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Genome-wide Expression Profiling Implicates a *MAST3*-Regulated Gene Set in Colonic Mucosal Inflammation of Ulcerative Colitis Patients

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD) presumably caused by dysregulated immune responses to the gut microbiota. Genetic association studies have implicated dozens of chromosomal regions or loci in IBD susceptibility. The next challenge is to explain the individual role of each of these modest effect loci in disease state. We have previously identified *MAST3* as an IBD susceptibility gene through genetic fine-mapping of the 19p linkage region. Testing *MAST3* in a reporter assay provided preliminary evidence that *MAST3* modulates the activity of inflammation-related transcription factor nuclear factor kappa B (NF- κ B). Here, we further characterized the function of *MAST3* through an examination of the influence of the modulation of *MAST3* expression on endogenous genome-wide expression patterns. More specifically, we looked at differential gene expression resulting from overexpression and knockdown of the *MAST3* gene in epithelial and macrophage cell lines. We highlight a group of genes whose expression is modulated by *MAST3* and correlate their expression with NF- κ B activity. Their expression was found to be enriched in inflamed mucosal tissue of UC patients, confirming the importance of these genes in IBD. These *MAST3*-regulated genes are central to mucosal immune responses. Among them are pro-inflammatory cytokines (e.g. *CCL20*, *IL8*), regulators of NF- κ B (e.g. *TNFAIP3*, *LY96*, *NFKBIA*), genes involved in interferon-induced defense against pathogen invasion (e.g. *IFIT1*, *ISG15*) and genes involved in cell adhesion and/or migration (e.g. *CD44*, *TMOD1*). Taken together, these results confirm *MAST3* as a modulator of the inflammatory response through regulation of immune gene expression in the gut of IBD patients.

Keywords

MAST3; Inflammatory Bowel Disease; NF- κ B

Introduction

Inflammatory bowel diseases (IBD) refer to Crohn's disease (CD) and ulcerative colitis (UC), two common inflammatory diseases of the gastrointestinal tract most prevalent in urban areas of North America and Europe. Current consensus assigns the causes of IBD to environmental factors - such as cigarette smoking, appendicitis, hygiene, and gut microbiota - combined to genetic predisposition that contribute to the development of a dysregulated immune response to the gut microbiota leading to mucosal inflammation (1). Genome-wide genetic association studies have identified 99 IBD susceptibility regions or loci and some of them contain multiple genes (2, 3). Targeted association studies, such as ours (4), have also identified several susceptibility genes. However, estimation of heritability explained by these loci suggests that many more remain to be discovered. The important challenge is to identify individual role of the IBD genes to better understand the molecular mechanisms underlying disease onset and chronic inflammation.

In a previous study, we identified the microtubule associated serine/threonine protein kinase-3 (*MAST3*) gene as a genetic risk factor for inflammatory bowel disease (IBD) through association fine-mapping of the chromosome 19p region (also known as linkage region IBD6) (4). *MAST3* is one of the least studied members of the *MAST* kinase family. Most members of the *MAST* family are ubiquitously expressed but their expression is highest in the brain (5). *MAST2*, the most studied member of this family, is involved in immune reactions, more specifically in the regulation of NF- κ B (6). Based on this knowledge, we performed a knockdown of *MAST3* in HEK293 cells and tested transcription factor NF- κ B activity through reporter assays. We showed that a knockdown of *MAST3* decreased significantly and specifically receptor TLR4 stimulated activity of NF- κ B (4).

In the current study, we aimed to further characterize the role of *MAST3* by modulating its expression, via overexpression and knockdown (KD) of the gene, in *ex vivo* cell culture systems and determining the impact on genome-wide endogenous gene expression. Specifically, we first compared transcriptional profiles of mock-transfected and *MAST3*-overexpressing HEK293 cells using genome-wide microarray analysis. Our genome-wide analysis highlighted a group of 28 *MAST3*-regulated genes whose expression levels were increased by 2-fold. In order to assess specificity of these results, we knocked down *MAST3* in immune cells (THP1) and determined the expression levels of a subset of the genes identified in the overexpression experiment. We observed that for 7/9 genes tested, the expression was significantly reduced and thus the results of our KD confirmed our previous overexpression model results. To assess the importance of the *MAST3*-regulated genes in the clinical presentation of IBD, we compared gene expression in colonic mucosal tissue of healthy controls and of UC patient non-inflamed and inflamed regions. We found a significant enrichment of the expression of the *MAST3* gene set in inflamed vs non-inflamed tissues (P=0.005).

The *MAST3*-regulated genes coordinate immune responses in intestinal inflammation. They include pro-inflammatory cytokines, regulators of NF- κ B, interferon induced genes, and genes involved in adhesion and cell migration and a majority of these genes are regulated by NF- κ B which suggests that *MAST3* controls their expression via the NF- κ B pathway.

Furthermore, the results of our expression study in patients biopsies suggest that *MAST3* can regulate the expression of a set of genes that discriminates inflamed from non-inflamed tissues and that the overexpression of these genes is part of the clinical manifestation of the disease.

Materials and Methods

Cell culture

THP1 cells (ATCC TIB-202) were cultivated in RPMI medium supplemented with 10% fetal bovine serum, 2-mercaptoethanol (final concentration 0.05 mM), and penicillin (100 units/mL)-streptomycin (100 ug/mL). TLR4 293-hTLR4A-MD2-CD14 HEK cells (InvivoGen #293-hTLR4md2cd14) were cultivated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL)- streptomycin (100 ug/mL), blasticidin (10 ug/mL) and HygroGold (50 ug/mL, InvivoGen) at 37°C in a 5% CO₂ atmosphere.

MAST3 overexpression model

At day 0, twelve aliquots of 1×10^6 TLR4 293-hTLR4A-MD2-CD14 HEK cells (InvivoGen, San Diego, CA, USA) were diluted in 2ml of complete medium (without antibiotics) and plated in individual wells of two 6-well plates. At day 1, cells were transiently transfected using 6ug of pCMV-XL6 vector containing *MAST3* cDNA (SC316595 Origene, Rockville, MD, USA) or 6ug of pCMV-AC-GFP vector (PS100010 Origene, Rockville, MD, USA) and 15 uL of lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were collected 24h later (at day 2) and frozen. For the time course assay, cells were similarly transfected, and collected and frozen at 0h, 8h, 12h and 24h after transfection and used for protein or RNA extraction.

MAST3 knockdown model

Small hairpin RNA (shRNA) was introduced into THP1 cells by spinoculation with Mission lentiviral particles (Sigma, St-Louis, MO, USA) according to manufacturer's instructions. At day 1, aliquots of 50,000 cells were diluted in 100 uL of complete medium. Cells were transduced with lentiviral transduction particules carrying a vector with shRNA targeting *MAST3* and a puromycin resistance gene (clone ID TRCCN0000200003 from Sigma, St-Louis, MO, USA, CCGGCGAGCCTTTCTGCCGACACAGCTCGAGCTGTGTCTGGCAGAAAGGCTCG TTTTGTG) or control transduction particules carrying a vector with non-target shRNA and a puromycin resistance gene (SHC002V Sigma, St-Louis, MO, USA) at MOI =10. Three independent aliquots of cells were transduced with *MAST3* shRNA and three others with non-target control shRNA. Cells with virus containing medium were centrifuged at 800 g for 30 minutes at 32 °C. Virus containing medium was aspirated and cell pellets were resuspended in 100 ul of complete media by gently pipetting the pellets up and down, and each resuspended pellet was transferred to its own well in a 96-well tissue culture plate. The plate was returned to the tissue culture incubator 37°C in a 5% CO₂ atmosphere. At day 4, puromycin (final concentration 0.5mg/mL) was added to the medium. At day 12, all cells in the non-transduced control wells were dead. Cells were further propagated in medium containing puromycin and pools of transduced THP1 clones were used for differentiation. The 6 pools of transduced THP1 cells (3 *MAST3* knockdowns, 3 controls) were each separated in aliquots. One was kept untreated, one was treated with phorbol myristate acetate (PMA; P1585 Sigma, St-Louis, MO, USA) (10 ng/mL) for 48h and the other ones were treated with PMA (10 ng/mL) for 48h and with lipopolysaccharide (LPS; L2654 Sigma, St-Louis, MO, USA) (10 ng/mL) for 3h, 6h or 24h. Cells were then collected for proteins and RNA extraction.

RNA extraction, quantification and reverse-transcription (RT) in cell models

RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was quantified using a Bioanalyzer 2100

(Agilent, Santa Clara, CA, USA). All samples had RIN above 9.5. RNA samples (2 ug per rxn) were reverse-transcribed (random hexamers) using the High Capacity cDNA Reverse Transcription kit (ABI, Carlsbad, CA, USA).

Proteins and Western Blots

Proteins were extracted from cell lysate using RIPA Buffer (899100 Thermo, Waltham, MA, USA) according to the manufacturer's instructions. Proteins were quantified using Pierce BCA Protein Assay Kit (23225 Thermo, Waltham, MA, USA) according to the manufacturer's instructions. The absorbance was read on a Synergy 2 plate reader (Biotek, Winoosky, VT, USA). Western blots were performed according to standard methods, antibodies used for immunoblotting were anti-MAST3 (ab64952, Abcam, Cambridge, MA, USA), anti-actin (ab3280, Abcam, Cambridge, MA, USA), and goat anti-rabbit-HRP (CLCC43007, Cederlane, Burlington, NC, USA).

qPCR

Gene expression was monitored with 10 ng of total cDNA and SybrGreen (Invitrogen Carlsbad, CA, USA) based qPCR using the Mx3005P Real-Time PCR system (Agilent, Santa Clara, CA, USA). Cycling conditions were set at 95°C for 10 minutes, then 40 cycles of 95°C for 30 seconds and 60°C for 1 minute and 1 cycle of 55°C for 30 seconds and 95°C for 30 seconds. Samples were run in duplicates. Gene expression levels were normalized to the *HPRT1* gene expression levels. Human gene specific primer sequences used were: *MAST3* forward (5'GCAGCGAAGTGGACTATGG3'), *MAST3* reverse (5'GATGGTATTCAGGAGAGATGGG3'), *HPRT1* forward (5'TGGCGTCGTGATTAGTGATG3'), *HPRT1* reverse (5'CAGAGGGCTACAATGTGATGG3') (Other sequences in Supplementary Table 4).

MAST3 model of overexpression: Whole-genome gene expression analysis

Twelve RNA samples from TLR4 293-hTLR4A-MD2-CD14 HEK cells (6 MAST3 overexpressed samples and 6 controls) were sent to Genome Quebec Innovation Center (Montreal, QC, Canada) (GQ) to be analyzed using the HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, CA, USA). Preliminary data was analyzed at GQ using the Genome Studio Software (Illumina, San Diego, CA, USA). We performed further quality control assessment and data normalization using the Lumi library included in the Bioconductor package (7, 8). First, data were adjusted by log₂ transformation and global mean normalization. Then, differentially expressed genes between cells overexpressing the *MAST3* gene and mock-transfected cells were determined using a one-tailed Welch's t-test calculated using the MeV software (9). P values were corrected for multiple testing using the adjusted Bonferroni correction. Genes with significant expression change ($P_{\text{adjusted}} < 0.05$) were further studied.

A query of the Database for Annotation, Visualization and Integrated Discovery (DAVID) (10) was used to identify functional pathways based on Gene Ontology biological processes.

NF-κB activity Assays

NF-κB activity was monitored using ELISA strip plates from the Transcription Factor kit for NF-κB p65 (89858 Thermo, Waltham, MA, USA) according to the manufacturer's instructions. For THP1 cells and TLR4 HEK cells, 6 and 20 ug of total protein lysate was used per well, respectively. For each of the three biological replicates at each point, luminescence from two independent wells was read on a Synergy 2 plate reader (Biotek, Winoosky, VT, USA).

Whole-Genome Expression in Patient Biopsies

Biopsies were collected from patients suffering from ulcerative colitis (UC) and healthy controls at Royal Victoria and Maisonneuve-Rosemont Hospitals in Montreal (QC, Canada). Patients had to be older than 18 year old and diagnosed with UC for at least 5 years. Patients under biological treatment (i.e.: Infliximab) were excluded from the study. Biopsies were collected during routine exams (patients) and during cancer screening exams (controls). Written informed consent was obtained from all participants and ethics approval was granted in each of the participating institutions. For patients, biopsies were taken from inflamed (on the margin of ulcerations) and noninflamed region. Biopsies were taken from the rectum (controls and most patients) and colon (some patients) and stored in RNAlater (Qiagen, Valencia, CA, USA) until RNA extraction. RNA was extracted in the following 24h using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer instructions. RNA was quantified using a Bioanalyzer 2100 from Agilent. RIN was above 8. One μ g of RNA was sent to GQ and whole-genome expression was evaluated using the Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA).

Statistical analyses for the enrichment analyses

Expression data from the biopsies was normalized using quantile normalization with the RMA algorithm within each analyzed pair of classes (control+ non-inflamed and non-inflamed+inflamed) (11, 12). Differential expression between each class was tested using Welch T tests. Data structure (inflamed and non-inflamed samples that came from the same individual and batches of Affymetrix (Santa Clara, CA, USA) chips hybridized on 2 separate occasions) was corrected using permutations (1000 permutations). Enrichment in the MAST3-regulated gene set was tested using Fisher's exact test.

For assessment of the differential expression of individual genes, P values were further adjusted for FWER using a Bonferroni-type correction that incorporates correlation within the data.

Results

Overexpression model identifies a MAST3-regulated gene set involved in immune response

In order to gain insight into the function of the IBD susceptibility gene *MAST3*, we overexpressed *MAST3* cDNA in HEK293 cells (chosen because these cells are easily and efficiently transfected) and analysed the resulting *MAST3* transcriptional signature using genome-wide cDNA microarray. The endogenous expression of *MAST3* is relatively low in these cells, and was measured by reverse transcription quantitative polymerase chain reaction (RT qPCR) to be several orders of magnitude lower than in cells overexpressing *MAST3*. *MAST3* protein levels at 24h post-transfection were increased by 60-fold in these cells (Supplementary Figure 1). Unsupervised hierarchical clustering of expression data clearly discriminates between cells overexpressing the *MAST3* gene and mock-transfected cell control (data not shown). In total, 195 probes showed significant ($P_{\text{corrected}} < 0.05$) differential expression (Supplementary Table 1). This represents 0.4% of all interrogated probes. The differentially expressed probes represent 146 different validated protein coding genes, 24 genes encoding small nuclear RNAs and 15 encoding hypothetical proteins. Of the 185 differentially expressed genes, 113 showed higher expression and 72 showed lower expression in cells overexpressing the *MAST3* gene compared to mock-transfected cells. Underexpressed genes (in cells overexpressing *MAST3*) consisted mostly of small nuclear RNAs and hypothetical proteins, so we decided to focus on the overexpressed genes. We were most interested in the top 28 genes with 2-fold or greater increases in expression

(Table 1). We validated the microarray expression data by testing the expression of 5 genes randomly chosen among the top 28 using RT qPCR (Supplementary Figure 2).

This set of 28 genes (Table 1) whose expression appears to be regulated by *MAST3* shows a significant enrichment ($P < 0.01$) of functional involvement in defense response (10-fold), inflammatory response (16-fold), response to wounding (11-fold), regulation of apoptosis (7-fold), and regulation of programmed cell death (7-fold) (Supplementary Table 2). The importance of this group of genes in the immune response can be further recognized in their known function based on the literature: they encode pro-inflammatory cytokines, regulators of the activity of NF- κ B, proteins involved in adhesion and cell migration, and proteins induced by interferon in response to pathogen invasion or tumor progression. Additionally, 61% of these genes are regulated by NF- κ B via functionally validated binding sites in their promoter (Figure 1 and Table 1) suggesting that *MAST3* may act through this pathway.

The median fold-increase in gene expression in the *MAST3*-regulated gene set (top 28 genes) in *MAST3* overexpressing cells compared to mock-transfected cells is 3. However, two genes are overexpressed by greater than 10-fold, *CCL20* ($P = 1.31 \times 10^{-10}$, 38-fold) and *IL8* ($P = 6.09 \times 10^{-6}$, 14-fold), suggesting that *MAST3* levels have a greater influence on the expression of these genes than the other upregulated genes. The *CCL20* chemokine is the only known ligand of the CCR6 receptor and CCR6 signaling is involved in the recruitment of dendritic cells, and other antigen presenting cells as well as CD4+T cells to the sites of epithelial inflammation (13). The chemokine IL-8 is produced predominantly in the lamina propria of the colon and its level correlates with mucosal inflammation (14).

Knockdown of *MAST3* expression in immune cells confirms gene expression profile

In order, to further study the effect of *MAST3*, we created a *MAST3* knockdown (KD). We were interested in confirming that the observed gene expression patterns from our overexpression model were reversed in the knockdown. Additionally, we wanted to study immune gene expression in an immune cell context so we selected monocyte-like THP1 cells. Our KD was created by transducing THP1 cells with *MAST3* small hairpin RNA (shRNA).

Interestingly, the KD has different effects on *MAST3* levels at different stages of cell differentiation and stimulation (Supplementary Figure 3). The KD has the greatest effect on *MAST3* levels in cells differentiated by phorbol 12-myristate 13-acetate (PMA) and stimulated by lipopolysaccharide (LPS) for 24h (a decrease of 75% of mRNA levels and of 92% of *MAST3* protein levels). In order, to maximize the effect of the *MAST3* KD on endogenous gene expression, we studied THP1 cells treated with PMA and LPS for 24h. We tested a subset of the *MAST3*-regulated gene set. Of the 9 genes tested, 7 are significantly underexpressed ($P < 0.05$) in our KD model compared to control and 2 show no significant difference (Table 2). Overall, this downregulation of gene expression in *MAST3* KD cells is consistent with the upregulation observed in the cells that overexpress the *MAST3* gene. Notably, *IL8* is highly expressed in PMA and LPS treated THP1 (4800-fold increase from unstimulated THP1 stage) and its expression level is reduced to 55% of the control expression in the KD ($P = 0.01$).

Our expression study in cells overexpressing the *MAST3* gene highlighted a group of upregulated genes that are regulated by transcription factor NF- κ B. We were interested in exploring the extent of the effect of a *MAST3* KD on the NF- κ B dependent endogenous gene expression but were somewhat limited by the HEK293 cell model (fibroblast like human embryonic kidney cells). The THP1 cell model allowed us to study the expression of additional NF- κ B regulated genes not necessarily expressed in HEK293 cells. We tested the expression of several interleukins, TGF β 1 and members of the NF- κ B pathway (Table 3).

As expected, based on our previous results, the KD of *MAST3* decreases the expression of a majority (4/6) of the pro-inflammatory genes tested. The KD also upregulates the expression of anti-inflammatory cytokine gene *IL10* to 210% of non target KD control ($P=0.003$). These additional results in our THP1 *MAST3* KD model support the results from the overexpression model and suggest a prominent pro-inflammatory role for *MAST3*.

NF- κ B activity correlates to *MAST3*-regulated gene expression

We were interested in studying the relationship between NF- κ B activity and the *MAST3*-regulated gene set expression. We have previously shown that a KD of *MAST3*, in LPS stimulated HEK293 cells that constitutively express the TLR4 receptor, decreases the activity of transcription factor NF- κ B (4). Thus, we first wished to confirm these findings in our two new cell models of overexpression in HEK cells and KD in THP1 cells. Then, we used the *IL8* and *CCL20* genes as proxies for the *MAST3*-regulated gene set to study the temporal correlation between NF- κ B activity and *MAST3* gene set expression.

We tested the activity of NF- κ B at different times post-transfection *MAST3* (Figure 2A). We observed a significant increase in activity specific to *MAST3* beginning 12 hours post-transfection. The activity of NF- κ B in cells overexpressing the *MAST3* gene, at 12 hours and 24 hours post-transfection is 159% ($P=0.01$) and 354% ($P=0.02$) that of mock-transfected cells, respectively. This increase in NF- κ B activity is correlated with significant *MAST3* mRNA (data not shown) and protein level increases (Supplementary Figure 1).

The activity of NF- κ B in our THP1 knockdown confirms the results of our previous study in HEK293 cells. A significant decrease in NF- κ B activity (70% of control, $P=0.02$) is detected in THP1 cells differentiated with PMA and treated with LPS for 6h (Figure 2B). Importantly, these results correlate with decreased *MAST3* mRNA (67.2% decrease) and protein (86% decrease) levels (Supplementary Figure 3).

The expression levels of *MAST3*-regulated genes *CCL20* and *IL8* genes are induced early and significant differences between *MAST3* overexpressed cells and control can be observed as soon as 8h post-transfection (Figure 2C and 2D). Yet, the most significant fold differences in expression between cells overexpressing the *MAST3* gene and mock-transfected cells are observed at 12 and 24h (10 and 32-fold increases respectively), and thus correlate temporally with the increases in NF- κ B activity.

MAST3-regulated gene set expression is enriched in inflamed tissues of UC patients

To establish the importance of the *MAST3*-regulated gene set in the clinical presentation of the disease, we tested its expression in human intestinal mucosa tissues from patient biopsies. In order to maximize our statistical power to detect differential expression by limiting noise, we defined the *MAST3*-regulated gene set as the group of genes that had been overexpressed by 3-fold or greater in our cell model of *MAST3* overexpression (Table 1, top 14 genes). We were mostly interested in answering two questions: 1. Are there differences in gene expression between intestinal tissue from healthy people and normal looking intestinal tissue from patients affected with UC? 2. Are there differences between normal looking intestinal tissues and inflamed tissue from UC patients? In order to answer these questions, we analyzed the differential gene expression profiles between biopsies of controls (BC) and patients' non-inflamed (BPN) and inflamed (BPI) regions of the colon and rectum. Specifically, we were interested in discovering whether there was an enrichment of our *MAST3*-regulated genes as part of the inflammatory process in IBD patients.

There were no detectable differences between BC ($n=16$) and BPN ($n=15$) (Table 4 and Supplementary Table 3). In contrast, we observed significant differences between BPN ($n=19$) and BPI ($n=15$). Of all the probes of the array, 34.5% (8498 out of 24607) showed

differential expression ($P < 0.05$). In our gene set, 71.4% of genes (10 out of 14) showed differential expression ($P < 0.05$), which represent a significant 2-fold enrichment ($P = 0.005$) (Table 4). After correction for multiple testing, we examined the expression patterns for individual genes. In general, expression of genes from the *MAST3*-regulated gene set was increased in the BPI compared to BPN. However, this trend did not extend to *MAST3* expression for which the difference between BPI and BPN did not reach statistical significance. For 5 genes the increase in expression was significant, *CD44* ($P = 0.001$), *TNC* ($P = 0.017$), *ISG15* ($P = 0.02$), *NFKBIZ* ($P = 0.02$) and *IL8* ($P = 0.031$) (Table 5).

Discussion

Genome-wide and targeted association studies have already identified numerous modest effect genes and estimations of cumulative heritability predict that they will continue to identify new genes for some time in the future. In order to use these genetic findings to benefit patients, functional studies are essential. The challenge is to explain the individual role of each of these genes in the pathogenesis of complex diseases.

Following the identification of *MAST3* as an IBD gene (4), we were interested in studying its functional effect in the context of the immune system. Since *MAST3* had not been studied extensively, we decided to use a non-targeted approach to examine the effect of perturbing the expression of *MAST3* (overexpression) in a well-characterized cell system. The hypothesis being that overexpression of *MAST3* will have an influence on expression of genes in related biological pathways. We report here our results that highlight a group of *MAST3*-regulated genes heavily involved in immune responses. These genes are functionally linked to NF- κ B as a majority of them are regulated and/or regulators of the NF- κ B pathway. We show that the activity of NF- κ B is correlated temporally with *MAST3*-regulated gene expression implying that *MAST3* acts on the NF- κ B pathway to trigger immune reactions through changes in gene expression. The *MAST3* gene set is upregulated in colonic biopsies of inflamed regions from patients with UC as compared to non-inflamed regions. This suggests that *MAST3* contributes to the dysregulation of the immune response to microbiota underlying IBD through modulation of gene expression.

To examine the *MAST3*-regulated gene set in a clinically relevant context, we studied genome-wide expression in colonic biopsies from control and UC patients. We observed a significant increase in the expression of the *MAST3*-regulated genes in inflamed vs non-inflamed tissues. Since 20% of the genes in this set are encoding proinflammatory cytokines, including the key chemokines CCL20 and IL8, this emphasizes the role of the inflammatory process in the initiation and/or maintenance of the disease state. Interestingly, IL8 is a chemokine that is important in innate immunity and highly regulated by *MAST3*. *IL8* expression has been shown to be increased in both UC and CD patients and IL8 mRNA and protein levels correlate to inflammation severity (14). Mutations in *NOD2*, the first gene associated to CD (15), were shown to regulate the expression of IL8 (16).

In addition, a few of the *MAST3*-regulated genes are involved in the regulation of the NF- κ B pathway which seems to also implicate *MAST3* in a feedback mechanism to prevent uncontrolled inflammation. *NFKBIA* is among the NF- κ B regulators within this gene set. *NFKBIA* encodes protein I κ B α which sequesters the NF- κ B complex in the cytoplasm therefore inhibiting its nuclear localization and transcription factor functions (17). Another of these regulators is *TNFAIP3* encoding the protein A20 which acts on the NF- κ B pathway through interaction with TRAF6 (18). Overexpression of A20 has been shown to block TLR4 activation of NF- κ B (19). MD-2, encoded by gene *LY96*, is the co-receptor to TLR4. The binding of LPS to TLR4 and subsequent NF- κ B activation is dependent on the

formation of the TLR4-MD-2 complex(20). IER3 has been shown to interact with NF- κ B and inhibits its activation, thus interfering with downstream gene expression (21).

Several IBD genes influence immune responses through modulation of the NF- κ B pathway. Notably, a recent study into the effect of the overexpression of IBD associated variant *NOD2*^{L1007finsC} on global gene expression underscores some NF- κ B regulated genes found in the MAST3-regulated gene set(22). *NOD2*^{L1007finsC} was found to upregulate the expression of pro-inflammatory cytokine gene *IL8* and *CXCL2* and of NF- κ B regulator genes *NFKBIA*, *IER3* and *TNFAIP3*. Our combined findings give weight to the hypothesis that the dysregulation of a limited number of biological pathways is involved in the pathogenesis of IBD. *NOD2* is the first identified and among the most systematically replicated CD genes. As opposed to *MAST3*, it is also among the most studied functionally. *NOD2* has been involved in the regulation of immune gene expression and epithelial barrier integrity (23). Here we show that another IBD risk factor, *MAST3*, is modulating the expression of genes and regulating the activity of NF- κ B in a similar way and thus that its variants could translate into similar cellular consequences. It is possible that there is an inflammation gene set that is regulated by several IBD genes such as *NOD2* and *MAST3*. The upregulation of the expression of this inflammation gene set in mucosal gut tissues could trigger the conditions for the development of IBD. In this context of interconnectivity between IBD genes, functional studies that aim to decipher disease-causing pathways are becoming essential companions to genetic studies in the quest to improve our understanding of the molecular mechanisms underlying IBD.

Among MAST3-regulated genes, a few - *TNF*, *TNFRSF9* and *CCL20/CCR6* - are located in IBD associated loci which also underlines the interconnectivity of the IBD genes. The *TNF* gene is on chromosome 6p21 in the major histocompatibility complex (MHC) region and has been associated to CD following a metaanalysis of genome-wide associated studies (24). The *TNF* gene encodes tumor necrosis factor α , a pro-inflammatory cytokine that induces cell proliferation and differentiation and that is found in increased amounts in patients suffering from IBD (25, 26). Biological therapies that target TNF have been used for several years to treat refractory IBD. Notably, the anti-TNF humanized antibody Infliximab has shown efficacy in reducing intestinal inflammation and maintaining remission, preventing fistulae, reducing the need for steroids treatment, reducing hospitalization and need for surgery, and improving quality of life (27–30). The *TNFRSF9* gene is located in a locus recently associated to UC (2). *TNFRSF9* encodes a cell surface transmembrane protein that is involved in cell differentiation and activation (31, 32). *TNFRSF9* has been shown to activate NF- κ B in a TRAF-dependent manner (33). *CCL20*, the most upregulated gene in our overexpression model, has not been associated to IBD. However, it encodes the sole ligand of receptor CCR6 which is encoded by a CD associated gene (24). *CCL20* and *CCR6* are involved in the regulation of mucosal immunity through the recruitment of antigen presenting cells to inflammation sites and homing of dendritic cells and CD4⁺ T cells to the lymphoid tissue of the gut (13). There is much interest in CCR6 because it is the only receptor expressed on every T_H17 cell subsets (34) and T_H17 driven immune responses are hypothesized to be responsible for CD pathogenesis(35).

Our genome-wide expression profiling is a first step into the investigation of the role of MAST3 in the immune response. Several signal transducers acting between TLR4 and NF- κ B are phosphorylated on serine and/or threonine. It is possible that the MAST3 kinase modulates the activity of NF- κ B through the phosphorylation and activation of one of these molecules. Also, MAST3 might physically interact and modulate the pathway through stabilization and chaperoning of a target. MAST2, a protein from the same family, has been shown to regulate NF- κ B through the formation of a complex with TRAF6 (6). Additional functional studies will be needed to pinpoint the precise target of MAST3 in the NF- κ B

pathway. In the meantime, this study reinforces the importance of this pathway in IBD and identifies an IBD risk factor, *MAST3*, as a regulator of NF- κ B activity and immune gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MAST3-regulated genes are involved in critical immune functions

Genes in the MAST3-regulated gene set (genes upregulated by 2-fold or more in *MAST3* overexpressed cells) were classified according to their function recovered from literature searches. The functions of the genes mainly cluster around the activity of NF-κB. Sixty-one percent of the 28 genes have experimentally confirmed NF-κB binding site in their promoter and are regulated by NF-κB. Thirty-two percent regulates the activity of NF-κB. Twenty-one percent are pro-inflammatory cytokines. Other functions include cell adhesion and migration and interferon mediated defense against pathogen invasion and tumor progression. Two genes out of the 28, *CALCB* and *SLC25A24* do not seem to be directly involved in immune functions and were not included in the diagram.

A.

B.

C.

D.

Figure 2. *MAST3* regulates gene expression through NF- κ B

A. The overexpression of *MAST3* increases the activity of NF- κ B. The graph shows the activity of NF- κ B tested in HEK293 cells that overexpress the *MAST3* gene at different times post-transfection (*MAST3* over). The increase in activity is significantly detected after 12h (*P=0.01). B. The knockdown of *MAST3* decreases the activity of NF- κ B. The graph shows the activity of NF- κ B tested in THP1 cells at different stages of differentiation and stimulation following different treatment. The decrease in activity is significantly detected in phorbol 12-myristate 13-acetate (PMA) differentiated cells after 6h of lipopolysaccharide (LPS) stimulation (*P=0.02). C. and D. Expression of *MAST3*-regulated genes *IL8* (C) and *CCL20* (D) post-transfection in cells overexpressing the *MAST3* gene. Expression is normalized to *HPRT1* expression. Error bar represent standard deviation of 3 independent biological replicates. KD *MAST3*: knockdown *MAST3*, KD NT: non target knockdown control.

Table 1 Differentially expressed genes (Fold increase>2) in the *MAST3* overexpression model

Gene	chr	P value	Fold increase	Function
<i>MAST3</i>	19	3.48E-04	42.00	
<i>CCL20</i>	2	1.31E-10	38.13	C, NP
<i>IL8</i>	4	6.09E-06	13.89	C, NP
<i>CD44</i>	11	1.13E-02	6.41	AM, NP
<i>IFIT2</i>	10	1.20E-03	6.23	I
<i>IER3</i>	6	4.05E-04	5.28	RN, NP
<i>NFKBIA</i>	14	4.41E-02	4.98	RN, NP
<i>IL32</i>	16	8.70E-04	4.60	C, NP
<i>ISG15</i>	1	6.82E-04	3.95	I
<i>TNFAIP3</i>	6	5.81E-04	3.83	RN, NP
<i>NFKBIZ</i>	3	8.69E-05	3.74	RN, NP
<i>IFIT1</i>	10	8.40E-06	3.66	I
<i>CD70</i>	19	5.98E-04	3.55	C, RN
<i>TNC</i>	9	3.97E-02	3.49	AM, NP
<i>SLC25A24</i>	1	3.82E-05	3.07	
<i>CXCL2</i>	4	5.62E-03	2.92	C, NP
<i>TNFRSF9</i>	1	1.40E-03	2.81	RN, NP
<i>IFIT3</i>	10	1.78E-04	2.80	I
<i>SGPP2</i>	2	3.89E-03	2.61	NP
<i>TNF</i>	6	2.77E-04	2.55	C, NP
<i>GAL</i>	11	2.53E-04	2.44	NP
<i>CD83</i>	6	5.69E-07	2.41	NP
<i>TNFRSF12A</i>	16	3.84E-03	2.35	RN, AM
<i>PMAIP1</i>	18	1.17E-03	2.28	I
<i>NFKB1</i>	4	2.87E-04	2.26	RN, NP
<i>LY96</i>	8	1.27E-02	2.22	RN
<i>CDK6</i>	7	5.01E-04	2.18	NP
<i>CALCB</i>	11	4.38E-03	2.17	
<i>TMOD1</i>	9	7.18E-05	2.00	AM

AM: cell adhesion and/or migration. chr: chromosome. C: pro-inflammatory cytokine. I: induced by interferons. NP: regulated by NF- κ B. RN: regulator of NF- κ B.

Table 2

Effect of the *MAST3* stable knockdown (KD) on expression of genes in stimulated THP1 cells confirms results from the overexpression model.

Gene	Effect of <i>MAST3</i> KD (% of expression of non target KD control)*	P
<i>IL8</i>	55%	0.01
<i>IER3</i>	41%	0.02
<i>CD70</i>	76%	0.03
<i>CCL20</i>	no detected effect	0.80
<i>ISG15</i>	75%	0.02
<i>LY96</i>	40%	0.01
<i>NFKB1A</i>	92%	0.02
<i>TNF</i>	no detected effect	0.47
<i>TNFAIP3</i>	75%	0.02

-: no significant effect of the KD of *MAST3* on expression.

* : THP1 cells were differentiated using PMA and treated with LPS for 3h (*IER3*, *TNF*) or 24h (other genes).

Table 3

MAST3 stable knockdown (KD) influences the expression of immune genes in stimulated THP1 cells.

Gene	Effect of <i>MAST3</i> KD (% of expression of non target KD control)*	P
IL10	210%	0.003
IL12B	200%	0.03
IL18	220%	0.04
IL1a	60%	0.01
IL1b	70%	0.03
TGFB1	65%	0.03
TRAF6	65%	0.002

* : THP1 cells were differentiated using PMA and treated with LPS for 6h (*IL10*, *IL1b*, *TGFB1*) or 24h (*IL12B*, *IL18*, *IL1A*, *TRAF6*).

Table 4

MAST3-regulated gene set enrichment tests in patient biopsies

	BC vs BPN		BPN vs BPI	
	MAST3-regulated gene set	All genes [*] on microarray	MAST3-regulated gene set	All genes [*] on microarray
P[*] < 0.05	3	7045	10	8498
Total	14	24066	14	24607
%	21.4	29.2	71.4	34.5
Enrichment P	0.825		0.005	

* Probe sets on the Affymetrix chip are used in lieu of genes. Some genes are represented by more than one probe set.

P*= the empirical P after 1000 permutations.

BC=biopsies from healthy control. BPN=biopsies from patients taken from non-inflamed region. BPI=biopsies from patients taken from inflamed region.

Table 5

Comparison of the expression of the MAST3-regulated genes between BPN and BPI

	Exp. BPN	Exp. BPI	P*
<i>CD44</i>	8.01	8.98	0.001
<i>TNC</i>	6.90	8.31	0.017
<i>ISG15</i>	9.02	9.96	0.02
<i>NFKBIZ</i>	10.56	11.74	0.02
<i>IL8</i>	5.89	7.66	0.027
<i>NFKBIA</i>	10.57	11.11	0.061
<i>TNFAIP3</i>	9.47	9.96	0.07
<i>IER3</i>	10.22	10.89	0.137
<i>CCL20</i>	10.33	11.57	0.27
<i>IFTT2</i>	8.12	8.56	0.505
<i>SLC25A24</i>	11.61	11.44	0.609
<i>CD70</i>	6.72	6.82	1
<i>IFTT1</i>	7.81	7.80	1
<i>IL32</i>	9.05	9.09	1

Exp.= mean expression. BPN=biopsies from patients taken from non-inflamed region. BPI=biopsies from patients taken from inflamed region. P*= Empirical P corrected for multiple testing.