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Regulation of cooperative function of the *Il12b* enhancer and promoter by the interferon regulatory factors 3 and 5

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ABSTRACT

The regulation of the *Il12b* gene, encoding the shared p40 subcomponent for IL-12 and IL-23, is critical for innate immune responses and subsequent T cell polarization. This gene is robustly induced upon Toll-like receptor (TLR) stimulation, wherein an enhancer located 10 kb upstream of the transcription start site is required for promoter activity; however, the underlying mechanisms that regulate this enhancer in cooperation with the promoter has remained elusive. We show here that the *Il12b* enhancer contains functional ISREs for recognition by interferon regulatory factors (IRFs), and provide evidence that TLR-activated IRF5 mediates cooperativity of the enhancer with the promoter which also contains ISREs. By contrast, IRF3 activated by cytosolic RIG-I-like receptor (RLR) signaling binds to these ISREs and causes gene suppression. Consistently, IRF5 binding is accompanied with chromatin remodeling of both regulatory regions and the formation of a productive transcriptional complex containing other transcription factors, whereas these events are inhibited by IRF3 binding. We show that the ISREs embedded in the enhancer are indeed critical for its activation by IRF5. We also adduce evidence that the 5′ sequences of the enhancer and promoter ISREs, all of which deviate from consensus ISREs, critically affect the function of IRF3. The dual commitment of these IRFs in the regulation of the *Il12b* enhancer and promoter is unique and may have implications for understanding the evolution of this gene.

1. Introduction

Complex gene regulation networks operate to ensure appropriate responses against distinct pathogens. Infection by pathogens triggers the activation of several types of signal transducing innate receptors, including membrane-bound Toll-like receptors (TLRs) and cytosolic receptors such as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) [1,2]. While the nuclear factor κB (NF-κB) is widely involved in the regulation of the target genes induced upon activation of these receptors [3,4], the interferon (IFN) regulatory factor (IRF) family of transcription factors also play critical roles in these gene regulatory networks [1,5].

The *Il12b* gene, which encodes the interleukin (IL)-12p40 subunit that is shared by IL-12 and IL-23, has been one of the best studied target genes induced in antigen-presenting cells (APCs) by TLR signaling in the context of the innate receptor-mediated instruction of T cell polarization against bacterial infections [6,7]. Indeed, the TLR-induced IL-12 and IL-23 is critical for the differentiation of naive T cells into T helper type 1 (Th1) cells and the maintenance of IL-17-producing Th17 cells, respectively [6–8]. The mouse *Il12b* promoter contains several *cis*-elements that recruit a number of transcription factors in response to TLR signaling, including IRF5, NF-κB, and CCAAT/enhancer-binding protein (C/EBP) [9,10].

The *Il12b* gene is also controlled by a TLR-inducible enhancer region located 10 kb upstream of the transcription start site; however, the underlying regulatory mechanism(s) for how the enhancer cooperates with the promoter still remains to be understood. TLR-induced gene transcription is accompanied with nucleosome remodeling and recruitment to the enhancer of octamer-binding transcription factor 1 (Oct-1) and Oct-2, which are constitutively expressed in APCs; hence, enhancer activation may involve binding of an unidentified, TLR-inducible transcription factor(s) that facilitate the recruitment of the Oct proteins [11].

We have shown that IRF5 upon activation by TLR signaling binds to the IRF-binding sites (IFN-stimulated response elements; ISREs) of the *Il12b* promoter, and is indispensable for the TLR-mediated activation of the *Il12b* gene [12]. More recently, we have

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shown that IRF3 activated by RLR signaling dominantly binds to the ISREs of the *Il12b* promoter over TLR-activated IRF5, thereby selectively suppressing the TLR-mediated gene induction and subsequent Th1- and Th17-type T cell responses [13]. However, the molecular basis of how the RLR-activated IRF3 functions as activator for type I IFN genes on the one hand and exerts a suppressive role on the *Il12b* promoter on the other remains unknown.

In this study, we identify new IRF-binding ISREs within the *Il12b* enhancer region and demonstrate that upon TLR stimulation IRF5, C/EBP and Oct transcription factors bind to the enhancer. We also show that, similar to the *Il12b* promoter, RLR-activated IRF3 dominantly binds to the enhancer in lieu of IRF5 and interferes with the enhancer binding of C/EBP and Oct factors. As such, IRF5 binding to ISREs of the *Il12b* enhancer and promoter results in cooperative activation of the gene, whereas IRF3 binding results in gene suppression. We also adduce evidence for the critical function of the unique ISRE sequences embedded in the *Il12b* enhancer/promoter and that the N-terminal DNA binding region (DBR) of IRF3 dictate the functional fate of this transcription factor on the *Il12b* gene. We discuss our findings in terms of the unique evolution of the regulatory mechanism of the *Il12b* gene by these IRFs.

2. Materials and methods

2.1. Reporter assay

HEK293T cells (3.0×10^4 cells) seeded in 48-well plates were transiently cotransfected with 5 ng of a *Renilla* luciferase plasmid and 100 ng of a reporter plasmid together with expression plasmids for IRF3–5D, IRF5A, IRF5N3C, IRF3N5C and/or c-Rel using X-tremeGENE 9 reagent (Roche). At 36 h after transfection, cells were harvested and luciferase activities were measured using the dual-luciferase reporter assay system (Promega). In all cases, the obtained data were normalized for transfection efficiency by dividing firefly luciferase activity by *Renilla* luciferase activity. Other materials and methods are in the Supplementary Information.

3. Results

3.1. Binding of IRFs to the Il12b enhancer upon TLR or RLR activation

It was shown previously that a 105-bp segment of DNA located 10 kb upstream of the transcription start site of the *Il12b* gene functions as a TLR-inducible enhancer [11]. Upon inspection of the enhancer sequence, we identified three new putative ISREs, which we termed e-ISRE1, e-ISRE2 and e-ISRE3; accordingly, we refer to the ISREs within the promoter as p-ISRE1 and p-ISRE2 throughout (Fig. 1A). We then examined by ChIP assay the binding of IRFs and other transcription factors to the enhancer region in mouse peritoneal macrophages following stimulation with either LPS for TLR4 activation or poly(I:C) for RLR activation. Interestingly, TLR4 stimulation resulted in the recruitment of IRF5, C/EBPβ and Oct-1/-2 transcription factors to the enhancer (Fig. 1B and Supplementary Fig. 1A). On the other hand, IRF3 was recruited in lieu of IRF5 upon RLR stimulation, but not C/EBPβ or Oct-1/-2, suggesting that the binding of IRF3 results in an abortive assembly of transcription factors on the enhancer (Fig. 1B and Supplementary Fig. 1A). Furthermore, when cells are stimulated by poly(I:C) followed by LPS stimulation, IRF3 binding is predominant over IRF5 and LPS-induced C/EBPβ and Oct-2 binding is attenuated (Fig. 1C and Supplementary Fig. 1B). These observations are reminiscent of the *Il12b* promoter regulation by these IRFs in that the IRF3 transcription factor activated by RLR stimulation functions as a suppressor of the promoter, where it interferes with the productive assembly of transcription factors that is otherwise induced by TLR signaling [13]. Consistent with this, a restriction enzyme accessibility assay revealed that the induction of chromatin

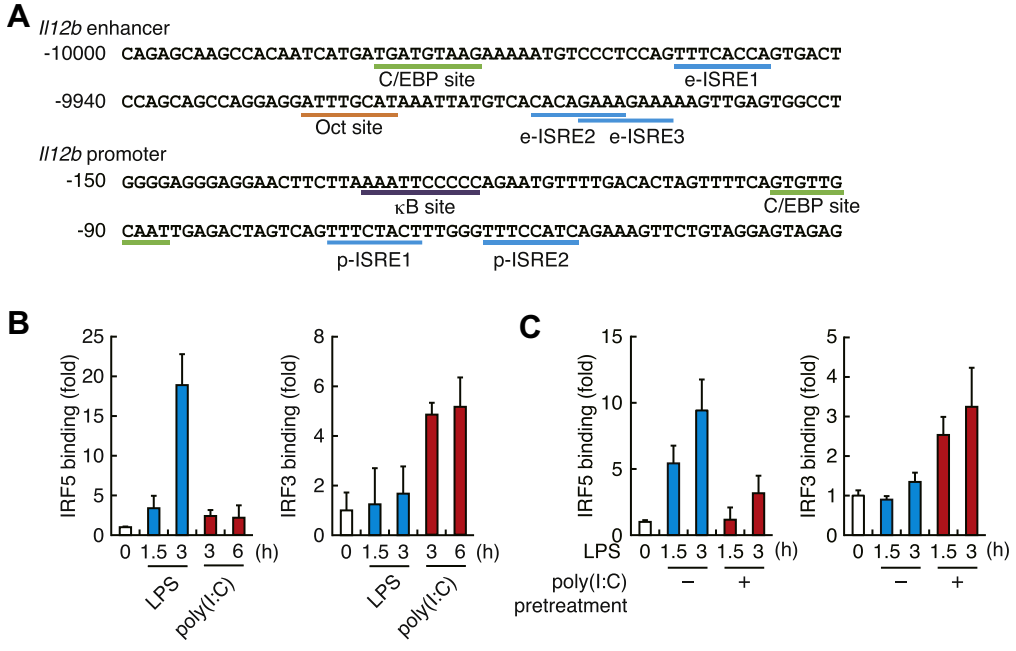

Fig. 1. Binding of IRFs to *Il12b* enhancer upon TLR or RLR activation (A) Schematic illustration of the murine *Il12b* enhancer and promoter regions, with three putative ISREs (e-ISRE1, e-ISRE2 and e-ISRE3; blue color) and two ISREs (p-ISRE1 and p-ISRE2; blue color), respectively, are shown. Oct (orange), C/EBP (green) and κB (purple)-binding sites are also shown. Numbers show the distance from the transcription start site of the *Il12b* gene. (B) Peritoneal macrophages from C57BL/6 mice were stimulated with poly(I:C) (5 μg/ml; red color) or LPS (100 ng/ml; blue color) for the indicated time periods. (C) Peritoneal macrophages from C57BL/6 mice were stimulated for the indicated time periods with LPS alone (100 ng/ml; blue color) or LPS with poly(I:C) (5 μg/ml; red color) pretreatment for 2 h. ChIP assay was performed using anti-IRF5 or anti-IRF3 antibody. Results shown are means ± SD of three independent experiments.