Enhanced interferon regulatory factor 3 binding to the interleukin-23p19 promoter correlates with enhanced interleukin-23 expression in systemic lupus erythematosus.

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Title: Enhanced interferon regulatory factor 3 binding to the IL-23p19 promoter correlates with enhanced IL-23 expression in systemic lupus erythematosus.

Running Title: IRF3-IL-23p19 promoter interaction in SLE.

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Conflict of Interest Statement

The authors have no financial conflict of interest with the work and results presented herein.
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Objectives: To examine the role of interferon regulatory factor 3 (IRF3) in the regulation of interleukin-23 (IL-23) production in the autoimmune condition systemic lupus erythematosus (SLE).

Methods: Bone marrow derived macrophages (BMDMs) were isolated from both wild type and IRF3−/− C57BL/6 mice. These cells were stimulated with the TLR3 agonist PolyI:C and IL-23p19 cytokine levels analyzed by enzyme-linked immunosorbent assay. IRF3 binding to the IL-23p19 gene promoter region in monocytes from patients with SLE and healthy control subjects was analyzed by chromatin immunoprecipitation (ChIP). Luciferase reporter gene assays were performed to identify key drivers of IL-23p19 promoter activity. TANK-binding kinase 1 (TBK1) protein levels were determined by Western blotting.

Results: Chromatin immunoprecipitation assays have demonstrated that IRF3 is stably bound to the human IL-23p19 promoter in monocytes, an association which increases following TLR3 stimulation. Significantly, SLE patients demonstrate increased levels of IRF3 bound to the IL-23p19 promoter compared to controls which correlated with enhanced IL-23p19 production in these cells. Investigations into TLR3-driven responses in monocytes derived from SLE patients revealed that the kinase TBK-1, which is critical for regulating IRF3 activity, is hyperactivated both in resting and TLR3 stimulated cells.

Conclusions: Our results demonstrate for the first time that SLE patients display enhanced IL-23p19 expression as a result of hyperactivation of TBK-1 resulting in increased binding of IRF3 to the promoter, providing novel insights into the molecular pathogenesis of SLE and the potential role for TLR3 in driving this response.
Interleukin-23 (IL-23) is a novel member of the IL-12 family of cytokines and is expressed predominantly by monocytes, macrophages and dendritic cells (DCs) in response to a variety of Toll-like receptors (TLRs). This cytokine plays a pivotal role in both immunity against pathogens and autoimmunity against self (1). IL-23 is a heterodimeric cytokine composed of a p19 subunit (which is unique to IL-23) and a p40 subunit (common to IL-12), held together by an interchain disulfide bond. Although IL-12 and IL-23 share the p40 subunit, the activity of each cytokine is highly unique (2), with IL-12 promoting the development of IFN-γ producing Th1 cells (3) and IL-23 promoting the expansion of a novel effector subset of CD4+ T cells known as Th17 cells (4,5,6). These IL-17 producing cells have been demonstrated to play a role in driving the pathogenesis of many autoimmune diseases including rheumatoid arthritis (7), multiple sclerosis (8), inflammatory bowel disease (9) and more recently systemic lupus erythematosus (10), with SLE patients having significantly increased levels of both Th17 cells and IL-17 (10,11,12). The increased levels of IL-17 production observed in SLE patients has been attributed to double negative (DN; CD4−/CD8−) T cells, which are expanded in the peripheral blood of these patients and are present alongside IL-17+ T cells in kidney biopsies of patients with lupus nephritis, suggesting a role for DN T cells and IL-17 overproduction in the pathogenesis of SLE (13). The essential role of the cytokine IL-23 in both driving and maintaining production of IL-17 has been highlighted using IL-23 deficient mice in which IL-17 production was found to be undetectable in their CD4+ T cells (1). In keeping with this, mice deficient in the IL-23 receptor did not mount an autoimmune response and were protected from developing lupus, highlighting the importance of this cytokine in the pathogenesis of this condition (14).
Although the regulation of the p40 gene has been well established, there is a limited understanding of the regulation of p19 expression in the context of autoimmunity and pathways that regulate it downstream of TLRs. Similar to IL-12p35 and p40, TLR signaling is also responsible for the expression of IL-23p19. Makela et al demonstrated a role for a number of TLRs in IL-23p19 expression in human dendritic cells and macrophages, including TLR 2, 3, 4, 5, 7 and 8 (15). With respect to regulating expression at the promoter level, IL-12 transcription involves binding of NF-κB, C/EBP and AP-1 to its promoter region (16-19) whereas IL-23p19 transcription appears to be regulated by various other molecules. The murine IL-23p19 gene promoter was shown to have three putative NF-κB binding sites, two of which play an essential role in TLR-induced IL-23p19 gene expression (17). Additionally it was demonstrated that SMAD3 and ATF2 activation are transcription factors which in combination with NF-κB are essential in IL-23p19 gene expression (20). Further investigation into the murine IL-23p19 gene promoter demonstrated this region to have putative binding sites for two interferon regulatory factor genes IRF3 and IRF7, with mutations in the IRF3 binding site leading to decreased IL-23p19 promoter activity (20).

Whilst these studies revealed the presence of IRF3/7 binding sites in the IL-23p19 promoter, analysis of functional roles were inconclusive. However recent work has given new insight into the mechanism regulating IL-23 production and the potential role for IRF3 in this process. Ro52, a well known autoantigen in SLE has recently been implicated as negative regulator of the IL-23/Th17 pathway (21). Ro52 is an E3 ubiquitin ligase that acts to limit the production of cytokines such as IFN-β and IL-23 downstream of pathogen recognition. Thus Espinosa et al. demonstrate that in the absence of Ro52, production of type I IFNs and IL-23 in response to TLR7 and 9, stimulation is greatly exaggerated, resulting in systemic autoimmunity (20). Given that
we have previously demonstrated IRF3 as a chief molecular target for Ro52 (22), these results suggest a role for IRF3 in the regulation of IL-23p19 expression. This transcription factor is activated following phosphorylation of multiple serines in its C terminal transactivation domain. The kinase responsible for regulating this, TANK-binding kinase-1 (TBK-1), is itself regulated by phosphorylation, which is activated downstream of double stranded RNA detection by either TLR3 or the intracellular pathogen recognition receptor RIG-I (23, 24).

In this study we have demonstrated that TLR3-induced IL-23p19 and IFN-β expression is also enhanced in Ro52-/- mice in a similar manner to that observed following TLR7 and TLR9 stimulation. As the molecular target for Ro52 is the transcription factor IRF3, our data indicated for the first time a novel role for IRF3 in the regulation of IL-23 production, which was supported by a lack of IL-23p19 production observed in macrophages derived from IRF3-/- mice. Chromatin immunoprecipitation assays have demonstrated that IRF3 is stably bound to the human IL-23p19 promoter, an association which increases following TLR3 stimulation. Significantly, increased levels of IRF3 bound to the IL-23p19 promoter was observed in SLE patients which correlated with enhanced IL-23p19 production. Investigations into TLR3-driven responses in monocytes derived from SLE patients revealed that the enhanced IRF3 binding in the SLE patients is as a result of an overactivated TBK-1-IRF3 signalling axis, providing novel insights into the molecular pathogenesis of SLE and the potential role for TLR3 in driving this response.
Materials and Methods

Cell Culture

Bone marrow-derived macrophages (BMDMs) were generated from bone marrow cells isolated from femurs of C57BL/6 wild-type (WT) and IRF3−/− mice (a kind gift of Dr Antonio Sica, Istituto Clinico Humanitas, Milan, Italy) as previously described (27). BMDMs were also generated from bone marrow cells isolated from femurs of C57BL/6 WT and Ro52−/− mice (obtained from Dr Marie Wahren-Herlenius, Rheumatology Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden). BMDMs were cultured in DMEM supplemented with 10% FCS, 100 µg/ml penicillin-streptomycin and 10ng/ml macrophage stimulating colony factor (MCSF) (from L929 cell supernatant). HEK293T cells were cultured in DMEM supplemented with 10% FCS and 100µg/ml penicillin-streptomycin. Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using a Ficoll gradient and cultured in DMEM supplemented with 10% FCS and 100 µg/ml penicillin-streptomycin. Human monocytes were extracted from PBMC’s by positive selection using CD14 beads (Miltenyi Biotec). Cells were allowed to recover overnight prior to stimulation.

Plasmids and reagents

The human IL-23p19 promoter designated p19promotionpGL3 was designed to incorporate a 1kb region of the promoter region containing the various transcription factor binding sites including NF-κB, IRF3 and IRF7. Flag-tagged pCMV-IRF3, Flag-tagged pCMV-IRF7, pEF-Bos-TRIF-Flag promoter constructs were a kind gift from Dr. Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA). Xpress™-
tagged Ro52 was a gift from Dr. David Rhodes (Cambridge Institute for Medical Research, Cambridge, UK). The TLR3 agonist polyinosine-polycytidylic acid (PolyI:C), TLR7 agonist Imiquimod, TLR4 agonist Lipopolysaccharide (LPS), and TLR9 agonist CpG were obtained from Invivogen.

**Study Subjects**

All SLE patients included in our studies met the American College of Rheumatology classification criteria [27] and were recruited from Beaumont Hospital, Dublin 9 and Belfast City Hospital, Belfast. All the SLE patients included in the study were females in an age range of 22-62 years. Age and sex matched healthy individuals were chosen as controls. The study protocol was approved by the institutional review boards of all involved institutions and written informed consent was obtained from all participating subjects.

**Reporter gene assays**

HEK293T cells were transiently transfected in triplicate for 18 hr with 50ng of the indicated reporter construct and cotransfected with either empty vector control or indicated constructs (50ng). All transfections were performed using Metafectene (Biontex) according to the manufacturer’s recommendations. Luciferase activity was standardized to Renilla luciferase plasmid activity to normalize for transfection efficiency. Results are expressed as mean relative stimulation from three separate experiments ± SEM.
Real-time PCR

RNA was extracted from cell cultures using an RNeasy kit (Qiagen) and reverse transcribed to cDNA using Omniscript reverse transcriptase (Qiagen) according to manufacturer’s recommendations. Quantitative real-time PCR was performed using SYBR Green Taq ReadyMix™ (Sigma) using the following primer pairs: murine IFN-β; sense, 5′-ATAAGCAGCTCCAGCTCCAA-3′, antisense, 5′-CTGTCTGCTGGTGGAGTTCA-3′ and murine IL-23p19; sense, 5′-AGCGGGACATATG AATCTACTAAGAGA-3′ and antisense, 5′-GTCCTAGTAGGGAGGTGTGAAGTTG-3′. Data was analysed on ABI Prism 7900 (Applied Biosystems) and was normalized to a GAPDH reference. Real-time PCR data was analysed using the 2^\(^{-\Delta\Delta C_{t}}\) method (29).

IL-23p19 and TNF-α ELISAs

Human and murine IL-23p19 ELISA kits were purchased from eBiosciences and used a p19 specific capture antibody, a biotin conjugated p40 specific detection antibody, and a recombinant human/mouse IL-23 as a standard. 3,3,5,5-tetramethylbenzidine (TMB) horseradish peroxide (HRP) substrate solution was used for colorimetric detection (Sigma). The murine TNF-α ELISA was obtained from R&D Systems and was performed according to manufacturer’s recommended guidelines.
Chromatin Immunoprecipitation Analysis

Chromatin Immunoprecipitation (ChIP) assays were performed using a commercial ChIP assay kit, as per the manufacturer’s instructions (Active Motif). Briefly, cells (1×10⁶ cells/ml) were cross-linked with 1% formaldehyde at room temperature for 10 min. Nuclei were isolated into 1% SDS buffer containing protease inhibitors and subjected to sonication to yield 200-500bp DNA fragments. Sonicated chromatin was immunoprecipitated with 1 µg of anti-IRF3 (Abcam ab11978) or control goat IgG (Santa Cruz Biotechnology) overnight at 4°C. Samples were washed, and then bound chromatin was eluted and incubated overnight at 65°C for reversal of cross-linking. After proteinase K digestion for 1h, DNA was extracted using a DNeasy kit (Qiagen). To determine the identity of the IRF3 target genes, ChIP DNA was further analysed by PCR using primers encompassing the regions of interest on the IL-23p19 promoter (Table 1). Input DNA was used as a positive control. PCR products were analyzed on 1.5% agarose gel by electrophoresis. Band intensities of products were analysed using GeneTools (Syngene). IgG control values were subtracted and the sample values were normalised to input DNA and are represented as percentage of input (10 × dilution).

Western Blot

To prepare whole cell lysates, cells were lysed in 1× loading buffer containing 63mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS and 0.0025% bromophenol blue. Equal quantities of whole cell lysates were resolved by electrophoresis on a denaturing SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Following immunoblotting, the membrane was developed using a chemiluminscent HRP substrate.
Statistical analysis

Changes in cytokine production, gene expression and luciferase promoter activity were analysed by Students T test. Significance was considered to be ≤ 0.05.

Results

Ro52/- mice demonstrate increased levels of IFN-β and IL-23p19 gene induction following TLR3 stimulation

Ro52/- mice demonstrate tissue inflammation and systemic autoimmunity as a result of deregulated IL-23/Th17 responses downstream of TLR7/9 stimulation (21) thus implicating Ro52 as a negative regulator of this pathway and possibly of IL-23p19 activity. We have previously shown that Ro52 negatively regulates IFN-β promoter activity following TLR3/4 activation by ubiquitinating IRF3 leading to proteasomal degradation of the transcription factor (22) and it is thought a similar molecular mechanism may be in place for the regulation of IL-23p19. To date TLR3-induced IL-23p19 expression in Ro52/- mice has not been explored. To investigate this, bone marrow derived macrophages (BMDMs) were isolated from wild-type (WT) and Ro52-deficient mice (KO) and were unstimulated or stimulated with the TLR3 agonist PolyI:C for 3, 6, and 24hr and RT-PCR carried out to investigate both IFN-β (Figure 1A) and IL-23p19 (Figure 1B) gene induction. In keeping with previous published studies, Ro52-deficient BMDMs demonstrated significantly higher levels of IFN-β mRNA expression
compared to WT BMDMs following TLR3 stimulation. Importantly, TLR3 stimulation of BMDMs resulted in significantly higher levels of IL-23p19 expression in Ro52-deficient BMDMs similarly to IFN-β.

**IRF3 is required for IL-23p19 expression downstream of TLR3**

As the molecular target for Ro52 is the transcription factor IRF3, we next sought to investigate a possible role for IRF3 in IL-23p19 production. BMDMs derived from wild-type (WT) and IRF3−/− C57BL/6 mice were untreated or stimulated for 6 and 24 hr with the TLR agonist PolyI:C. We observed significant levels of IL-23p19 production in response to TLR3 stimulation in the WT BMDMs but remarkably IRF3−/− BMDMs failed to produce any detectable IL-23p19 following TLR3 stimulation demonstrating that there is an absolute requirement for IRF3 in IL-23p19 production (Figure 1C). Importantly TLR3 drove TNF-α production in the IRF3−/− BMDMs (Figure 1D) indicating that the cells were responding to TLR3 stimulation and are not impaired in their ability to transduce signals. To evaluate in detail the role for IRF3 in IL-23p19 expression, we investigated the human IL-23p19 promoter region and examined its activity. Sequence analysis of the human IL-23p19 promoter region using the MatInspector Search program revealed 2 putative NF-κB binding sites, which have previously been demonstrated to be essential for TLR-induced murine IL-23p19 promoter activity (16). In addition to this, the human promoter region also contains putative binding sites for IRF3 and 7 which shared a high level of conservation between both mouse and human. To investigate human IL-23p19 promoter activity, we designed a luciferase reporter plasmid incorporating a 1kb region of the human IL-23p19 promoter designated p19prompGL3. This 1kb region contains the two κB sites and the
two putative IRF binding sites. To investigate whether IRF3 and Ro52 regulated the human IL-23p19 promoter in an analogous manner to the murine promoter, HEK293T–TLR3 cells were transiently transfected with this vector and then challenged with increasing amounts of IRF3. IRF3 drove the human IL-23p19 promoter in a dose dependant manner highlighting its importance in promoter activity (Figure 1E). Having previously demonstrated a role for Ro52 in the negative regulation of IFN-β promoter activity, we next sought to investigate if Ro52 could inhibit IRF3- driven IL-23p19 promoter activity directly (22). Increasing amounts of Ro52 was observed to inhibit the ability of IRF3 to induce the IL-23p19 promoter dose-dependently (Figure 1F), in a similar manner to the IFN-β promoter again implicating a similar role for Ro52 in regulating these cytokines through IRF3.

IRF3 binds the IL-23p19 promoter in human monocytes, an association which increases with TLR3 stimulation.

To evaluate the mechanism by which IRF3 is driving IL-23p19 production following TLR3 stimulation, we next investigated whether the transcription factor IRF3 physically interacted with the human IL-23p19 promoter in monocytes using chromatin immunoprecipitation (ChIP). Cells from the human monocytic cell line THP1 were untreated or stimulated with PolyI:C for 60min. ChIP was performed using an IRF3-specific antibody and PCR carried out on the immunoprecipitates using primers spanning the various transcription factor binding sites identified in the IL-23p19 promoter region (Table 1). Region A incorporated the NF-κB site in the promoter, region B the putative IRF7 site and region C the putative IRF3 site. As shown in Figure 2A, IRF3 bound to both the endogenous and TLR stimulated IL-23p19 promoter
exclusively through the IRF7 and 3 (regions B and C) sites but not to the NF-κB site (region A). Interestingly, IRF3 was constitutively present at the IRF3 and IRF7 sites in the human IL-23p19 promoter, with PolyI:C stimulation inducing a strong increase in IRF3 association at the IRF3 site (Figure 2A and 2B). This result is consistent with studies carried out by Al-Salleeh and Petro (19) in which it was reported that IRF3 is constitutively present at the IL-23p19 promoter in RAW264.7 cells and murine splenic macrophages, with this association increasing in RAW264.7 cells following TMEV infection for 6hr. The ability of IRF3 to bind the human IL-23p19 promoter was next confirmed in primary monocytes isolated from peripheral blood. PCR amplification of the resulting immunoprecipitates using primers flanking the IRF3 site of the IL-23p19 promoter region revealed IRF3-binding activity on the human IL-23p19 promoter. Again IRF3 was constitutively bound at IRF3-site on the IL-23p19 promoter in human peripheral monocytes, with PolyI:C stimulation increasing this association after 3hr (Figure 2C and 2D). Importantly our results demonstrate a novel interaction of IRF3 with the human IL-23p19 promoter, an association that increases upon TLR3 stimulation.

SLE patients show increased IRF3 binding to the IL-23p19 promoter and increased IL-23 p19 production.

Previous studies have reported enhanced production of cytokines involved in the Th17 pathway such as IL-6, IL-12, IL-17 and IL-23p19 in SLE patients (10). In keeping with these reports, we observed enhanced IL-23p19 levels in plasma derived from SLE patients compared with healthy controls (Figure 3A). In addition to this, mRNA levels of IL-23p19 and p40 have also been reported to be significantly higher in SLE patients
compared to healthy controls (10). Given our findings that IRF3 plays an important role in regulating IL-23p19 production, we sought to investigate the IRF3-IL-23p19 promoter interaction in these patients. To examine the role of IRF3 in driving this response, ChIP analysis was performed to investigate levels of IRF3 binding to the promoter in monocytes derived from SLE patients and age and sex matched healthy controls. Monocytes from SLE patients showed significantly increased binding of IRF3 (Figure 3B left panel) to the endogenous IL-23p19 promoter compared to healthy controls, which correlated to the enhanced production of IL-23 observed by these cells from these patients (Figure 3C left panel)

TLR3 stimulation enhanced IRF3 binding to the IL-23 p19 promoter and increased IL-23p19 production in SLE patients.

The activity of IRF3 is regulated by the upstream kinase TBK-1, which itself becomes phosphorylated when active. Therefore, to provide a molecular explanation for our observations, we investigated TBK-1 activity in both resting and TLR3 stimulated monocytes. TBK-1 is hyperphosphorylated (indicative of activation) in SLE patients and its activity is further enhanced in response to PolyI:C stimulation in monocytes derived from SLE patients compared with healthy controls (Figure 3D). As TLR3 can drive IRF3 binding to the IL-23p19 promoter, in addition to endogenous levels of IRF3 binding to the IL-23p19 promoter, we also investigated IRF3 binding in PolyI:C stimulated monocytes from both SLE patients and controls. As expected, SLE patients were observed to have increased levels of IRF3 binding following stimulation when compared to PolyI:C stimulated control cells (Figure 3B right panel). Production of IL-23p19 was also measured in the supernatants from these monocytes by ELISA. SLE
patients showed significantly higher levels of IL-23p19 production following TLR3 stimulation when compared to healthy controls (Figure 3C right panel). Taken together, these results indicate that activation of IRF3 in monocytes via TLR3 stimulation leads to increased IRF3 binding to the IL-23p19 promoter which corresponds to the enhanced levels of IL-23p19 observed in SLE patients, thus implicating this factor as an important player in both IL-23p19 regulation and SLE. Importantly our results indicate that the enhanced IL-23p19 expression we observe in these patients is as a result of deregulation of the TBK1-IRF3 signalling axis.

Discussion

The current study demonstrates a role for the autoantigen Ro52 as a negative regulator of IL-23p19 activity through targeting the transcription factor IRF3, implicating for the first time a role for IRF3 in IL-23p19 production. In the absence of this factor, abrogation of IL-23p19 production is observed, highlighting the essential role for this transcription factor in the production of this cytokine. We have demonstrated that IRF3 is bound constitutively to the IL-23p19 promoter in human monocytes, an association which was enhanced in SLE patients. In addition, a positive correlation between the level of IRF3 binding to the IL-23p19 promoter and the level of IL-23p19 production has been observed. Our results further indicate that the enhanced IRF3 binding in the SLE patients is as a result of overactivated TBK-1-IRF3 signalling axis, suggesting this pathway may be a potential target for the treatment of this disease.
Previous studies have reported that IL-23 is important for the development, expansion, and proliferation of the IL-17 producing T cell population (1), with increased levels of IL-17 and also IL-23 having being observed in the serum of SLE patients. These increases in IL-17 lead to the production of various proinflammatory mediators, resulting in inflammation and ultimately the organ damage characteristic of SLE. The Ro52 knockout mouse, which develops autoimmune dermatitis consistent with a lupus-like syndrome, has been shown to be a negative regulator of IFN production and interestingly, IL-23p19 production following TLR7 and TLR9 stimulation (20). The effect of Ro52 on the transcriptional activity of IRF3 has been well documented with Ro52 substantially decreasing IRF3 transcriptional activity through ubiquitination and ultimately its degradation (22). Whilst TLR7 and TLR9 are widely accepted to be important in driving the production of type I IFNs and thus contributing to the pathogenesis of SLE, a role for TLR3 in SLE is less well defined. In this study, we demonstrate enhanced IL-23p19 expression in Ro52\(^{-/-}\) cells stimulated with the TLR3 agonist PolyI:C. This induction was seen to increase slightly as a result of the loss of Ro52 at 6 hr, suggesting that Ro52 acts early on this pathway. However the observation that differences in IL-23p19 gene induction are markedly increased at 24hr would suggest Ro52 regulates additional pathways or potentially a positive regulatory loop, such as IFN-induced up regulation of IRF7. Importantly however, taken together our results strongly suggest a key role for IRF3 as a primary driver of these responses and may contribute to the pathogenesis of this disease. In support of this, studies in lupus-prone New Zealand Black (NZB) mice have demonstrated that administration of PolyI:C exacerbates disease progression as a result of enhanced type I IFN production (30).

Expression of IL-23p19 is thought to be strictly controlled at the transcriptional level by multiple transcription factors, with NF-κB being the most widely characterized of these.
Two proximal NF-κB sites have been described in the murine IL-23p19 promoter, both of which are required for TLR induced IL-23p19 expression. cRel binds these sites in the gene promoter following TLR stimulation, an association which was shown to be essential for activation of the IL-23p19 promoter. Investigation into the evolutionary conservation of these DNA sequences between mouse and human in the IL-23p19 gene promoter demonstrated complete conservation throughout the two species in these regions, suggesting a shared functionality. In addition to cRel, the murine IL-23p19 promoter region contains putative binding sites for multiple transcription factors including AP-1, ATF2, SMAD3 and members of the IRF family (IRF3 and 7). In addition to NF-κB, the transcription factors AP-1, ATF2 and SMAD3 have also been observed to be essential for activation of the promoter with mutations in these sites resulting in loss of IL-23p19 promoter activation (17, 20). In our report, we have investigated the requirement for IRF3 in IL-23p19 expression and have found that in the absence of IRF3, TLR3 is unable to drive its expression in bone marrow derived macrophages. In addition ChIP analysis demonstrates that IRF3 was constitutively associated with the IL-23p19 promoter, an association that increased following PolyI:C treatment of cells. Thus our results indicate an absolute requirement for IRF3, a previously unappreciated aspect of its regulation. Previous reports had shown that down regulating IRF3 via shRNA had no effect on IL-23p19 promoter activity (20), although mutating the IRF3 binding site in the promoter resulted in complete loss of inducible expression. These results suggest that partial loss of IRF3 observed in this study is not sufficient to completely inhibit IL-23p19 expression or potentially that additional transcription factors, such as IRF7, may compensate for IRF3 in this setting. Our results indicate that IRF3 is an integral part of the enhancesosome required to drive IL-23p19 and loss of this transcription factor results in complete abrogation of expression of IL-23.
Given the role of IL-23 in autoimmune disease it is not surprising that enhanced levels of this cytokine, and by extension Th17 cells, are observed in SLE patients, with mRNA levels of IL-23p19 correlating with disease severity. Our results indicate that increased IRF3 binding to the promoter of IL-23p19 is responsible for this enhanced expression. Critically our results indicate a positive correlation between IL-23p19 expression and IL-23p19 promoter binding, supporting the idea that the IL-23/IL-17 axis may act as a potential biomarker of disease progression in SLE (10, 26). To provide a molecular explanation for our findings we have investigated the activation state of the upstream kinase TBK1 and have found that it is hyperactivated in monocytes derived from SLE patients. Interestingly TLR3-induced activation of TBK1 is also enhanced in the SLE monocytes, further supporting the hypothesis that signaling downstream of the nucleic acid sensing pathways may contribute to the pathogenesis of SLE. Thus overactivation of TBK1 in patients and the downstream consequences of this finding (i.e. enhanced IL-23 production) give further support for TBK1 being an ideal target for therapeutic intervention. Use of specific inhibitors of TBK1, such as MPI-485520 (Myrexis, Inc, UT) will be useful in testing this hypothesis in future studies.

This report provides novel insights into the regulation of IL-23p19 expression and critically demonstrates that IRF3 is absolutely required for TLR3-induced expression of IL-23. Importantly this is the first report that demonstrates that the TBK1/IRF3 axis is dysregulated in SLE patients thus resulting in enhanced IRF3 binding to the IL-23p19 promoter and increased protein expression. Overall our results further support TBK1 as an important therapeutic target for the treatment of SLE given its ability to regulate IRF3 activity and hence type I interferon and IL-23 production.
References


Table 1: Primer sequences used for ChIP analysis of the human IL-23p19 promoter

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<th>Transcription factor binding site</th>
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<tr>
<td>NF-κB site (Region A)</td>
<td>FP: 5’-ACCACACATACTCATTCCCCC-3’</td>
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<td></td>
<td>RP: 5’-CTTTATACCAGCGGTGACTC-3’</td>
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<td>IRF7 site (Region B)</td>
<td>FP: 5’-TCCCGATTCTCCAAGTTCC-3’</td>
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<td>RP: 5’-GCCACCCCCAACCTAATTTC-3’</td>
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<td>IRF3 site (Region C)</td>
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<td>RP: 5’-GCATGAAGGAACTTGGAC-3’</td>
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**Figure 1:** BMDMs derived from wild type (WT) and Ro52−/− mice were left untreated or stimulated with PolyI:C (20μg/ml) for 3, 6 and 24hr. mRNA expression of IFN-β (A) and IL-23p19 (B) was determined by RT-PCR. (n=3) Bars represent mean± SD. * p≤ 0.05 significantly different compared to WT. BMDMs from both WT or IRF3−/− mice were left untreated or stimulated with PolyI:C (20μg/ml) for 6 and 24hr. Following this, supernatants were collected and (C) IL-23p19 and (D) TNFα levels were investigated by ELISA (n=3). Bars represent mean± SD. * p≤ 0.05 significantly different compared to untreated cells. † p≤ 0.05 significantly different compared to WT. IL-23p19 reporter gene analysis was carried out by transiently transfecting HEK293T cells with 50ng of the IL-23p19 promoter and cotransfecting with increasing amounts of IRF3 (E) or cotransfecting with 50ng of IRF3 (F) with increasing amounts of Ro52 (50, 100 and 150ng) (n=3). Bars represent mean± SD. * p≤ 0.05 significantly different compared to empty vector control.

**Figure 2:** THP1 cells were untreated or stimulated with PolyI:C (20μg/ml) for 60min. IRF3 binding to transcription factor binding sites of the IL-23p19 promoter was analysed (A) by ChIP analysis. PCR was carried out using primers encompassing the NF-κB site (designated A on the gel), IRF3 site (designated B) and the IRF7 site (designated C) of the IL-23p19 promoter (n=3). Optical densitometry was performed on these gels (B). Bars represent mean± SD. * p≤ 0.05 significantly different compared to untreated cells. (C) IRF3 binding to the IL-23p19 promoter was assessed in human monocytes by ChIP analysis similarly to (A) (n=4). PCR was carried out using primers encompassing the IRF3 site of the IL-23p19 promoter alone. Optical densitometry was performed on these gels (D). Bars represent mean± SD. * p≤ 0.05 significantly different compared to untreated cells.
Figure 3: (A) IL-23p19 cytokine production in plasma derived from both healthy controls and SLE patients was analysed by ELISA (n=8) with each point representing a single donor. The horizontal lines represent the mean value in each group. *p ≤ 0.05 significantly different compared to control individuals. (B) Monocytes from healthy controls and SLE patients were untreated (left panel) or stimulated (right panel) with PolyI:C (20µg/ml) for 3hr. IRF3 binding to transcription factor binding sites of the IL-23p19 promoter was analysed (B) by ChIP analysis (n=8). *p ≤ 0.05 significantly different compared to control individuals. (C) IL-23p19 production in supernatants from these monocytes was investigated by ELISA (n=8). *p ≤ 0.05 significantly different compared to control individuals (D) Protein levels of phospho-TBK1 were analysed in unstimulated and TLR3 stimulated (20µg/ml PolyI:C for 30min) monocytes derived from healthy controls and SLE patients by Western Blot (n=3).
Figure 1: BMDMs derived from wild type (WT) and Ro52<sup>−/−</sup> mice were left untreated or stimulated with PolyIC (20μg/ml) for 3, 6 and 24hr. mRNA expression of IFN-β (A) and IL-23p19 (B) was determined by RT-PCR. (n=3) Bars represent mean ± SD. * p<0.05 significantly different compared to WT. BMDMs from both WT or IRF3<sup>−/−</sup> mice were left untreated or stimulated with PolyIC (20μg/ml) for 6 and 24hr. Following this, supernatants were collected and (C) IL-23p19 and (D) TNFα levels were investigated by ELISA. (n=3) Bars represent mean ± SD. * p<0.05 significantly different compared to untreated cells. † p<0.05 significantly different compared to WT. IL-23p19 reporter gene analysis was carried out by transiently transfecting HEK293T cells with 50ng of the human IL-23p19 promoter and cotransferring with increasing amounts of IRF3 (E) or cotransferring with 50ng of IRF3 (F) with increasing amounts of Ro52 (50, 100 and 150ng) (n=3). Bars represent mean ± SD. * p<0.05 significantly different compared to empty vector control.
Figure 2: THP1 cells were untreated or stimulated with Poly I:C (20μg/ml) for 60mins. IRF3 binding to transcription factor binding sites of the IL-23p19 promoter was analyzed (A) by ChIP analysis. PCR was carried out using primers encompassing the NF-κB site (designated A on the gel), IRF3 site (designated B) and the IRF7 site (designated C) of the IL-23p19 promoter (n=3). Optical densitometry was performed on these gels (B). Bars represent mean ± SD. * p<0.05 significantly different compared to untreated cells. (C) IRF3 binding to the IL-23p19 promoter was assessed in human macrophages by ChIP analysis similarly to (A) (n=4). PCR was carried out using primers encompassing the IRF3 site of the IL-23p19 promoter alone. Optical densitometry was performed on these gels (D) Bars represent mean ± SD. * p<0.05 significantly different compared to untreated cells.
Figure 3: (A) IL-23p19 cytokine production in plasma derived from both healthy controls (CTL) and SLE patients was analysed by ELISA (n=8) with each point representing a single donor. The horizontal lines represent the mean value in each group. *p<0.05 significantly different compared to control individuals. (B) Monocytes from healthy controls (CTL) and SLE patients were untreated (left panel) or stimulated (right panel) with Poly I:C (20μg/ml) for 3hr. BRD3 binding to transcription factor binding sites of the IL-23p19 promoter was analysed (B) by ChIP analysis (n=8). *p<0.05 significantly different compared to control individuals. (C) IL-23p19 production in supernatants from these monocytes was investigated by ELISA (n=8). *p<0.05 significantly different compared to control individuals. (D) Protein levels of phospho-TBK1 were analysed in monocytes unstimulated and TLR3 stimulated (20μg/ml Poly I:C for 30min) derived from healthy controls (CTL) and SLE patients by Western Blot (n=3).