Toll-Like Receptor 7 (TLR7) Mediated Transcriptomic Changes on Human Mast Cells

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Background: Mast cells are skin immune sentinels located in the upper dermis, where wheal formation and sensory nerve stimulation take place. Skin inflammation is occasionally accompanied by mast cell-driven responses with wheals, angioedema, or both. Immunoglobulin E (IgE) antibodies are regarded as typical stimuli to drive mast cell activation. However, various causative factors, including microbial infections, can drive IgE-independent mast cell response. When infected, the innate immunity orchestrates an immune response by activating receptor signaling via Toll-like receptors (TLRs). Objective: In this study, we determined the effect of TLR7 stimulation on mast cells to investigate the possible mechanism of IgE-independent inflammatory response. Methods: Human mast cell (HMC) line, HMC-1 cells were treated with TLR7 agonist and the morphologic alteration was observed in transmission electron microscopy. Further, TLR7 agonist treated HMC-1 cells were conducted to RNA sequencing to compare transcriptomic features. Results: HMC-1 cells treated with TLR7 agonist reveals increase of intracellular vesicles, lipid droplets, and ribosomes. Also, genes involved in pro-inflammatory responses such as angiogenesis are highly expressed, and Il12rb2 was the most highly up-regulated gene. Conclusion: Our data suggest that TLR7 signaling on mast cells might be a potential therapeutic target for mast cell-driven, IgE-independent skin inflammation. (Ann Dermatol 33(5) 402~408, 2021)

Keywords: Mast cell, Skin inflammation, Toll-like receptor 7

INTRODUCTION

Mast cells play a crucial role in skin immune response by expressing important stimulatory receptors on the cell surface. Classical mast cell activation occurs through the high affinity immunoglobulin E (IgE) receptor, FcεRI. Activation occurs when adjacent receptors, occupied by receptor-bound IgE, are crosslinked by re-exposure to the original or a crossreactive bivalent or multivalent antigen. Many inflammatory skin diseases are mediated by such IgE-dependent mast activation, including atopic dermatitis and acute urticaria. Besides classical IgE-mediated activation, it is emerging that the novel mast cell activation that are not only independent of IgE crosslinking but also express unique cytokine secretion profiles. The diverse stimulatory receptors expressed on mast cells implicate multiple roles of mast cells in the pathogenesis underlying skin inflammation.

Importantly, mast cells respond to a wide range of pathological and environmental stimuli owing to their expression of Toll-like receptors (TLRs). TLRs are pattern-recognition receptors that initiate innate immune responses via the recognition of pathogen-associated molecular patterns (PAMPs). TLRs can also sense endogenous molecules that are released after cellular stress or tissue injury, known as danger-associated molecular patterns (DAMPs).
of TLRs in immune cells leads to the synthesis of various pro-inflammatory cytokines and chemokines via transcriptional regulation. Also, TLRs are considered cellular sensors for detecting exogenous and endogenous ligands in primary sensory neurons to initiate itchy sensation (pruritus) that is associated with skin infections and tissue injuries. For example, TLR7 expression is a subset of primary sensory neurons that co-express itch-signaling components such as the transient receptor potential vanilloid subtype 1, which play an important role in itchy sensation. Other studies show that TLR7 agonists effectively mimic psoriatic skin inflammation in mouse with increase of dermal mast cells. Thus, TLR-7 stimulus could be a mediator in the development of skin inflammation. Considering that mast cells predominantly reside in skin as a skin immune sentinel, we hypothesized that mast cells may act as a key player in the pathogenesis of infectious skin inflammation via TLR7 activation. In this study, we investigated transcriptome portrait of mast cells in stimulation with TLR7 and provided possible therapeutic targets in skin inflammatory disease.

MATERIALS AND METHODS

All procedures were approved by the Ewha Womans University College of Medicine Animal Care and Use Committee (EUM 20-024).

Cell culture

The human mast cell (HMC) line, HMC-1, was grown in Iscove’s Modified Dulbecco’s Medium (IMDM; GIBCO, Grand Island, NY, USA) containing 10% FBS, streptomycin (100 μg/ml), and penicillin (100 U/ml). The cells were stimulated with 2 μg/ml imiquimod (R837; Invivogen, San Diego, CA, USA) for 24 hours to examine the effects of TLR7 ligand on HMC-1 cells. The cultured HMC-1 cells were visualized by toluidine blue staining.

Reverse transcription polymerase chain reaction (RT-PCR)

For the analysis of TLR7 gene expression, HMC-1 cells cultured in the presence or absence of imiquimod for 24 hours were harvested and homogenized in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 μg) was transcribed into complementary DNA using a reverse transcription reagent (ELPIS-Biotech Inc., Daejeon, Korea) according to the manufacturer’s instructions. TLR7 (201 bp) and internal control gene GAPDH (192 bp) were amplified using the primers listed in Table 1. The band pixel densities of TLR7 were divided by the pixel densities of the corresponding GAPDH bands for quantitation using UN-SCAN-IT-gel 6.1 software (Silk Scientific Inc., UT, USA).

Table 1. Primers used for reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR

| Primer | Sequence | Product size (bp) |
|--------|----------|------------------|
| Tlr7   | F: 5’-CTTGAGGGCAACAACATCT-3’ | 201 |
|        | R: 5’-GTTAGGAGGGCTGTGACATT-3’ | |
| Muc22  | F: 5’-GGCCACTGACGTTTCTATCCA-3’ | 121 |
|        | R: 5’-GGCCGTGAAGTCCATTCCAG-3’ | |
| Ern2   | F: 5’-TCGAAGGACCAATGTACGTCA-3’ | 117 |
|        | R: 5’-GGATGGTGAATGGCAGTTTCAT-3’ | |
| Rps17  | F: 5’-GTTCGCACCAAAACCGTGAAG-3’ | 339 |
|        | R: 5’-GTTGGACAGACTGCCGAAGT-3’ | |
| Ern2   | F: 5’-GTGGGCTCTTACCTCGCTTTC-3’ | 124 |
|        | R: 5’-CCCCGAGAGTGTCTGGGA-3’ | |
| Kcnt1  | F: 5’-GACCCCGTCCTTCCAGAACG-3’ | 173 |
|        | R: 5’-ACGGCCACCAATGTAGAGCA-3’ | |
| Nlrp3  | F: 5’-CTGTGATCCCCTAATAGGAGT-3’ | 191 |
|        | R: 5’-CTGTAGTCTCCATTAGGAGT-3’ | |
| Ccl4   | F: 5’-CTCTGCTGTATCCAGGAGATC-3’ | 279 |
|        | R: 5’-CTCACTGTCGCTCATACATCA-3’ | |
| Il12rb2| F: 5’-AAAAATAGTGGTGCCAGAAGQ-3’ | 211 |
|        | R: 5’-GGGGGAGGAGGCTGTCTG-3’ | |
| Gapdh  | F: 5’-GGTAAAGTGGATATTGTTGCCATCA-3’ | 192 |
|        | R: 5’-GGAGGGATCTCGCTCCTGGAAGTGGTG-3’ | |

F: Forward, R: Reverse.

Transmission electron microscopy (TEM)

HMC-1 cells were cultured in the presence or absence of imiquimod (2 μg/ml) for 24 hours at a concentration with 10^6 cells/ml in 3 ml media. After centrifugation, the cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Specimens were washed in 0.1 M phosphate buffer and post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 hour, dehydrated with ethanol, and embedded in epoxy resin. Ultrathin sections, approximately 60∼70 nm thick, were cut by an EM UC7 ultramicrotome (Leica, Wetzlar, Germany) using a diamond knife. Sections were contrasted with uranyl acetate followed by lead citrate and observed with H-7650 TEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

RNA extraction, library construction, and sequencing

RNA was extracted from control HMC-1 cells and imiquimod-treated HMC-1 cells (Qiagen, Hilden, Germany) and subsequently column purified with RNeasy mini kit (Qiagen). Purified RNA was treated with DNase I (New England Biolabs, Ipswich, MA, USA) to remove genomic DNA. RNA quantity and quality were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with an RNA integrity number ≥8. cDNA libraries were
prepared with 1 μg of starting total RNA using a TruSeq RNA library Prep kit v2 (Illumina, San Diego, CA, USA), and transcriptome sequencing was performed using a NovaSeq S4 Reagent kit and Illumina NovaSeq with 151 bp paired-end reads per sample (Macrogen, Seoul, Korea).

**Sequence annotation and identification of differentially expressed genes**

FASTQ-formatted sequencing data were demultiplexed to assign reads to the originating sample. The raw sequences were quality checked using FastQC v.0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the low-quality bases with quality scores < 30 and the adapter contamination were removed by Trimmomatic v.0.38 (https://www.usadellab.org/cms/?page=trimmomatic) using the parameters ‘LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36’. Trimmed reads were mapped to the human genome reference (UCSC hg19) using TopHat v2.0.13 (https://ccb.jhu.edu/software/tophat/index.shtml). The total mapped read numbers for each transcript were determined and normalized to detect fragments per kilobase of exon per million fragments mapped (FPKMs) using TopHat v2.0.13 (https://cole-trapnell-lab.github.io/cufflinks/releases/v2.2.1/). Genes with more than one zero FPKM value out of the analyzed samples were excluded to filter potentially significant gene expressions. For differentially expressed gene (DEG) analysis, the values of log2 (FPKM+1) were calculated, and then normalized by quantile. Transcripts with absolute fold-change values larger than 2 with a p-value ≤ 0.05 were included in the analysis as DEG. Hierarchical clustering analysis was performed using complete linkage and Euclidean distance as a measure of similarity to display the DEG expression patterns. All DEG data analysis was conducted using R 3.2.2 (https://www.r-project.org).

**Gene ontology and enrichment analysis**

Functional groups and pathways encompassing the DEGs were identified based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID v.6.8; https://david.ncifcrf.gov) software. The threshold was set as modified Fisher Exact p-value (EASE score) ≤ 0.05.

**Quantitative reverse transcription PCR (qRT-PCR)**

For validation of DEG, HMC-1 cells were cultured in the presence or absence of imiquimod for 24 hours followed by extraction of RNA using TRIzol (Thermo Fisher Scientific). Complementary DNA was synthesized using reverse transcription reagent (ELPIS-Biotech) according to the manufacturer’s instructions. Real-time PCR analysis was performed on a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA) using a SensiFAST SYBR Hi-ROX kit (Bioline, London, UK). All gene expression values (Muc22, Ern2, Rps17, Epha1, Kcnt1, Nlrp3, Ccl4, and Il12rb2) were normalized to the GAPDH reference gene using the primers listed in Table 1.
Fig. 2. Transcription differs between control human mast cell (HMC)-1 cells and imiquimod-treated HMC-1 cells. (A) A heatmap of hierarchical clustering indicates differentially expressed gene (rows) between control HMC-1 cells and imiquimod-treated HMC-1 cells (fold-change $>2$, $p<0.05$). Red indicates upregulation and blue indicates downregulation. (B) Functional enrichment analysis of highly regulated genes in control HMC-1 cells and imiquimod-treated HMC-1 cells were annotated in the GO category of biological process (left). Distribution of positively regulated genes was annotated in the GO category of biological process (right).
Table 2. Twenty-five most upregulated transcripts in imiquimod-treated human mast cell (HMC)-1 cells compared to untreated cells

| Gene_ID | Gene_symbol | Gene_description | Fold-change |
|---------|-------------|------------------|-------------|
| 3595    | IL12RB2     | Interleukin 12 receptor, subunit beta 2 | 3.16        |
| 26824   | RNU11       | RNA, U11 small nuclear | 5           |
| 8339    | H2BC8       | H2B clustered histone 8 | 4.28        |
| 6218    | RPS17       | Ribosomal protein S17  | 3.96        |
| 8875    | VNN2        | Vanin 2          | 3.79        |
| 100507679 | MUC22     | Mucin 22        | 2.96        |
| 57582   | KCNT1       | Potassium sodium-activated channel subfamily T member 1 | 2.81        |
| 103344718 | HOTS      | H19 opposite tumor suppressor | 2.84        |
| 150946  | GAREM2      | GRB2-associated regulator of MAPK1 subtype 2 | 2.81        |
| 146227  | BEAN1       | Brain-expressed associated with NEDD4 1 | 2.67        |
| 2041    | EPHA1       | EPH receptor A1  | 2.67        |
| 728113  | ANXA8L1     | Annexin A8 like 1 | 2.64        |
| 103091864 | SNHG22    | Small nucleolar RNA host gene 22 | 2.57        |
| 6351    | CCL4        | C-C motif chemokine ligand 4 | 2.55        |
| 23632   | CA14        | Carbonic anhydrase 14 | 2.54        |
| 9478    | CYP1B1      | Cytochrome P450 family 1 subfamily B member 1 | 2.47        |
| 83643   | CCDC3       | Coiled-coil domain containing 3 | 2.43        |
| 2626    | GATA4       | GATA binding protein 4 | 2.43        |
| 10595   | ERN2        | Endoplasmic reticulum to nucleus signaling 2 | 2.39        |
| 2117    | ETV3        | ETS variant transcription factor 3 | 2.38        |
| 9945    | GFTP2       | Glutamine-fructose-6-phosphate transaminase 2 | 2.38        |
| 1829    | DSG2        | Desmoglein 2     | 2.37        |
| 10859   | LILRB1      | Leukocyte immunoglobulin-like receptor B1 | 2.37        |
| 114548  | NLRP3       | NLR family pyrin domain containing 3 | 2.34        |

Fig. 3. Validation of genes upregulated in response to imiquimod. The mRNA expression of Muc22, Em2, Rps17, Epha1, Kcnt1, Nltp3, Ccl4, and Il12rb2 in control human mast cell (HMC)-1 cells and imiquimod-treated HMC-1 cells was analyzed by quantitative reverse transcription polymerase chain reaction. Values are presented as the mean ± standard error of the mean (*p < 0.05).
involved in angiogenesis—half of the six categories were positive regulation of angiogenesis, angiogenesis, and vascular endothelial growth factor production (Fig. 2B, right).

**RT-qPCR results validate RNA sequencing**

We selected eight genes involved in various inflammatory process such as angiogenesis, cell adhesion, migration, and vascular permeability for validation. Expression of Muc22, Ern2, Rps17, Epha1, Kcnt1, Nlrp3, Ccl4, and Il12rb2 was confirmed in both control HMC-1 and imiquimod-treated HMC-1 cells using RT-qPCR. We found that expression of all eight genes increased in imiquimod-treated HMC-1 cells, confirming the results of the RNA sequencing analysis. Although the fold-changes of upregulated genes did not appear substantial by RNA sequencing, RT-qPCR validated the fold-changes. In particular, Il12rb2, the most highly expressed gene when analyzed by RNA sequencing, was also most upregulated by quantitative PCR (Fig. 3).

**DISCUSSION**

In this study, we demonstrated that TLR7 activation stimulates mast cells, leading to the upregulation of genes involved in the inflammatory process. Specifically, we showed that mast cells activate in response to TLR7 stimulation with imiquimod, phenotypically presented by the increases in vesicles, lipid droplets, and ribosomes. Furthermore, transcriptome sequencing indicates that TLR7 activation increases the expression of genes involved in pro-inflammatory responses such as angiogenesis.

Mast cells can sense a plethora of agents by expressing various receptors including TLRs. TLRs are divided into two groups according to their subcellular localization and respective PAMPs: (i) cell surface TLRs (TLR1, 2, 4, 5, 6, and 10) that recognize microbial membrane components such as lipids, lipoproteins, and protein; and (ii) intracellular TLRs (TLR3, 7/8, and 9) that recognize viral or endogenous nucleic acids. Infections by microorganisms such as bacteria or viruses could be an underlying cause in inflammatory skin disease. Besides, some viral infection occur acute urticaria together with the onset of viral symptoms. Thus, TLR signals might be an important mediasor of infectious inflammatory skin disease.

TLR7 was originally identified as recognizing imidazoquinoline derivatives such as imiquimod and resiquimod, and guanine analogs such as loxoribine. Intradermal injection of imiquimod, R848, and loxoribine induced scratching in wild-type mice, which was reduced in Thr7−/− mice, indicating that TLR7 is an itch mediator and a potential therapeutic target for anti-itch treatment in skin diseases. On the other hand, imiquimod-treated mice showed psoriasis-like skin inflammation with increase of dermal mast cells, suggesting that a TLR7 agonist could be direct stimulator of mast cells in psoriatic skin inflammation.

Our results strongly support that TLR7 activation on mast cells can induce an inflammatory response. The obvious increase of intracellular components such as vesicles, lipid droplets, and ribosomes in imiquimod-treated HMC-1 cells indicate that active biological processes respond to TLR7 stimulation. Transcription analysis showed upregulation of pro-inflammatory genes when TLR7 activated mast cells, particularly in angiogenesis. Importantly, most skin inflammatory diseases are characterized by excessive angiogenesis. Among 25 top-upregulated genes, we particularly validated eight genes involved in various inflammatory process that possibly contribute to angiogenic immunopathogenesis. Mucins (MUCs) are family of glycoproteins that are present in the mucus coating of epithelial surface and are used as ligands for cell adhesion and also promote angiogenesis. MUC gene expression has been found to be altered in inflammatory states. TLR7 stimulated mast cells showed upregulation of one of MUCs coding gene, Muc22 and positive regulator of mucin gene expression, Ern2. The upregulation of Rps17 gene which provides instructions for making ribosomal proteins support outstanding increase of ribosomes in TLR7-stimulated mast cells. Epha1 gene is involved in cell migration, adhesion, and angiogenesis. In addition, upregulated Kcnt1, Nlrp3, and Ccl4 are expected to contribute to promote inflammatory process such as immune cell activation and migration. In particular, Nlrp3 is expected to increase vascular permeability by producing active form of interleukin (IL)-1β. Of note, the most highly upregulated gene, Il12rb2, implied that TLR7 signaling may enhance IL-12 response in mast cells. Upregulation of the Il12rb2 gene is associated with several diseases including leprosy, and is thought to contribute to the inflammatory response and host defense mechanism. In fact, IL-12 is key player in the regulation of Th1 response which are orchestrated mainly by macrophage and dendritic cells in viral infection. Our result indicate that mast cells also could be direct responder in addition to T cells in IL-12 abundant microenvironment. Enhanced expression of receptor for IL-12 could activates downstream pathway such as NF-κB that ultimately induce pro-inflammatory cytokines. Interestingly, several mast cell-derived pro-inflammatory cytokines have been implicated in Th17 cell-skewing inflammation, including IL-1β and IL-6. Therefore, mast cells could be responsible for producing pro-inflammatory cytokines as the consequence of increased IL-12 response when TLR7 stimulated. The specific outcome of IL-12 effect on mast cells should be handled within our next expanded research. Also, our
findings should be broaden into early and rate response in actual mast cell degranulation. Considering that other chro-
nic inflammatory autoimmune disease such as psoriasis
can be initiated by excessive activation of endosomal TLRs,
particularly TLR7, TLR8, and TLR9 19, TLR7 signaling in
mast cells is able to expand our point of view on immune
network between inflammatory skin disease.
In summary, we demonstrated that TLR7 directly mediate
mast cell activation and the TLR7 or IL-12 signaling could
be candidates in the search for therapeutic targets for skin
inflammatory disease.

CONFLICTS OF INTEREST
The authors have nothing to disclose.

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DATA SHARING STATEMENT
The data that support the findings of this study are avail-
able from the corresponding author upon reasonable re-
quest.

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