The gene encoding the Wiskott-Aldrich syndrome (WAS) protein is transcriptionally repressed following stimulation of peripheral blood mononuclear cells with muramyl dipeptide.

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The nucleotide-binding oligomerization domain protein 2, NOD2, is the pattern recognition receptor for muramyl dipeptide, a component of the cell wall of both gram-positive and gram-negative bacteria (1, 2). Sensing of muramyl dipeptide by NOD2 triggers signal transduction downstream of the RIP2 kinase to transcriptionally activate a diverse innate immune gene expression program (3-5). Single nucleotide variants in NOD2 are the strongest genetic risk factors for developing Crohn's disease (6, 7), an inflammatory bowel disease (8). By mining a published dataset (9), we found that among the genes whose expression changed most significantly in peripheral blood mononuclear cells (PBMC) stimulated with muramyl dipeptide, genome-wide, was the gene encoding the Wiskott-Aldrich syndrome (WAS) protein (10), a regulator of actin polymerization in hematopoietic cells (11). The WAS gene is mutated in patients with Wiskott-Aldrich syndrome (12), a genetic disorder characterized by thrombocytopenia, eczema, immunodeficiency and lymphoid malignancies (13), and whose symptoms include bloody diarrhea (14, 15). WAS RNA message was transcriptionally repressed following exposure of PBMC to muramyl dipeptide. These data link together a gene, that when mutated in humans leads to gastrointestinal symptoms not dissimilar to those found in patients with Crohn's disease, with the ligand for the gene whose product encodes the single most significant genetic variant conferring risk for development of Crohn's disease, and further suggest that the actin cytoskeleton may be a central target of gene expression changes effected by NOD2 pattern recognition of MDP.

Keywords: Wiskott-Aldrich syndrome protein, NOD2, muramyl dipeptide, WAS

1 A hallmark of the innate arm of the immune system is the ability to respond to a
2 molecular pattern associated with danger from a pathogen (pathogen-associated molecular
3 pattern, or PAMP) using a wide variety of cellular machinery known as pattern recognition
4 receptors that detect these patterns and initiate cellular responses in the form of signal
5 transduction (16-18). This is in comparison to the presentation of peptide antigens associated
6 with specific pathogens by the adaptive arm of the immune system, displayed by the major
7 histocompatibility complex proteins MHC-I (19, 20) and MHC-II (21) and recognized by T- and
8 B-cell receptors for cytolytic and humoral responses, respectively (22, 23). PAMPs are
9 “generic” in that they are representative of a variety of different pathogens, with a shared
10 characteristic being that these molecular patterns are not otherwise found in the human body
11 (16). These include flagellin, a component of the flagellate structure used by some bacteria to
12 conduct locomotion, recognized by the Toll-like receptor 5 (TLR5) (24), and cyclic dinucleotides,
13 produced and utilized by bacteria as second messengers in their endogenous signaling
14 systems, recognized by in humans by cGAS and STING (25-27). A component unique to
15 bacteria but shared by both gram-negative and gram-positive bacteria is muramyl dipeptide, a
16 molecular pattern composed of two amino acids, L-alanine and D-isoglutamine, conjugated at
17 the amino-terminus with N-acetyl muramic acid, a carbohydrate moiety (28). This simple
18 molecular structure induces potent signaling after detection by NOD2, the cellular pattern
19 recognition receptor for MDP. The details of MDP recognition by NOD2 have remained
20 controversial but studies have localized the recognition capacity of NOD2 for MDP to its leucine-
21 rich region (LRR) domain (29, 30).

26 Crohn’s disease is an inflammatory bowel disease (8) which causes significantly
27 gastrointestinal tissue damage in the form of ulceration which can penetrate through the
28 mucosa and into the muscular wall and includes the presence of granuloma (31), and a
prominent symptom of Crohn’s disease includes diarrhea which can be bloody (8, 31).

Genome-wide association studies identified three separate single-nucleotide polymorphisms (SNPs) in NOD2 as the strongest risk factors for the development of Crohn's disease (6, 7); further variants were subsequently identified (34). Each of these originally identified SNPs is localized to the leucine-rich region of NOD2, the region of NOD2 important for the pattern recognition of MDP (29, 30). While Crohn's disease has historically been thought of as an autoimmune disease (35-38), there is sufficient evidence to point to underlying immune deficiencies in patients with Crohn's disease (39-44). It has been difficult to translate the effect of NOD2 polymorphisms into functional differences in immune signaling and cytokine production/secretion (45, 46) as this can differ in based on cell type and type of variant the patient possesses (47-50), in experimental settings based on the species studied (e.g., mouse versus human) (46, 49), and based on the manner in which the polymorphisms are modeled (e.g., complete knock-out of the NOD2 locus versus modeling of the human polymorphisms in the endogenous murine NOD2 locus) (32, 51).

In order to understand in an unbiased and systematic fashion changes in the gene expression program of human blood after sensing of muramyl dipeptide, we mined a published microarray dataset (9) containing transcriptome data from the blood of non-affected, control subjects, in the resting state and after stimulation with muramyl dipeptide. We found that the gene encoding the Wiskott-Aldrich syndrome protein, or WAS, was among the genes whose expression changed most significantly following treatment of peripheral blood mononuclear cells with muramyl dipeptide. Levels of WAS mRNA were significantly reduced in PBMC treated with MDP as compared to PBMC in the resting state. Muramyl dipeptide sensing by NOD2, and differential expression of the Wiskott-Aldrich syndrome gene following MDP stimulation of peripheral blood mononuclear cells link pattern recognition, the actin cytoskeleton, and disparate genetic risk factors, NOD2 and WAS, leading to development of gastrointestinal pathology.

Methods

We used dataset GSE68765 (9) for this analysis in conjunction with GEO2R. GSE68765 (9) was generated by Begun et al. using Illumina HumanHT-12 V4.0 expression beadchip technology with n=6 untreated peripheral blood mononuclear cells (PBMC) and n=5 PBMC stimulated with muramyl dipeptide.

The Benjamin and Hochberg method of p-value adjustment was used for ranking of differential expression but raw p-values were used for assessment of statistical significance of global differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used.

A statistical test was performed to evaluate the significance of difference between mRNA expression levels of WAS in resting state PBMC as compared to PBMC treated with muramyl dipeptide using a two-tailed, unpaired t-test with Welch's correction. Only p-values less than 0.05 were considered statistically significant.

Results

We mined published transcriptome data generated by microarray analysis of peripheral blood mononuclear cells, in the resting state and after treatment with the pathogen-associated molecular pattern and NOD2 ligand, muramyl dipeptide (9).

The gene encoding the Wiskott Aldrich syndrome protein (WASp) is differentially expressed in peripheral blood mononuclear cells stimulated with muramyl dipeptide.

We identified a transcript encoded by the Wiskott Aldrich syndrome (WAS) gene as among the genes whose expression was most different in peripheral blood mononuclear cells stimulated with muramyl dipeptide as compared to peripheral blood mononuclear cells in the resting state. When sorting each of the transcripts measured by microarray in peripheral blood

mononuclear cells by the magnitude of change in gene expression with and without muramyl dipeptide stimulation, WAS ranked 84 out of 47323 transcripts. Differential expression of WAS mRNA in PBMC treated with MDP was statistically significant (Table 1; $p=5.7E-06$).

Peripheral blood mononuclear cells treated with muramyl dipeptide express significantly less mRNA for the WASp gene than PBMC in resting state.

We extracted exact mRNA expression values for the WAS transcript we identified as differentially expressed. Peripheral blood mononuclear cells stimulated with muramyl dipeptide expressed decreased quantities of messenger RNA for the Wiskott-Aldrich syndrome protein as compared to peripheral blood mononuclear cells in the resting state. Decreased expression of WAS mRNA by MDP-treated PBMC was statistically significant (Figure 1; $p<0.0001$).

Thus, we found that the gene encoding the Wiskott-Aldrich syndrome protein was differentially expressed following stimulation of peripheral blood mononuclear cells with muramyl dipeptide, with a change in expression greater than 99.8% of the transcriptome. Moreover, we found that stimulation of PBMC with MDP lead to a significant decrease in expression of mRNA encoded by the WAS gene.

Discussion

In this study, we mined published microarray data (9) containing transcriptome data from peripheral blood mononuclear cells in the resting state and with muramyl dipeptide stimulation to identify in an unbiased and systematic fashion the genes whose expression most significantly changed upon pattern recognition of the MDP, the pathogen-associated molecular pattern and ligand for the gene product of the most significant genetic risk factor for the development of Crohn's Disease, NOD2. We found that the Wiskott-Aldrich syndrome protein was among the genes most differentially expressed in peripheral blood mononuclear cells upon treatment with

1 muramyl dipeptide, with a significant decrease in expression of the WAS gene following
2 treatment of PBMC with MDP.

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4 The Wiskott-Aldrich syndrome protein (WASp) is a regulator of actin polymerization in
5 the hematopoietic system, associating with the cytoskeleton through the Actin-related protein
6 Arp2/3 complex (52-54). In addition to its Arp2/3 interaction domain, it possesses five domains
7 including a PH, or pleckstrin homology domain, the GTPase binding domain or CRIB motif, a
8 cofilin homology domain, a WIP, or WASp interactive protein domain, and a proline rich region
9 found in cytoplasmic T-cell kinases such as Itk (55-58). Binding of the Rho-family GTPase
10 Cdc42 to the GTPase binding domain leads to a conformation change in WASp that exposes its
11 C-terminus, enabling interaction with the actin cytoskeleton (10). Significantly, mice null for the
12 Wiskott-Aldrich syndrome protein develop colitis (59). T-cell activation by anti-CD3 ϵ stimulation
13 is compromised in purified cells from these WASp $-/-$ mice (59). Subsequent studies found that
14 WASp is recruited to lipid rafts following T-cell activation through ligation of the T-cell receptor
15 (TCR) and CD28 and that T-cells from patients with Wiskott-Aldrich syndrome possess defects
16 in lipid raft movement to the immunological synapse and fail to adequately proliferate following
17 TCR/CD28 ligation (60). Other blood cells abnormalities found in patients with Wiskott-Aldrich
18 syndrome include deficits in dendritic cell polarization and cell motility, specifically in
19 translocation (61), an absence of podosomes, a structure critical for cell adhesion, in
20 macrophage from patients expressing truncated versions of WASp (62), and compromised
21 monocyte migration in response to the chemokine monocyte chemoattractant protein-1, as well
22 as a rounded shape to the normally polarized F-actin cytoskeleton in monocytes from WAS
23 patients (63). Each of these blood cell abnormalities is tied to abnormal cellular architecture
24 with defects in the cytoskeleton as their molecular basis.

1 These data demonstrate that stimulation of peripheral blood mononuclear cells with the
2 ligand for the gene product of the most significant genetic risk factor for development of Crohn's
3 disease, NOD2, leads to transcriptional repression of a gene, WAS, possessing specific
4 expression in the hematopoietic system, which when itself is mutated in humans leads to a
5 hybrid autoimmunity / immune deficiency (64, 65) with gastrointestinal dysfunction as a
6 prominent symptom. These data reinforce an important role for the hematopoietic compartment
7 in the induction of colitis and inflammatory bowel disease-like pathology, directly demonstrate
8 that changes in expression of the WAS gene, a regulator of the actin cytoskeleton, are a
9 component of the muramyl dipeptide-induced gene expression program, suggest that changes
10 in cellular state and function modulated by the pattern recognition of muramyl dipeptide by
11 NOD2 may involve the cytoskeleton (66, 67) and allude to a common molecular mechanism
12 important for gastrointestinal pathology found in patients with Wiskott-Aldrich syndrome and
13 Crohn's disease involving the actin cytoskeleton.
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Rank	ID	p-value	t	B	Gene	Gene name
84	ILMN_1760027	5.7E-06	-7.6655996	4.33621	WAS	Wiskott-Aldrich syndrome

Table 1: The gene encoding the Wiskott-Aldrich syndrome protein is differentially expressed in peripheral blood mononuclear cells following stimulation with muramyl dipeptide.

The rank of differential expression, probe ID, p-value of differential expression relative to 47323 transcripts globally, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, the gene and gene name and listed in this chart.

Figure 1: Stimulation of peripheral blood mononuclear cells with muramyl dipeptide leads to transcriptional repression of the gene encoding the Wiskott-Aldrich syndrome protein.

The mRNA expression levels of the WAS gene in untreated PBMC and PBMC stimulated with muramyl dipeptide are graphically represented with mean values marked and p-value listed.