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IRF5 Risk Polymorphisms Contribute to Interindividual Variance in Pattern Recognition Receptor-Mediated Cytokine Secretion in Human Monocyte-Derived Cells

Matija Hedl and Clara Abraham

Monocyte-derived cells display highly variable cytokine secretion upon pattern recognition receptor (PRR) stimulation across individuals; such variability likely affects interindividual inflammatory/autoimmune disease susceptibility. To define mechanisms for this heterogeneity, we examined PRR-induced monocyte-derived cell cytokine secretion from a large cohort of healthy individuals. Although cytokine secretion ranged widely among individuals, the magnitude of cytokine induction after individual nucleotide-binding oligomerization domain 2 (Nod2) and TLR2 stimulation (a cohort of 86 individuals) or stimulation of multiple TLRs (a cohort of 77 individuals), either alone or in combination with Nod2, was consistent intr-individually across these stimuli. Nod2 and TLRs signal through IFN regulatory factor 5 (IRF5), and common IRF5 polymorphisms confer risk for autoimmunity. We find that cells from rs2004640 IRF5 risk-associated allele carriers secrete increased cytokines upon individual or synergistic PRR stimulation in a gene dose- and ligand dose-dependent manner in both monocyte-derived dendritic cells and monocyte-derived macrophages. IRF5 expression knockdown in IRF5 risk allele carrier cells significantly decreases PRR-induced cytokines. Moreover, we find that IRF5 knockdown profoundly decreases Nod2-mediated MAPK and NF-κB pathway activation, whereas the PI3K and mammalian target of rapamycin pathways are not impaired. Finally, the IRF5 rs2004640 polymorphism is a major determinant of the variance (r² = 0.53) in Nod2-induced cytokine secretion by monocyte-derived cells from different individuals. We therefore show a profound contribution of a single gene to the variance in interindividual PRR-induced cytokines. The hyperresponsiveness of IRF5 disease-associated polymorphisms to a wide spectrum of microbial triggers has broad implications on global immunological responses, host defenses against pathogens, and inflammatory/autoimmune disease susceptibility. The Journal of Immunology, 2012, 188: 5348–5356.

The interplay between microbial and genetic susceptibility factors is particularly important for development of autoimmune/inflammatory diseases. Interindividual differences in host responses to microbes confer some of the largest risk effects in autoimmunity; pattern recognition receptor (PRR) pathway associations are one such example. For instance, the sensor of bacterial peptidoglycan nucleotide-binding oligomerization domain 2 (Nod2) is associated to Crohn’s disease (1), a subtype of inflammatory bowel disease (IBD). Although IBD has traditionally been associated with dysregulation of responses to bacteria (2), recent studies show that viruses also play a role in modulating intestinal inflammation (3); therefore, polymorphisms in PRR pathways that modulate both bacterial and viral responses may have a particularly significant role in IBD risk. Additional evidence for microbial responses contributing to autoimmune/inflammatory diseases include identification of microbial signature pathways (e.g., type I IFN) (4) and genetic associations in microbial response pathways including IFIH1 in type 1 diabetes mellitus (5) and IRF5 in multiple autoimmune/inflammatory diseases (including systemic lupus erythematosus [SLE], IBD, rheumatoid arthritis, Sjögren’s syndrome, primary biliary cirrhosis, systemic sclerosis, and multiple sclerosis) (6–11). An important consequence of PRR activation by bacterial and viral products is induction of cytokine secretion, and autoimmune/inflammatory diseases are characterized by dysregulated cytokines (1, 12, 13).

In this study we sought to define the nature and etiology of differences in PRR-mediated cytokine induction across a large cohort of healthy individuals. We performed our study in healthy individuals given the increased or dysregulated cytokine production in autoimmune/inflammatory diseases such as IBD and SLE (1, 12, 13), and that immune-modulating medications administered for these diseases can alter cytokine regulation. We and others have found that PRR stimulation of primary human monocyte-derived cells shows large interindividual variability in the magnitude of cytokine induction (14–16). We now establish that this variability is consistent within a given individual over time, in monocyte-derived dendritic cells (MDDC) and monocyte-derived macrophages (MDM), over a wide range of Nod2 and TLR2 ligand concentrations, as well as between distinct cytokines. Importantly, we establish that the rs2004640 disease-associated risk allele in the IRF5 gene region leads to significantly increased individual and synergistic Nod2- and TLR-induced cytokine secretion and is a major contributor to the variance in PRR-induced cytokine secretion across individuals. Significantly, IRF5 knockdown in human MDDC results in decreased PRR-mediated MAPK and NF-κB pathway activation and cytokine secretion. That select IRF5 polymorphisms dramatically modulate Nod2-
and TLR-induced cytokine protein secretion and are a uniquely major determinant of the variance in Nod2-induced cytokine secretion between individuals highlights one mechanism whereby these polymorphisms may contribute to autoimmune/inflammatory diseases.

Materials and Methods

Patient recruitment and genotyping

Informed consent was obtained per protocol approved by the Institutional Review Board at Yale University. We recruited participants with no personal or family history of autoimmune/inflammatory disease, including psoriasis, SLE, rheumatoid arthritis, multiple sclerosis, type I diabetes mellitus, Crohn’s disease, and ulcerative colitis, or a history of HIV. Additional self-reported information collected included gender, race, and age. Our cohort included individuals of self-declared European (88%), African (7%), Asian (2%), or other ancestries (3%). Two separate cohorts of 86 and 77 individuals were recruited for Nod2/TLR2 dose-response studies in MDDC and MDM, and Nod2/TLR synergy studies in MDDC, respectively. We selected 35 individuals to overlap between the two cohorts so as to examine a subset of the responses at two distinct times. Based on this treatment design, MDDC from 128 nonoverlapping individuals were treated with 1 μg/ml muramyl dipeptide (MDP). Twenty-five Crohn’s disease-risk polymorphisms (10, 17–19) were initially selected for analysis based on their location in regions containing genes with known or potential roles in mediating PRR responses. Genotyping was performed by TaqMan SNP genotyping (Applied Biosystems, Foster City, CA) or Sequenom platform (Sequenom, San Diego, CA); individuals were sequenced for the IRF5 CCGGG-indel polymorphism at the Yale University Keck facility.

Primary MDM and MDDC cell culture

Monocytes were purified from human PBMCs by positive CD14 selection (Miltenyi Biotec, Auburn, CA), tested for purity and cultured for 7 d with IL-4 (40 ng/ml) and GM-CSF (40 ng/ml) (R&D Systems, Minneapolis, MN) (for MDDC differentiation) or 10 ng/ml M-CSF (for MDM differentiation).

MDM stimulation

Cultured MDM or MDDC from a cohort of 86 individuals were treated with increasing doses of MDP (Bachem, King of Prussia, PA) or PamCys (Calbiochem, La Jolla, CA) for 24 h. MDDC from a cohort of 77 individuals were treated with a single dose of MDP, PamCys, lipid A (Peptides International, Louisville, KY), flagellin, CL097, CpG, or polyinosinic-polycytidylic acid (Invivogen, San Diego, CA) for 24 h alone or in combination. Following the treatment of the cells, supernatants were frozen and processed in large batches. Supernatants were assayed for TNF-α, IL-8 (BD Biosciences), IL-12p40, and IL-10 (R&D Systems) or IL-1β (Pierce Biotechnology, Rockford, IL) by ELISA.

Constructions and transfection of small interfering RNAs

Scrambled small interfering RNA (siRNA) (300 nM) or siRNA against IRF5 (300 nm; Dharmaco, Lafayette, CO) were transfected into MDDC using Amaxa Nucleofector technology (Amaxa, San Diego, CA). Cells were cultured for an additional 48 h and then treated as indicated.

Phosphoprotein activation

Phosphorylation of MAPKs, Akt, p70-S6K, and IκBα was determined by flow cytometry using Alexa Fluor 647-labeled phospho-ERK, phospho-p38, phospho-JNK, phospho-Akt, phospho-p70-S6K, and phospho-IκBα (Cell Signaling Technology, Danvers, MA) along with isotype controls.

mRNA expression analysis

Following stimulation, total RNA was isolated, reverse transcribed, and quantitative PCR was performed as in Li et al. (20) on the ABI Prism 7000 (Applied Biosystems). Each sample was run in duplicate and normalized to GAPDH. Primers sequences are available upon request.

Statistical analysis

Correlation was calculated using the Spearman rank correlation coefficient. Significance for differences in cytokine protein, cytokine mRNA, and phosphoprotein induction was assessed using a t test. A p value of <0.05 was considered significant. The effects of polymorphisms on PRR-induced cytokine secretion were analyzed by ordinal logistic regression, and a forward stepwise strategy was used to select the polymorphisms remaining in the final model. As the pseudo-r² obtained from ordinal logistic regression cannot be directly interpreted as a measure of percentage of variance, we performed linear regression applying an additive model between the three genotypes and used r² to determine interindividual variance in cytokine secretion. Haplotype phasing was performed utilizing PLINK package version 1.07 (21).

Results

Nod2 stimulation results in highly variable cytokine expression across individuals

To define the spectrum of interindividual differences in PRR responses in primary monocyte-derived cells we used well-powered cohorts of healthy individuals. Given the prominent role of Nod2 in host responses to microbes and Crohn’s disease susceptibility (1), we examined the range and magnitude of cytokine induction to Nod2 stimulation by its ligand MDP (22, 23) in MDDC. MDDC phenotype was verified by absence of B cell and T cell markers (Supplemental Fig. 1A), upregulation of the surface markers HLA-DR, CD1c, CD1d, CD11b, CD11c, CD33, CD40, CD45RO, CD54, CD80, CD83, and CD86, and downregulation of CD14 relative to monocytes (Supplemental Fig. 1B, 1C). We stimulated cells from 128 healthy controls with 1 μg/ml MDP for 24 h and measured TNF-α secretion. Unstimulated cells show very low baseline cytokine secretion, and MDP stimulation induced a wide range of TNF-α secretion between individuals, ranging from no increase to 1440-fold increase over no treatment (Fig 1A). To better assess the response distribution, we reanalyzed the values using log2 transformation (Fig 1B), and we use log2-transformed data in our subsequent figures. Interestingly, cytokine secretion levels cluster into two groups: a group with very low TNF-α secretion to MDP stimulation, and a group that shows a normalized distribution over moderate to high TNF-α secretion levels (Fig 1B). Similar to our results, prior studies using monocytes identify low and high responders to TLR stimuli (14–16). We found poor correlation between the magnitude of TNF-α induction and age (data not shown). Whole blood studies observed decreased cytokines upon TLR stimulation when specifically comparing separate cohorts of older (>65 y) to younger (21–32 y) individuals (15). The lack of age to cytokine level correlation in our study is likely due to the low number of older individuals.

Magnitude of cytokine secretion shows intra-individual correlation across multiple cytokines and different cell types

Importantly, upon Nod2 stimulation the magnitude of induction for TNF-α and other proinflammatory cytokines, including IL-12p40 (Fig. 1C, 1D), IL-8, and IL-1β (Fig. 1D), significantly correlates within each individual. These cytokines were chosen because they are upregulated in a number of autoimmune/inflammatory diseases, including Crohn’s disease (2, 24), and they are secreted upon MDP stimulation of monocyte-derived cells (14, 25–27). Because anti-inflammatory cytokines suppress proinflammatory cytokines, we questioned whether these two cytokine types inversely correlate upon Nod2 stimulation. Interestingly, we find that IL-10 induction levels are similar to those of proinflammatory cytokines in each individual (Fig. 1D). To assess whether results were similar in other cell types, we examined MDM from a subset of 86 individuals and found excellent correlation in the Nod2-induced TNF-α levels secreted by MDM and MDDC from the same individual (Fig. 1E). Importantly, we found significant intra-individual correlation in MDP-induced cytokine secretion in MDDC isolated upon repeated blood draws (Fig. 1F, 1G). Taken together, Nod2 stimulation induces differential intra-individual cytokine secretion, and the magnitude of induction of different cytokines in different monocyte-derived cells and over time is highly correlated within each individual.
The magnitude of cytokine secretion shows correlation across Nod2 and TLR2 ligand doses

We next questioned whether higher MDP doses abolish the interindividual differences in secreted cytokine levels upon Nod2 stimulation. We therefore stimulated MDDC from a cohort of 86 individuals with MDP doses of 1, 10, and 100 μg/ml MDP. Although there is an MDP dose-dependent induction of TNF-α (Fig. 2A), we observe excellent correlation in the magnitude of cytokine secretion between the MDP doses within each individual (Fig. 2B). Therefore, individuals that cluster in the lower range of cytokine secretion with low MDP dose stimulation also cluster in the lower range of cytokine secretion upon higher MDP dose stimulations.

The Gram-positive bacterial cell wall contains both the Nod2 ligand peptidoglycan and the TLR2 ligand lipotechoic acid such that Gram-positive infection activates both receptors. Therefore, we investigated PamCys-mediated TLR2 stimulation of MDDC. Similar to Nod2 stimulation, we observe interindividual variability upon TLR2 stimulation of MDDC with a PamCys dose-dependent induction of TNF-α (Fig. 2C). Moreover, there was excellent correlation in the degree of TNF-α secretion between the PamCys doses within each individual (Fig. 2D). Importantly, we observe significant correlation in TNF-α induction levels between Nod2 and TLR2 stimulation in each individual (r = 0.86) (Fig. 2E). Given this striking correlation, we considered a genetic contribution to the interindividual variability in cytokine secretion in pathways downstream of PRRs.

A polymorphism in the IRF5 gene shows strong association with interindividual variation in TNF-α production

We assessed a subset of Crohn’s disease-associated polymorphisms in regions that included genes likely to regulate PRR signaling with respect to cytokine induction. Because allele frequencies vary between ancestry groups, we focused this analysis on MDDC from our most represented ancestry within the overall cohort, 111 individuals of European ancestry. We used regression to examine TNF-α secretion by MDDC upon stimulation with 1 μg/ml MDP. Of the 25 polymorphisms examined, we observed highly significant evidence for the effects of only the association examined within the IRF5 region, rs2004640 (uncorrected p value = 3.7 × 10^{-13}, corrected p value = 9.2 × 10^{-13}) (Table I). Importantly, the r^2 or variance explained by the IRF5 rs2004640 genotype on TNF-α secretion is 0.53. This indicates that the IRF5 rs2004640 genotype is a major genetic factor modulating the interindividual variance in host cytokine induction upon Nod2 stimulation. We therefore decided to more closely examine the relationship of the IRF5 disease-associated polymorphisms with PRR-induced cytokine secretion.

The disease-associated rs2004640 and CGGGG-indel IRF5 risk alleles show significantly increased cytokine secretion following Nod2 or TLR2 stimulation

Previous studies found that the commonly distributed rs2004640 T allele is associated with multiple autoimmune diseases (6–11). The rs2004640 T allele, located at the intron/exon border of exon 1B in the IRF5 gene, is proposed to create a splice site resulting in expression of the variant exon 1B IRF5 mRNA transcript and increased IRF5 mRNA expression (8, 9). IRF5 disease associations have been shown in multiple ancestries, including African American, Chinese Han, Japanese, and European ancestries (7, 9, 28, 29), such that we initially included individuals of all ancestries collected in our study to examine the functional immunological outcomes of this polymorphism. We find that MDDC (Fig. 3A) and MDM (Fig. 4) from rs2004640 T risk allele carriers signifi-
cantly increase TNF-α protein induction upon MDP and Pam3Cys stimulation at 1 μg/ml. We observe a dramatic gene dose effect, with cells from GT heterozygotes demonstrating cytokine secretion intermediate between TT and GG homozygotes (Figs. 3A, 4). We next asked whether increasing the doses of MDP and Pam3Cys could overcome the wide differential in TNF-α secretion between different genotypes. Although the greatest differential between the rs2004640 alleles was observed at low MDP and Pam3Cys doses, a significant gene dose effect remained even at the highest PRR ligand concentrations examined (Figs. 3A, 4). Importantly, at low ligand doses, cells from individuals homozygous for the protective GG allele secreted negligible cytokine amounts in MDDC (Fig. 3A) and MDM (Fig. 4). Note that untreated cells secrete negligible levels of cytokines, and the effects of the rs2004640 genotype on cytokine secretion are observed specifically upon PRR stimulation of MDDC (Supplemental Fig. 2A). Reanalysis of the correlation between MDDC (Supplemental Fig. 2A) and the correlation of the fold TNF-α induction by MDDC shows correlation across Nod2 and TLR2 ligand doses. Human MDDC (expressed as fold increase in TNF-α over untreated cells (log2-transformed)) upon Nod2 or TLR2 stimulation compared with the cytokine levels of untreated cells ± SEM. (B and D) Spearman correlation coefficients of induction of TNF-α (log2 transformed) across indicated MDP and Pam3Cys doses. ††p < 1 × 10⁻⁵. (E) Correlation of the fold TNF-α induction comparing 1 μg/ml MDP and 1 μg/ml Pam3Cys stimulation with Spearman correlation coefficient indicated. Gray dot (indicated by arrow) represents 27 clustered individuals who showed minimal cytokine response following stimulation. r, Spearman coefficient.

Table I. Contribution of Crohn’s disease-associated polymorphisms to Nod2-induced cytokine secretion in MDDC

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chr.</th>
<th>Risk Allele</th>
<th>Frequency in European Controls</th>
<th>Observed Allele Frequency</th>
<th>Minor Allele Homozygote (n)</th>
<th>Major Allele Homozygote (n)</th>
<th>Corrected p Value</th>
<th>r²</th>
<th>Positional Candidate Gene of Interest</th>
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<td>rs2476601</td>
<td>1</td>
<td>p13</td>
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<td>96</td>
<td>13</td>
<td>&gt;0.05</td>
<td>4.00 × 10⁻³</td>
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<td>0.814–0.833</td>
<td>0.860</td>
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<td>25</td>
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<td>2p16</td>
<td>T</td>
<td>0.412–0.432</td>
<td>0.437</td>
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<td>59</td>
<td>19</td>
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<td>T</td>
<td>0.786–0.825</td>
<td>0.766</td>
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<td>42</td>
<td>5</td>
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<td>1.43 × 10⁻²</td>
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<td>T</td>
<td>0.042–0.044</td>
<td>0.081</td>
<td>93</td>
<td>18</td>
<td>0</td>
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<td>1.96 × 10⁻⁴</td>
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<td>8q5</td>
<td>T</td>
<td>0.592–0.617</td>
<td>0.536</td>
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<td>9q15</td>
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<td>0.620–0.690</td>
<td>0.649</td>
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<td>50</td>
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<td>0.662</td>
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<td>45</td>
<td>15</td>
<td>&gt;0.05</td>
<td>4.43 × 10⁻³</td>
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<td>17q22</td>
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<td>0.468</td>
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<td>29</td>
<td>9.2 × 10⁻¹¹</td>
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<td>0.492–0.518</td>
<td>0.550</td>
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<td>C</td>
<td>0.230–0.392</td>
<td>0.306</td>
<td>52</td>
<td>50</td>
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<td>rs904739</td>
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<td>0.671</td>
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<td>0.058</td>
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<td>0.045</td>
<td>101</td>
<td>10</td>
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<td>6.00 × 10⁻³</td>
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<tr>
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<td>C</td>
<td>0.043–0.150</td>
<td>0.049</td>
<td>100</td>
<td>11</td>
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<td>&gt;0.05</td>
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<td>insC</td>
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<td>0.131</td>
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<td>rs740495</td>
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<td>0.233–0.261</td>
<td>0.243</td>
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<td>11</td>
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<td>rs2413583</td>
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<td>0.865</td>
<td>82</td>
<td>28</td>
<td>1</td>
<td>&gt;0.05</td>
<td>3.28 × 10⁻⁴</td>
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Regression analysis was conducted to examine potential PRR response-regulating genes associated to Crohn’s disease for the effects on TNF-α secretion upon 1 μg/ml MDP stimulation of MDDC derived from 111 European ancestry healthy controls. The p values were adjusted by Bonferroni correction for multiple comparisons. Our cohort represents normal healthy controls. Therefore, some disease-associated or less frequent polymorphisms may not be well represented. For this reason, polymorphisms in genes such as Nod2 known to regulate cytokines upon MDP stimulation (22, 23), may not be adequately powered in this cohort to detect differences, particularly when the effects are seen mainly in minor allele homozygote carriers.

*The risk allele is for independent Crohn’s disease-associated polymorphisms (or single nucleotide polymorphisms in LD) (6, 17).

Chr., Chromosome; Het., heterozygotes; Homoz., homozygotes.
The cytokine responses from Fig. 2 were stratified based on the genotype of the individuals for rs2004640 (A) or CGGGG-indel (B) in IRF5. The data are represented as fold TNF-α induction (log2 transformed) upon dose-dependent Nod2 (left) or TLR2 (right) stimulation stratified on the IRF5 genotype ± SEM. **p < 0.01, ***p < 0.001, †p < 1 × 10^{-4}, ††p < 1 × 10^{-5}.

FIGURE 4. The rs2004640 IRF5 T risk allele is associated with increased TNF-α induction upon Nod2 or TLR2 stimulation of primary human MDM. The cytokine responses from MDM derived from the same induction (log2 transformed) upon dose-dependent Nod2 (left) or TLR2 (right) stimulation stratified on rs2004640 IRF5 genotype ± SEM. **p < 0.01, ***p < 0.001, †p < 1 × 10^{-4}, ††p < 1 × 10^{-5}.

FIGURE 3. The disease-associated rs2004640 and CGGGG-indel IRF5 risk alleles result in increased TNF-α secretion upon Nod2 or TLR2 stimulation of primary human MDDC. The cytokine responses from Fig. 2 were stratified based on the genotype of the individuals for rs2004640 (A) or CGGGG-indel (B) in IRF5. The data are represented as fold TNF-α induction (log2 transformed) upon dose-dependent Nod2 (left) or TLR2 (right) stimulation stratified on the IRF5 genotype ± SEM. **p < 0.01, ***p < 0.001, †p < 1 × 10^{-4}, ††p < 1 × 10^{-5}.

IRF5 knockdown significantly reduces PRR-mediated cytokine induction from carriers of high risk IRF5 variants

IRF5 deficiency results in decreased cytokines upon stimulation with \( \alpha \)-glycolyl-MDP (30) and TLR ligands (31, 32). To examine whether the production of cytokines in human MDDC is directly regulated by IRF5, we silenced IRF5 (Fig. 5A) in individuals that were homozygous for the rs2004640 T allele and CGGGG-insertion allele (high cytokine producers) using siRNA. We observe that decreasing IRF5 expression in cells from IRF5 risk allele carriers results in significantly attenuated RNA (Fig. 5B) and protein (Fig. 5C) expression of TNF-α, IL-12p40, IL-8, IL-1β, and IL-10 upon Nod2 or TLR2 stimulation, indicating that IRF5 is crucial for modulating cytokine responses through these receptors.

IRF5 knockdown significantly reduces MDP-mediated MAPK and NF-κB activation in MDDC

We next questioned how IRF5 regulates pathways leading to cytokine induction. MAPK and NF-κB participate in cytokine induction downstream of PRRs (33), but interestingly the activation of these pathways was not affected in B cells from IRF5-deficient mice upon PRR stimulation (31). However, to our knowledge, how IRF5 regulates these and other pathways in dendritic cells, including primary human MDDC, has not been studied. We therefore used siRNA to IRF5 to investigate the consequences of decreased IRF5 expression on Nod2-initiated signaling pathways in primary human MDDC. We find that in contrast to the mouse B cell studies, IRF5 silencing upon Nod2 stimulation of MDDC resulted in significant attenuation of ERK, p38, and JNK activation (Fig. 6A). IRF5 knockdown cells also showed significantly reduced NF-κB pathway activation upon Nod2 stimulation as evidenced by decreased IkB phosphorylation (Fig. 6B). We and others have found that PI3K and one of its downstream targets, mammalian target of rapamycin (mTOR), also regulate Nod2-mediated cytokine induction (25, 34), such that we examined the effect of IRF5 silencing on these pathways. We find that neither the activation of the PI3K substrate Akt nor of the mTOR substrate p70-S6K was significantly attenuated upon IRF5 knockdown in MDDC (Fig. 6C). Taken together, we find that IRF5 is necessary for optimal Nod2-mediated activation of both the MAPK and NF-κB pathways and that IRF5 knockdown in high-risk IRF5 variant-carrier cells decreases the elevated PRR-mediated cytokine induction observed in these cells.

Disease-risk IRF5 polymorphisms show decreased cytokine production upon individual or synergistic stimulation of a broad range of PRRs

To determine whether disease-associated IRF5 polymorphisms affect cytokine secretion by a broad range of PRRs, we isolated MDDC from a cohort of 77 individuals and examined cytokine secretion after stimulation with various PRR ligands. We selected PRR ligand doses that are commonly used and that result in a similar range of cytokine induction. We observed a high degree
of intraindividual correlation across Nod2, TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 ligand stimulation (Fig. 7A). Therefore, when MDDC from a given individual produce high levels of cytokines in response to stimulation of one PRR, they similarly do so in response to stimulation of other PRRs. As low doses of Nod2 and TLR ligands synergize to induce more robust cytokine induction (20, 27), the synergy of Nod2 with each of the above PRRs was examined. We observed high intraindividual correlation in levels of cytokine secretion upon stimulation of MDDC with MDP combined with each of the individual TLR ligands examined (Fig. 7B). Finally, stratification on rs2004640 (Fig. 7C) and CGGGG-indel (Fig. 7D) IRF5 polymorphisms revealed that the risk allele carriers secrete significantly higher levels of TNF-α upon stimulation with TLR2, TLR4, TLR5, TLR7, and TLR9 alone (Fig. 7C, 7D, left) or in combination (Fig. 7C, 7D, right) with Nod2, indicating that these IRF5 polymorphisms affect TNF-α secretion by a broad range of PRRs. Similar results were observed for IL-12p40, IL-8, IL-1β, and IL-10. Moreover, similar results were observed when analyzing European ancestry-only individuals (Supplemental Fig. 3B). To corroborate our results, we stratified our data on two additional IRF5 disease-associated polymorphisms, rs7808907 and rs4728142, and found similar results with the disease-risk alleles for these IRF5 polymorphisms (Supplemental Fig. 4A–D).

#### FIGURE 5. IRF5 knockdown reduces cytokine mRNA expression and protein secretion following Nod2 or TLR2 stimulation. MDDC from individuals homozygous for both IRF5 rs2004640 T allele and CGGGG-insertion allele (n = 8) (high cytokine producer cohort) were transfection with scrambled or IRF5 siRNA. (A) IRF5 mRNA expression was assessed 48 h after transfection to ensure efficacy of knockdown. The data are represented as the fold mRNA expression compared with cells transfected with scrambled siRNA (represented by the dotted line at 1) ± SEM. Forty-eight hours after transfection cells were treated with 1 µg/ml MDP or 1 µg/ml Pam3Cys for (B) 4 h or (C) 24 h. The data are represented as the fold (B) mRNA expression or (C) protein expression (log2 transformed) of TNF-α, IL-12p40, IL-8, IL-1β, and IL-10 compared with untreated cells transfected with scrambled siRNA ± SEM. **p < 0.01, ***p < 0.001, ‘p < 1 × 10−4. A, Change; M, MDP; P, Pam3Cys; scr, scrambled siRNA.

#### FIGURE 6. IRF5 knockdown reduces MAPK and NF-kB pathway activation following Nod2 stimulation. (A–C) MDDC were transfected with scrambled or IRF5 siRNA and stimulated with 100 µg/ml MDP for 10 min. Left, Shown are representative flow cytometry plots with mean fluorescence intensity values as indicated for (A) phospho-MAPK (n = 6–8), (B) phospho-IκBα (n = 6), or (C) phospho-Akt and phospho-p70S6K (n = 11–14). Shaded region indicates untreated cells, solid dark line indicates scrambled siRNA-transfected cells treated with MDP, and dotted line indicates IRF5 siRNA-transfected cells treated with MDP. Right, Summarized data are represented as the fold phospho-protein induction normalized to untreated cells (represented by the dotted line at 1) ± SEM. *p < 0.05, **p < 0.01, †p < 1 × 10−4. scr, Scrambled siRNA.

**The IRF5 rs2004640 polymorphism is a major contributor to interindividual variance of cytokine expression**

The four IRF5 markers examined in our cohort are contained in the same haplotype block and are in strong linkage disequilibrium (Supplemental Fig. 4E), similar to observations in other studies (9, 10) and the International HapMAP CEU (European ancestry) data. To more thoroughly analyze the effects of IRF5 polymorphisms, we increased the density of mapping in the IRF5 region to include a total of 20 markers that have been assessed in prior studies (6–11, 17, 35, 36) (Supplemental Fig. 4E). To assess the relative importance of the 20 IRF5 variants in the observed interindividual cytokine variation, we analyzed each of the variants individually by regression with respect to their effect on TNF-α secretion. We found that the rs2004640 SNP had the most significant effect on the magnitude and interindividual variance of TNF-α secretion (Table II). We next questioned whether the 20 IRF5 polymorphisms, or a subset of these polymorphisms, in combination led to an increased contribution to cytokine variance.
relative to the contribution of the single polymorphisms. Using a forward stepwise regression model on all 20 polymorphisms, we found that rs2004640 fully accounts for the *IRF5* contributions to TNF-α secretion upon 1 μg/ml MDP stimulation of MDDC. Taken together, *IRF5* polymorphisms are a major determinant of the interindividual variance in cytokine expression.

**Figure 7.** Cytokine secretion upon PRR stimulation and Nod2/TLR synergistic stimulation of primary human MDDC shows excellent interindividual correlation, and the disease-associated rs2004640 and CGGGG-indel *IRF5* risk alleles result in increased TNF-α secretion upon stimulation of multiple PRRs. Human MDDC (n = 77) were stimulated with 1 μg/ml MDP, 1 μg/ml Pam3Cys, 0.1 μg/ml polyinosinic-polycytidylic acid, 0.01 μg/ml lipid A, 0.5 ng/ml flagellin, 0.1 μg/ml CL097, or 0.1 μg/ml CpG DNA for 24 h alone (A) or in combination (B). Supernatants were assayed for TNF-α. (A and B) Correlation in fold TNF-α induction (log2 transformed) between PRR stimulation alone or in combination as calculated by Spearman correlation coefficients. (C and D) The cytokine responses were stratified based on the genotype of the individuals for rs2004640 (C) or CGGGG-indel (D) in *IRF5*. The data are represented as fold TNF-α induction (log2 transformed) upon individual (left) and combined (right) Nod2 and TLR stimulation stratified on the *IRF5* genotype ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, †p < 1 × 10⁻⁴, ††p < 1 × 10⁻⁵. r, Spearman coefficient.

**Table II.** Analysis of effects of multiple polymorphisms in the *IRF5* region on MDP-induced TNF-α secretion

<table>
<thead>
<tr>
<th>IRF5 SNP</th>
<th>Expected Minor Allele Frequency</th>
<th>Observed Minor Allele Frequency</th>
<th>Major Allele Homoz. (n)</th>
<th>Het. (n)</th>
<th>Minor Allele Homoz. (n)</th>
<th>p Value</th>
<th>r²</th>
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Regression analysis was conducted on 20 markers in the *IRF5* region individually in European ancestry individuals (n = 111) to examine effects on TNF-α secretion upon 1 μg/ml MDP stimulation of MDDC.

As reported in dbSNP version 135 (October 12, 2011) and Refs. 8–10.

Het., Heterozygotes; Homoz., homozygotes.
**Discussion**

The dramatic effects of IRF5 genotypes on PRR responses observed in this study reflect several unique features of IRF5. First, although numerous genetic loci have demonstrated associations across multiple autoimmune disorders, IRF5, together with the PTPN22 and IL23R polymorphisms, confers uniquely high disease odds ratios (6–11). Second, IRF5 polymorphisms are unique in their effects on gene expression; in a genome-wide expression quantitative trait loci screen, cis-acting IRF5 polymorphisms demonstrated one of the most significant correlations to mRNA expression in the entire transcriptome (37). Finally, the frequencies of both the risk T and the nonrisk G allele of IRF5 rs2004640 range from 0.44 to 0.56 (8–10); therefore, the distinct TT, TG, and GG rs2004640 genotypes are commonly distributed throughout the population. This common distribution, combined with the dramatic effect of rs2004640 on PRR-induced cytokines in MDDC in a gene-dose-dependent manner, accounts for the marked contribution of IRF5 rs2004640 to interindividual variance in cytokine secretion. Taken together, utilizing monocyte-derived cells from a large cohort of individuals, we demonstrate that rs2004640, a common disease-associated polymorphism in IRF5, confers increased responsiveness to a broad array of PRRs and is a major genetic contributor to the variation in PRR-induced cytokine secretion observed between individuals.

Although the IRFs have generally been associated with viral responses and secretion of type I IFN, IRF5 contributes to induction of additional cytokines by other microbial ligands (31, 32), implicating IRF5 in responses to multiple classes of pathogens in human and mouse cells. PRR stimulation results in IRF5 phosphorylation and translocation into the nucleus (38). In the nucleus, IRF5 binds to promoters of proinflammatory cytokine genes, thereby inducing their expression (31, 32, 38). Consistent with its contributions to cytokine secretion from innate cells, IRF5 influences subsequent Th1 and Th17 responses (32) and assists in responses to and/or clearance of various pathogens (30, 39, 40). At the same time, IRF5 deficiency leads to decreased cytokines and amelioration of disease progression in a mouse model of SLE (41).

A recent study identifies an IRF5 haplotype in SLE patients that is associated with higher anti-dsDNA and anti-Ro Abs and increased serum IFN-α (35). This haplotype, consisting of rs2004640, rs3807306, rs10488631, and rs2280714, accounts for four distinct mechanisms of regulating IRF5 (9, 35). Although stratifying our results on this haplotype showed a highly significant contribution of the previously implicated (35) TATA and TACA (rs2004640, rs3807306, rs10488631, and rs2280714) haplotypes (p value = 5.64 × 10−12) to MDP-induced TNF-α secretion, these results did not improve on the rs2004640 genotype alone (Table II).

In our study we also show that lowering IRF5 expression in human MDDCs from IRF5-risk allele carriers (high cytokine producers) dramatically decreases secretion of multiple cytokines following Nod2 and TLR2 stimulation (Fig. 5C). Moreover, we determine that decreased IRF5 expression in MDDC results in decreased Nod2-mediated MAPK and NF-κB pathway activation, whereas PI3K and mTOR pathway activation was not significantly impaired (Fig. 6). In contrast to our findings, a previous study in B cells from IRF5-deficient mice examining MAPK activation did not demonstrate a defect in this pathway upon PRR stimulation relative to WT B cells (31). This discrepancy highlights fundamental functional differences between mouse B cells and our studies in primary human MDDC, as well as consequences to new pathways examined.

Polymorphisms in other genes have been associated with modifying cytokine secretion upon stimulation of selected PRRs (1, 42). However, we find that the commonly distributed rs2004640 IRF5 risk-allele variant has the most profound influence on the variance in interindividual PRR-induced cytokine secretion among not only the polymorphisms we examined (Table I), but, to our knowledge, among the other common disease risk polymorphisms reported to date. The fine-tuning of cytokine secretion following infection likely significantly influences susceptibility to both infection and autoimmune/inflammatory disease. The highly correlated hyperresponsiveness of PRR-induced cytokines and IRF5 disease-associated polymorphisms implicates a wide spectrum of microbial triggers in autoimmune disease pathogenesis and has broad implications in global immunological responses, host defenses against pathogens, and disease susceptibility.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


**Supplementary Figure 1. MDDC surface markers.** Freshly isolated monocytes or MDDC (n=8) were stained for the indicated surface markers. (A) Shown are representative flow cytometry plots indicating the absence of CD3 (T-cell marker) and CD19 (B-cell marker) on isolated monocytes, and presence of CD11c expression. PBMCs serve as a positive control for these markers. Percentages are indicated in the quadrants. (B) Shown are representative flow cytometry histogram plots of markers regulated on MDDC relative to the parent monocyte. Shaded peak, isotype control; dotted line, monocytes; solid line dark, MDDC. (C) Shown is a table with mean fluorescence intensity (MFI) values indicating the per cell expression level and a table with percentages of cells expressing surface markers + SEM. Significance in upregulation/downregulation of markers from monocyte to MDDC is shown. MDDC phenotype is characterized by upregulation of surface markers HLA-DR, CD1c, CD1d, CD11b, CD11c, CD33, CD40, CD45RO, CD54, CD80, CD83 and CD86, and downregulation of macrophage marker CD14.

**Supplementary Figure 2. The rs2004640 IRF5 T risk allele is associated with increased cytokine induction upon Nod2 stimulation of primary human MDDC and MDM.** (A) Human MDDC (n=86) were left untreated or stimulated for 24 h with 1 μg/ml of MDP (M). Absolute levels of TNF-α secretion are shown stratified on rs2004640 IRF5 genotype. (B) Data from Figure 1E are represented using blue dots (rs2004640 IRF5 GG carriers), pink dots (rs2004640 IRF5 GT carriers) or green dots (rs2004640 IRF5 TT carriers). Clustered individuals who showed minimal cytokine response following Nod2 stimulation and are overlying one another graphically are
depicted with a solid circle/pie chart (indicated by arrow) and represent 31 (22 GG, 8 GT, 1 TT) carriers. ***, p<0.001; †, p<1x10^{-4}; ††, p<1x10^{-5}. r, Spearman rho coefficient.

Supplementary Figure 3. The rs2004640 IRF5 T risk allele is associated with increased TNF-α induction upon Nod2 or TLR stimulation of primary human MDDC in European ancestry individuals. Cytokine secretion from MDDC derived from the European ancestry only individuals within the overall cohort from (A) Figure 3 (n=77) or (B) from Figure 7 (n=70), was stratified by rs2004640 IRF5 genotype. The data are represented as fold TNF-α induction (log2 transformed) upon (A) dose-dependent Nod2 (left) or TLR2 (right) stimulation, or (B) upon individual (left) or combined (right) Nod2 and TLR stimulation, stratified on the IRF5 genotype + SEM. *, p<0.05; **, p<0.01; ***, p<0.001; †, p<1x10^{-4}; ††, p<1x10^{-5}.

Supplementary Figure 4. Multiple polymorphisms in the IRF5 region modify TNF-α secretion upon Nod2 or TLR stimulation of primary human MDDC. PRR-induced cytokine secretion from Figure 2 and Figure 7 were stratified based on the genotype of the individuals for the rs7808907 (A, B) or rs4728142 (C, D) IRF5 alleles. The data are represented as fold TNF-α induction (log2 transformed) upon (A, C) dose-dependent Nod2 (left) or TLR2 (right) stimulation as per Figure 2, or (B, D) upon individual (left) and combined (right) Nod2 and TLR stimulation as per Figure 7 stratified on the IRF5 genotype + SEM. (E) Shown is a pairwise r^2 plot for our European ancestry cohort for twenty markers in the IRF5 region. The intensity of the shading is proportional to r^2. *, p<0.05; **, p<0.01; ***, p<0.001; †, p<1x10^{-4}; ††, p<1x10^{-5}.
**Supplementary Figure 2**

**A**

![Graph A: TNF-α (pg/ml) over untreated cells (log2) vs Treatment.](image)

**B**

![Graph B: Fold increase in TNF-α over untreated cells (log2) vs Fold increase in TNF-α over untreated cells (log2).](image)

- **Supplementary Figure 2: MDDC and MDM**

  - **Figure A**: Bar graph showing the fold increase in TNF-α (log2) over untreated cells. The x-axis represents different treatments, and the y-axis represents TNF-α levels (log2). The bars are color-coded as follows: GG (n=22), GT (n=30), and TT (n=34).

  - **Figure B**: Scatter plot with a trend line indicating the correlation between fold increase in TNF-α and fold increase in TNF-α over untreated cells (log2). The correlation coefficient (r) is 0.91.
Supplementary Figure 3

A

European ancestry individuals

Fold increase in TNF-α over untreated cells (log2)

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B

Fold increase in TNF-α over untreated cells (log2)

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Fold increase in TNF-α over untreated cells (log2)

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Supplementary Figure 4

A

rs7808907

B

rs4728142

C

rs4728142

D

rs4728142

E