation step; one-step activation of NLRP3 inflammasomes can also occur. Priming involves NF- κ B mediated transcriptional upregulation of inflammasome components, such as NLRP3, and proIL-1 cytokine transcripts. Activation is initiated by interaction with pathogen or damage-associated molecular patterns (PAMPs or DAMPs) and leads to the formation of the inflammasome complex, activation of caspase-1, and secretion of IL-1 cytokines, including IL-1 β and IL-18 (Swanson, Deng, 2019). We observed that SP-treated CD16+ monocytes displayed upregulation of the NFKB2 and NLRP3 transcripts, as well as differential exon usage in caspase-1. This suggests that SP treatment of CD16+ monocytes could impact either the priming or activation of the NLRP3 inflammasome. This concept is supported by data showing that NK1R signaling activated NF- κ B in HEK293 cells (Lai, Lai, 2008), and induced IL-1 β secretion in conjunction with LPS in rat microglia (Martin et al., 1993).

The pro-inflammatory effect of NK1R signaling that we observed in CD16+ monocytes is also highlighted by the upregulation of genes involved in inflammatory responses. These include IRAK1, which is involved in IL-1 mediated activation of the transcription factor NF- κ B (Liu et al., 2007), as well as OSCAR and CD300c, both of which interact with TLRs to induce the release of pro-inflammatory cytokines (Merck et al., 2006, Simhadri et al., 2013). In addition, we observed upregulation of transcripts involved in canonical glycolysis, cell adhesion and negative regulation of apoptosis (data not shown). Similar pathways (i.e. inflammation, lipid metabolism, cell adhesion and apoptosis) were impacted in a clinical trial that studied the effect of NK1R inhibition on plasma protein levels of HIV infected individuals (Spitsin, Tebas, 2017), which supports the importance of NK1R signaling in inflammation during HIV infection. Notably, glycolysis has also been implicated in the regulation of the NLRP3 inflammasome in human monocytes and macrophages (Lee et al., 2019) (Moon et al., 2015) (Finucane et al., 2019). Taken together, these data indicate that NK1R signaling may be involved in NLRP3 inflammasome activity and downstream inflammatory activity in mature monocytes but the precise nature of these effects remain undetermined.

4. Conclusion

We examined the effect of SP treatment on the transcriptional profile and alternative splicing of monocyte subsets. We found that NK1R signaling has a predominantly pro-inflammatory effect in CD16+ monocytes that may be mediated via activation of NF- κ B and subsequent activation of the NLRP3 inflammasome pathway. These data support the hypothesis that activation of the NK1R pathway could play an important role in disease and/or conditions involving the expansion of CD16+ monocytes, such as HIV, especially in the context of neuroinflammation and HAND. This suggests that NK1R inhibition may have useful anti-inflammatory effects but additional studies are needed to evaluate the therapeutic potential of this pathway in CNS infection and neuroinflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations:

BBB	blood brain barrier
CNS	central nervous system
DAMP	damage-associated molecular pattern
DEG	differentially expressed gene
ERK	extracellular signal-regulated kinase
FDR	false discovery rate GO: gene ontology
GO BP	gene ontology biological process
HAND	HIV-associated neurocognitive disorder
HIV	human immunodeficiency virus
IFN-γ	interferon gamma
IL	interleukin
IRAK1	interleukin 1 receptor associated kinase 1
LPS	lipopolysaccharide

NF-κB	nuclear factor- κ B
NK1R	neurokinin-1 receptor
NLRP3	nucleotide-binding domain and leucine-rich repeat containing protein 3
OSCAR	osteoclast-associated receptor
PAMP	pathogen-associated molecular pattern
PKC	protein kinase C
SP	substance P
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha

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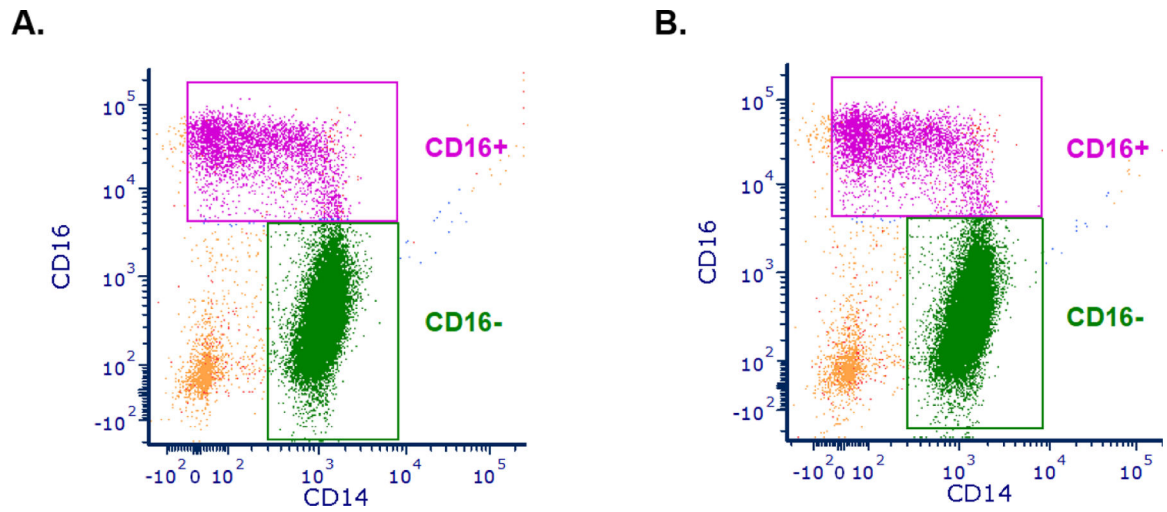


Figure 1. Cell sorting of SP-treated and vehicle-treated control monocytes.

Monocytes were sorted based on the CD14 and CD16 surface markers into CD14+CD16- (CD16-) and CD14+CD16+ (CD16+) monocytes. Representative images for **A.** SP-treated and **B.** vehicle-treated control samples are shown. We observed no significant differences in the abundance of CD16+ monocytes between SP-treated and vehicle-treated control samples (Mann Whitney test, $p > 0.99$).

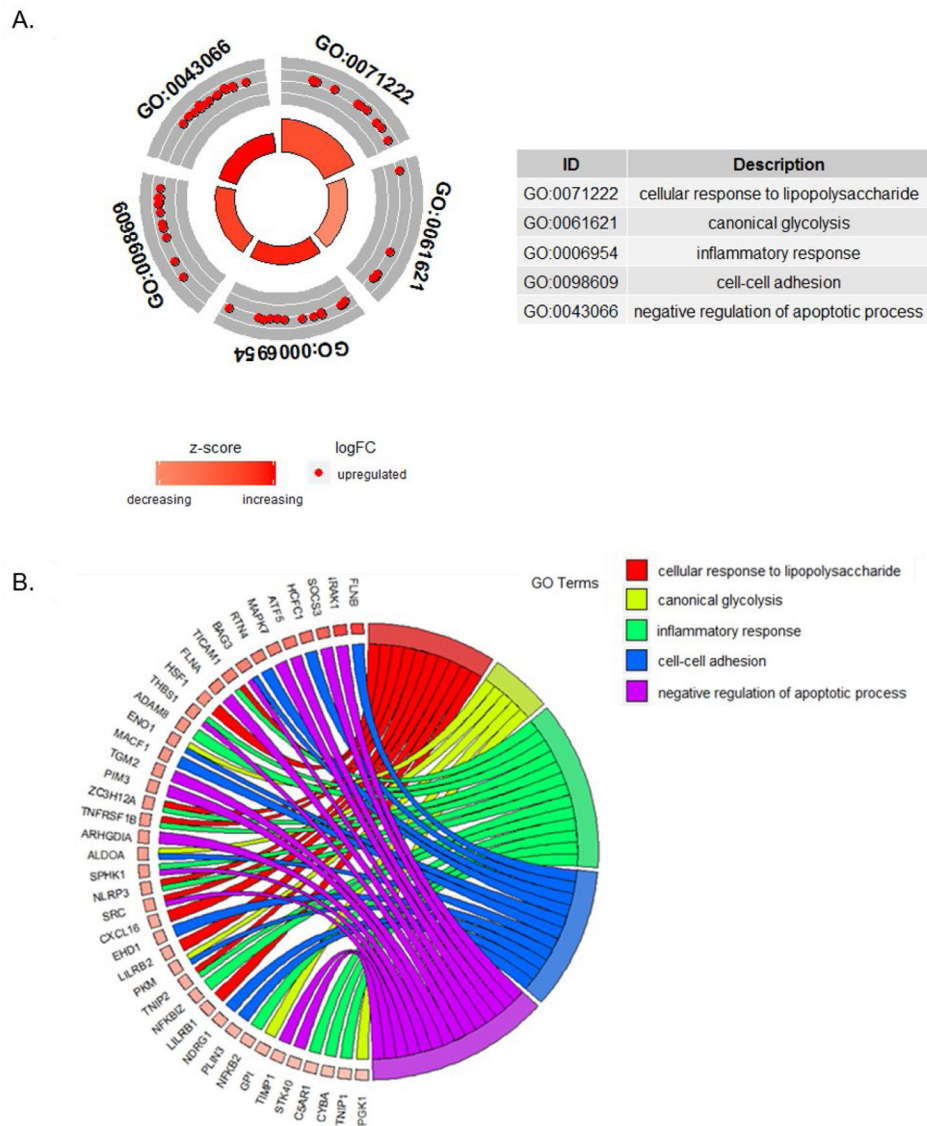


Figure 2. Gene Ontology Biological Processes analysis in SP-treated CD16+ monocytes. Gene ontology analysis based on 152 differentially expressed genes (DEGs) in SP-treated CD16+ monocytes shows 5 significantly upregulated Gene Ontology Biological Processes (GO BP). **A.** GOCircle showing the 5 significant GO BP annotations. Red circles show upregulated genes, whereas downregulated genes would have been depicted in blue. The size of the inner circle shows the statistical significance ($-\log_{10}$ adjusted P-value). The table shows the GO BP annotations and their descriptions. **B.** GOChord plot showing the relationship between DEGs and GO BP annotations. Genes are linked to their assigned terms.

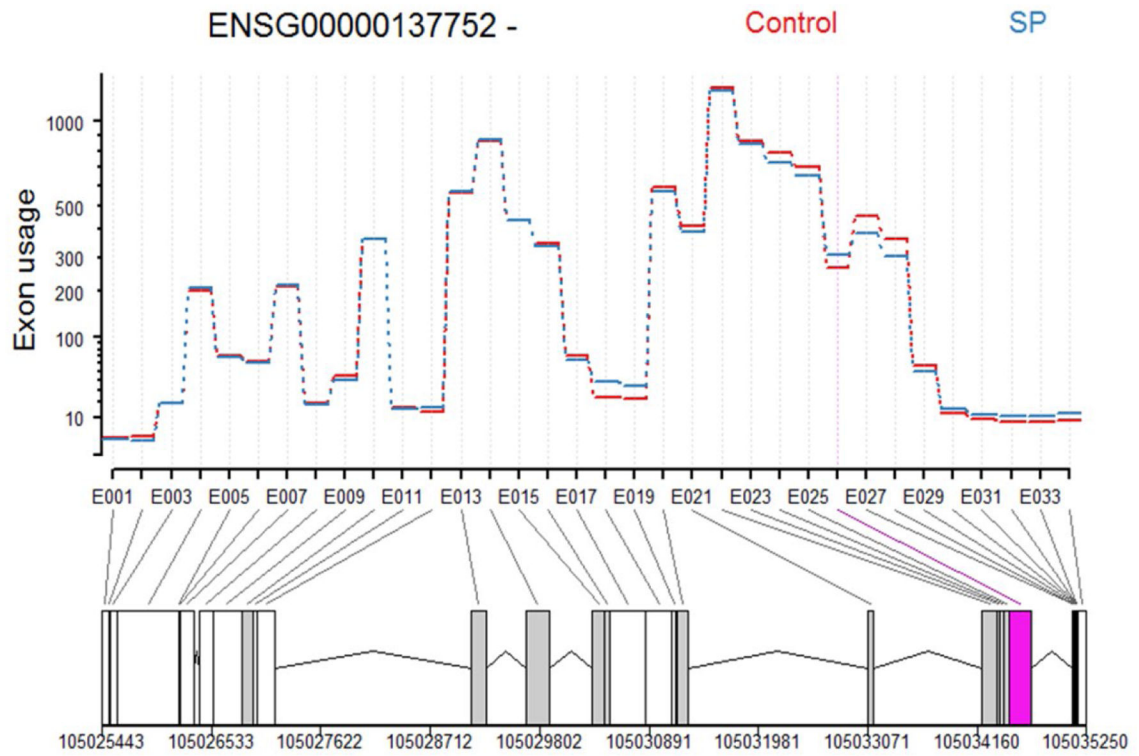


Figure 3. Transcript model of the differential exon usage within CASP1 in SP-treated CD16+ monocytes.

In CD16+ monocytes SP treatment results in alternative splicing of caspase-1 (CASP1), where the use of exon 026 shows an increase of 0.21 in log₂ Fold Change. The upregulated isoform corresponds to the protein coding ENST00000527979.5 transcript.

Table 1.

Antibody panel for flow cytometry.

Antigen	Fluorophore	Host species	reactivity	Clone	Company	Catalog number
CD14	APC	Mouse	Human	M5E2	BD Biosciences	555399
CD16	PE/Cy7	Mouse	Human	3G8	BioLegend	302016
CD235a	FITC	Mouse	Human	HIR2	BD Biosciences	559943
CD15	FITC	Mouse	Human	HI98	BioLegend	301904
CD3	FITC	Mouse	Human	UCHT1	BioLegend	300406
CD56	FITC	Mouse	Human	HCD56	BioLegend	318304
CD19	FITC	Mouse	Human	HIB19	BD Biosciences	555412

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