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The Feedback Phase of Type I Interferon Induction in Dendritic Cells Requires Interferon Regulatory Factor 8

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SUMMARY

Dendritic cells (DCs) produce type I interferons (IFNs) in greater amounts than other cells, but the mechanisms remain elusive. Here we studied the role of a transcription factor, IRF8, in DC induction of type I IFNs. Upon newcastle disease virus (NDV) infection, bone marrow-derived plasmacytoid and conventional DCs induced IFN transcripts, exhibiting two-phase kinetics. The second, amplifying phase represented an IFN feedback response that accounted for much of IFN protein production. Induction of second phase transcription required IRF8. Mouse cytomegalovirus (MCMV) and Toll-like receptor-mediated IFN induction in DCs also required IRF8. Chromatin immunoprecipitation analysis showed that IRF7, IRF8, and RNA polymerase II were recruited to the IFN promoters upon stimulation. Moreover, sustained RNA polymerase II recruitment to the promoters critically depended on IRF8. Together, these data indicate that IRF8 magnifies the second phase of IFN transcription in DCs by prolonging binding of basic transcription machinery to the IFN promoters, thereby playing a role in innate immunity.

INTRODUCTION

Type I interferons (IFNs) are produced immediately after pathogen infection, profoundly influencing the nature of innate and adaptive immunity (Biron, 2001). Although many types of cells produce type I IFNs, dendritic cells (DCs) are the highest IFN producers (Asselin-Paturel et al., 2001; Siegal et al., 1999), accounting for the early establishment of innate immunity (Asselin-Paturel and Trinchieri, 2005; Banchereau et al., 2004). Type I IFN encoding genes belong to a multigene family consisting of more than 10 *Ifna* genes and a single *Ifnb* gene, all clustered in a ~500 kb long region in chromosome 4 in the mouse (Pestka et al., 2004). IFN induction is mediated through Toll-like receptor (TLR) signaling, which simulates sequential activation of adaptors and kinases of the interleukin (IL)-1 receptor-associated kinase (IRAK), inhibitor of kappaB kinase (IKK), and Tnf receptor-associated factor (TRAF) families, ultimately leading to the activation of transcription factors, interferon regulatory factor 3 (IRF3), interferon regulatory factor 7 (IRF7), and nuclear factor of kappa light chain gene enhancer in B cells (NF-κB) (Honda and Taniguchi, 2006; Kawai and Akira, 2006). Besides TLR signaling, activation of RNA helicase pathways such as retinoic acid-inducible gene I (RIG-I) leads to activation of IRF3 and type I IFN induction (Honda and Taniguchi, 2006; Yoneyama et al., 2005).

Mechanisms underlying type I IFN gene induction have been extensively studied in non-DCs, including fibroblasts (Civas et al., 2002; Marie et al., 1998; Sato et al., 2000). These studies showed that IFN production involves initial induction of *Ifnb* and *Ifna4* transcripts that are mediated by IRF3 and IRF7. These IRFs are phosphorylated, dimerized, and translocated into the nucleus to activate the two IFN genes (Sharma et al., 2003). This initial event then leads to the next stage of transcription where additional *Ifna* encoding genes and *Irf7* are induced in an IFN-dependent manner (Marie et al.,1998; Tailor et al., 2006). A recent study indicates that IRF7, not IRF3, is essential for the initial IFN induction both in DCs and non-DCs (Honda et al., 2005b).

DCs are a heterogeneous population of cells with diverse functions. Although it was previously thought that plasmacytoid DCs (pDCs) are responsible for much of type I IFN production, more recent studies showed that conventional DCs (cDCs) also produce considerable amounts of type I IFNs (Diebold et al., 2003; Kato et al., 2005). pDCs seem to have several distinct characteristics that might be relevant to high IFN production. For

example, the TLR complexes containing MyD88 and IRF7 are retained in pDCs longer than in other cells (Honda et al., 2005a). In addition, pDCs express IRF7 at high amounts, possibly enabling enhanced IFN gene activation (Dai et al., 2004). Furthermore, the RNA helicase-RIG I pathway appears dispensable in pDCs (Kato et al., 2005).

Despite intense interest, molecular mechanisms by which pDCs and in some cases cDCs express high amounts of type I IFNs have remained poorly understood. Although several reports indicated an IFN feedback as an important mechanism of IFN production in DCs, evidence is inconsistent, making it uncertain as to the significance of the feedback loop (Gautier et al., 2005; Honda et al., 2005b; Kerkmann et al., 2003). Another unsolved question is the role of IRF8 in DC induction of type I IFN genes. IRF8 is an immune system-specific member of the IRF family (Levi et al., 2002; Tailor et al., 2006; Tamura and Ozato, 2002). It is a nuclear protein expressed at high amounts in pDC and other DC subtypes (Tamura et al., 2005a). We previously showed that *Irf8*$^{-/-}$ DCs produced little type I IFNs and that reintroduction of IRF8 rescued type I IFN induction in *Irf8*$^{-/-}$ DCs (Schiavoni et al., 2002; Tamura et al., 2005a; Tsujimura et al., 2003b). In addition, IRF8 is essential for the development of DC subsets, particularly pDCs and CD8α^{+} DCs, although another member, IRF4, also contributes to pDC development, albeit to a minor extent (Aliberti et al., 2003; Schiavoni et al., 2002; Tamura et al., 2005a; Tsujimura et al., 2003a, 2003b). Because of its prominent role in DC development, the contribution of IRF8 to IFN induction in differentiated DCs has not been fully elucidated so far.

The present study reveals two notable aspects of IFN transcription that are different between DCs and other cell types. First, many *Ifna* subtypes are induced immediately in the first phase prior to the initiation of IFN feedback response. IRF8 is required for the second, amplifying phase of IFN induction both in pDCs and cDCs, although IRF8 is not required for the activation of IRF3 or IRF7. Our results indicate that the role of IRF8 is to help prolong the recruitment of basal transcription machinery to the IFN promoters, a role not shared by IRF7 or IRF3, which supports characteristically high IFN transcription in DCs.

RESULTS

Two-Phase Kinetics of Type I IFN Induction in DCs: The Absence of the Second Phase Peak in *Irf8*$^{-/-}$ DCs

To study the mechanism of type I IFN gene induction in DCs, bone marrow-derived *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs generated in the presence of Flt3L were exposed to newcastle disease virus (NDV) for 1 hr. Cells were washed and then incubated in fresh media for up to 24 hr. *Ifna* transcripts induced after viral exposure were measured by quantitative (q) RT-PCR with a primer set that detected all *Ifna* subtypes (Figure 1A). The transcript induction in *Irf8*$^{+/+}$ DCs followed two-phase kinetics, exhibiting two peaks. The first relatively small peak was seen at 1 hr, followed by the greater second peak at 7 hr. Strikingly, *Irf8*$^{-/-}$ DCs did not elicit the second peak, although they elicited the first peak similar to that in *Irf8*$^{+/+}$ cells in terms of timing and transcript expression. In Figure 1B, the amount of IFNA proteins secreted from *Irf8*$^{+/+}$ DCs rose sharply during initial 11 hr and plateaued thereafter. In contrast, IFNA produced in *Irf8*$^{-/-}$ DCs remained near background expression, in line with previous reports (Schiavoni et al., 2002; Tamura et al., 2005a; Tsujimura et al., 2003b). Given that IFN protein production was virtually absent in *Irf8*$^{-/-}$ DCs, despite the presence of the first transcript peak, it is likely that much of IFN protein production in DCs is accounted for by the second phase of IFN transcript expression. In Figure 1C, *Ifnb* transcript expression displayed similar two-phase kinetics in *Irf8*$^{+/+}$ DCs. Again, the second peak was completely missing in *Irf8*$^{-/-}$ DCs, although the first peak in *Irf8*$^{-/-}$ DCs was higher than that in *Irf8*$^{+/+}$ DCs.

The two-phase kinetics observed above is reminiscent of two-step IFN induction described for non-DCs (Civas et al., 2002; Marie et al., 1998; Sato et al., 2000). In non-DCs, *Ifnb* and *Ifna4* are the first subtypes expressed in the early stage, and other, non-a4 *Ifna* subtypes are expressed in the second stage. To assess whether *Ifna* subtypes were induced in a similar, hierarchical order in DCs, expression of non-a4 *Ifna* transcripts was examined. Semiquantitative RT-PCR rather than qRT-PCR was employed in these experiments, because selection of primers that could detect individual non-a4 *Ifna* transcripts in qRT-PCR was not practical. As seen in Figure 1D, non-a4 *Ifna* transcripts were induced at 1 hr along with induction of *Ifnb* and *Ifna4* both in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs. In *Irf8*$^{+/+}$ DCs, the expression of non-a4 *Ifna* mRNA transcripts markedly increased at 7 hr, along with an increase in *Ifna4* and *Ifnb* mRNAs. In *Irf8*$^{-/-}$ DCs, however, neither non-a4 *Ifna*, *Ifna4*, nor *Ifnb* transcripts were detected at 7 hr and thereafter. These data indicate that contrary to non-DCs, multiple *Ifna* subtypes are induced in the first phase in DCs, and their expression is enhanced in the second phase. Determination of 25 *Ifna* transcript sequences expressed at 1 hr in DCs confirmed the abundant presence of non-a4 *Ifna* IFN species in the first phase (Table S1 in the Supplemental Data available online).

The Role of IRF8 in Type I IFN Expression in cDCs

We noted that ~20%–30% of *Irf8*$^{+/+}$ DCs were pDCs carrying B220, Siglec-H, and Ly49Q, whereas cells with these markers were virtually nonexistent in *Irf8*$^{-/-}$ DCs (Figure S1). Because the failure of *Irf8*$^{-/-}$ DCs to elicit the second IFN peak may be attributed to the absence of pDCs in the culture, we tested whether IFNs were induced in cDCs after NDV stimulation. MACS fractionation of total DCs from *Irf8*$^{+/+}$ mice yielded a clean cDC population in which B220^{+} pDC were 0.1%. The other, remaining population was enriched with pDCs (~70% B220^{+}) (Figure S2). *Ifna* transcripts were induced both in cDCs and pDC-enriched populations, both showing two peaks, similar to those in total DCs. Similarly, *Ifnb* transcripts were expressed in the both cDCs and pDC-enriched cells, generating again two peaks resembling those in total DCs. These results

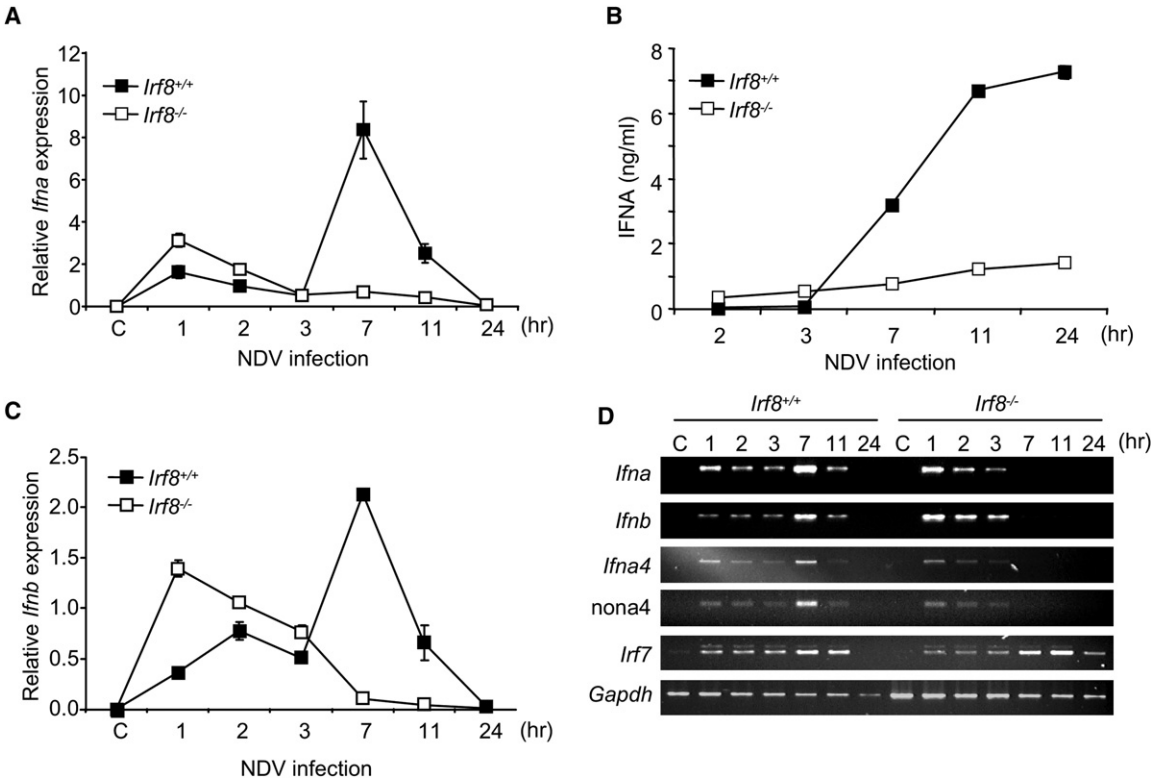

Figure 1. Two-Phase Kinetics of Type I IFN Transcript Induction in DCs

(A) DCs from *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ mice were infected with NDV for 1 hr and washed, and *Ifna* mRNA expression was measured at indicate times (hr) by qRT-PCR with pan-*Ifna* primers. Values represent the average of three determinations ± SD. Seven separate experiments yielded similar data.

(B) IFNA protein production in above cells was measured by ELISA. Data are representative of three experiments.

(C) *Ifnb* mRNA expression in above cells was measured with Ifnb primers as in (A).

(D) Semiquantitative RT-PCR was performed for *Ifna4* and non-a4 *Ifna* transcripts in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ cells at indicated times after NDV infection as in (A). Data are representative of two experiments.

show that type I IFN genes are induced both in cDC and pDC after NDV stimulation and that the absence of the second peak in *Irf8*$^{-/-}$ DCs was not due the absence of pDCs but rather was due to the absence of IFN mRNA expression.

Because Flt3L and GM-CSF promote development of different DC subsets (Gilliet et al., 2002; Tamura et al., 2005a), IFN induction was also studied in GM-CSF-derived DCs (Figure S3). After NDV stimulation, *Irf8*$^{+/+}$ DCs from GM-CSF culture expressed much lower *Ifna* transcripts and proteins than those from the Flt3L culture. More importantly, IFN induction in *Irf8*$^{-/-}$ DCs from GM-CSF culture were at a background amount (Figure S3). These results are in agreement with the previous observations that Flt3L, but not GM-CSF, supports development of IFN-producing cells (Tamura et al., 2005b).

It was important to test whether the requirement of IRF8 is limited to the NDV system or whether this requirement is a more general aspect of DC IFN induction. To address this question, we infected *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs with the murine cytomegalovirus (MCMV). Results in Figure 2A show that whereas *Irf8*$^{+/+}$ DCs induced *Ifna* transcripts at high amounts that peaked at 7 hr, *Irf8*$^{-/-}$ DCs expressed virtually no IFN transcripts. *Ifnb* expression exhibited essentially the same pattern, where the transcripts were almost undetectable in *Irf8*$^{-/-}$ DCs. Consequently, little IFN proteins were produced in *Irf8*$^{-/-}$ cells in contrast to *Irf8*$^{+/+}$ cells that yielded a ng/ml order of IFNs. Vesicular stomatitis virus (VSV), another virus we tested, led to much lower expression of IFN transcripts than NDV and MCMV. Nevertheless, *Irf8*$^{+/+}$ DCs expressed measurable amounts of IFN transcripts, whereas *Irf8*$^{-/-}$ DCs induced *Ifna* transcripts at amounts barely above detection (data not shown). In Figures 2B and 2C, ligands for TLR 3, 7, and 9 (poly IC, R848, and CpG, respectively) were tested for IFN induction. These ligands constitute major components of RNA and DNA viruses. In *Irf8*$^{+/+}$ DCs, CpG DNA led to the highest induction of both *Ifna* and *Ifnb* transcripts and proteins, although other ligands also stimulated IFN transcript induction. In contrast, essentially no IFN transcripts were expressed in *Irf8*$^{-/-}$ DCs in response to these TLR ligands. These results show that the defect in expressing type I IFN in *Irf8*$^{-/-}$ DCs is not a specialized feature limited to certain stimuli, but it represents a fundamental, mechanistic deficit of the *Irf8*$^{-/-}$ DCs. Unlike what was observed with the NDV stimulation, the above stimuli elicited a single major IFN transcripts peak and did not display two clear peaks. It is likely that these stimuli

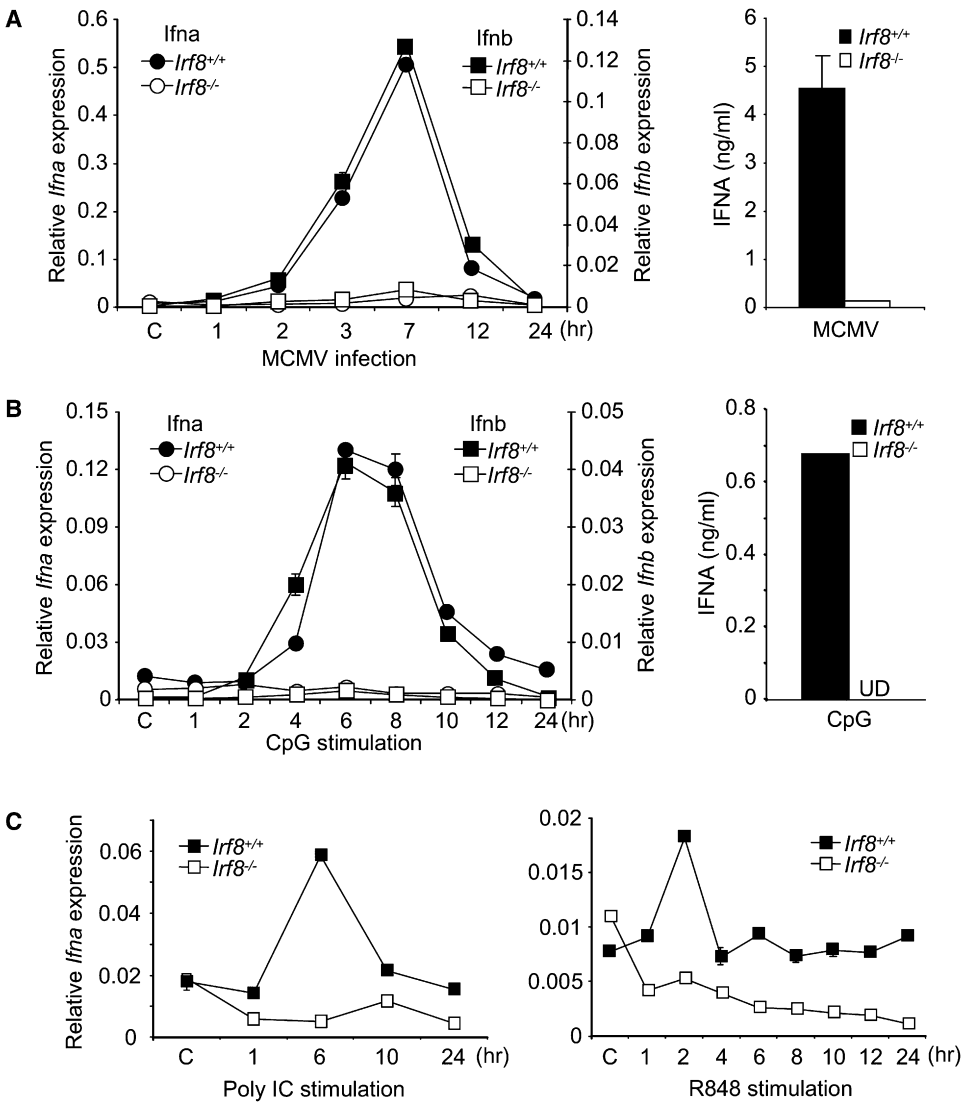

Figure 2. Absence of IFN Induction in *Irf8*$^{-/-}$ DCs after MCMV Infection and TLR Signaling

(A) DCs were stimulated with MCMV for 1 hr, and induction of *Ifna* (circle) and *Ifnb* (square) transcripts and IFNA protein production was measured as in Figure 1. Values represent the average of thee assays ± SD.

(B and C) DCs were stimulated with indicated TLR ligands, and *Ifna* (circle) and *Ifnb* (square) transcripts and proteins were detected as above. The ligands were present for the entire incubation time. Abbreviation: UD, IFN production was undetectable in *Irf8*$^{-/-}$ DCs. Values in (B) represent the average of three assays ± SD, and all data were reproduced in two separate assays.

generated two peaks in a shorter, overlapping interval, indicating that IFN induction kinetics may differ depending on types of stimuli.

The Second Peak Represents an IFN Feedback Response

We surmised that the second peak elicited by NDV was an equivalent of the IFN feedback loop described for non-DCs. To ascertain whether this idea holds true, we tested IFN transcript induction in DCs from *Ifnar1*$^{-/-}$ mice that would not generate the feedback loop because of the lack of type I IFN receptor. DCs generated from *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ mice, both in the BALB/c background, contained similar percentages of pDCs (Figure 3A). In Figure 3B, although *Ifnar1*$^{+/+}$ DCs showed a typical two-phase IFN mRNA induction upon NDV addition, *Ifnar1*$^{-/-}$ DCs produced no detectable second peak. The first peak in *Ifnar1*$^{-/-}$ DCs, although observed reproducibly, was lower than in *Ifnar1*$^{+/+}$ DCs, possibly because of prior IFN priming in *Ifnar1*$^{+/+}$ DCs. These data indicate that the second IFN peak, missing in *Irf8*$^{-/-}$ DCs, is accounted for by an IFN feedback response, which provides a major mechanism of enhanced IFN induction in DCs.

The above results raised the possibility that *Irf8*$^{-/-}$ DCs were deficient in responding to the IFN feedback signal. To test this possibility, we examined expression of several classical IFN-responsive genes (Figure 3C). Upon IFNA addition, 2′-5′ oligo (A) synthetase (2-5OAS), interferon

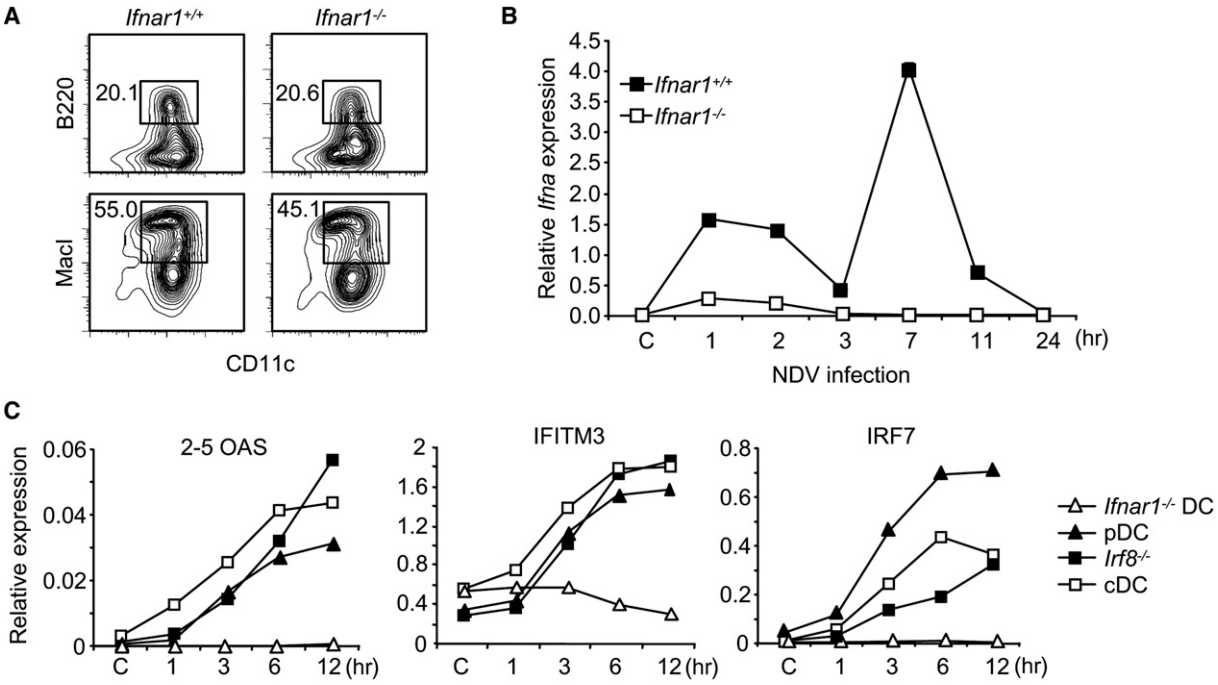

Figure 3. The Absence of the Second Peak in *Ifnar1*$^{-/-}$ DCs after NDV Infection

(A) Flow cytometry detection of pDCs in *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ DCs. B220^{+} and CD11b^{+} (MacI) cells are boxed in each panel.

(B) *Ifna* transcripts in above cells were measured as in Figure 1A.

(C) cDCs and pDC-enriched cells from *Irf8*$^{+/+}$ mice and DCs from *Irf8*$^{-/-}$ or *Ifnar1*$^{-/-}$ mice were stimulated with 500 U/ml of IFNA, and expression of indicated genes was monitored by qRT-PCR at indicated times. All data in Figure 3 were reproduced in at least two experiments.

induced transmembrane protein 3 (IFITM3), and IRF7 were expressed in *Irf8*$^{-/-}$ DCs along with *Irf8*$^{+/+}$ pDCs and *Irf8*$^{+/+}$ cDCs, indicating that *Irf8*$^{-/-}$ DCs respond to IFN signals. Only *Ifnar1*$^{-/-}$ DCs failed to express these genes. Supporting the idea that *Irf8*$^{-/-}$ DCs are capable of responding to the IFN feedback signal generated by viral stimulation, the above genes were also induced after NDV infection in both *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs (Figure S4).

To further delineate defects in *Irf8*$^{-/-}$ DCs, we asked whether IRF3 and IRF7 are activated in *Irf8*$^{-/-}$ DCs. To this end, nuclear translocation of the two proteins was examined, because it represented an endpoint event of TLR signaling, initiating IFN gene transcription. For IRF7, we analyzed DCs expressing IRF7 cDNA fused to the green fluorescent protein (GFP), because available antibodies were not suitable for immunostaining of endogenous IRF7. As seen in Figure 4A, IRF7-GFP was distributed mostly in the cytoplasm prior to infection. One hour after stimulation, intense fluorescent signals were observed in the nucleus (except for nucleoli), overlapping with DNA stain, both in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs, attesting to nuclear translocation in both DCs. In the majority of cells, there was residual IRF7-GFP in the cytoplasm after NDV stimulation, suggesting incomplete translocation or rapid reshuffling. Nuclear translocation of IRF3 was studied by immunoblot analysis of the cytoplasmic and nuclear fractions prepared before and 1 hr after NDV stimulation (Figure 4B). There was little cross contamination between the two fractions, as verified by the selective presence of c-cbl and YY1 in the cytoplasmic and nuclear fractions, respectively. Prior to NDV addition, IRF3 was detected mostly in the cytoplasmic fraction in *Irf8*$^{+/+}$ DCs, although the nuclear fraction also contained some IRF3. In *Irf8*$^{-/-}$ DCs, IRF3 was almost exclusively in the cytoplasm prior to infection. After stimulation, the nuclear IRF3 markedly increased both in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs, along with the reduction in the cytoplasm. In agreement with these results, immunostaining of endogenous IRF3 showed clear nuclear translocation after NDV stimulation (Figure 4C). These data indicate that the TLR signaling cascade functioned normally in *Irf8*$^{-/-}$ DCs to activate IRF3 and IRF7. It should be noted here that IRF8 resided almost exclusively in the nucleus and its expression did not change before and after NDV stimulation for 7 hr, as would have been expected (Figures 4B and 4D).

Exogenous IRF8 Amplifies IFN Gene Expression in Fibroblasts

Whereas type I IFNs are produced in many cells including fibroblasts, IRF8 is expressed only in the cells of hematopoietic lineage. It was of interest to test whether ectopic expression of IRF8 in fibroblasts enhances IFN expression. To this end, NIH 3T3 fibroblasts were transduced with a retroviral vector for IRF8, and *Ifna* and *Ifnb* transcript induction was examined after NDV stimulation (Figures 5A and 5B). Cells transduced with control, empty vector generated a relatively large first *Ifna* peak at 2 hr, followed by a much lower second peak seen between 10 hr and 16 hr.

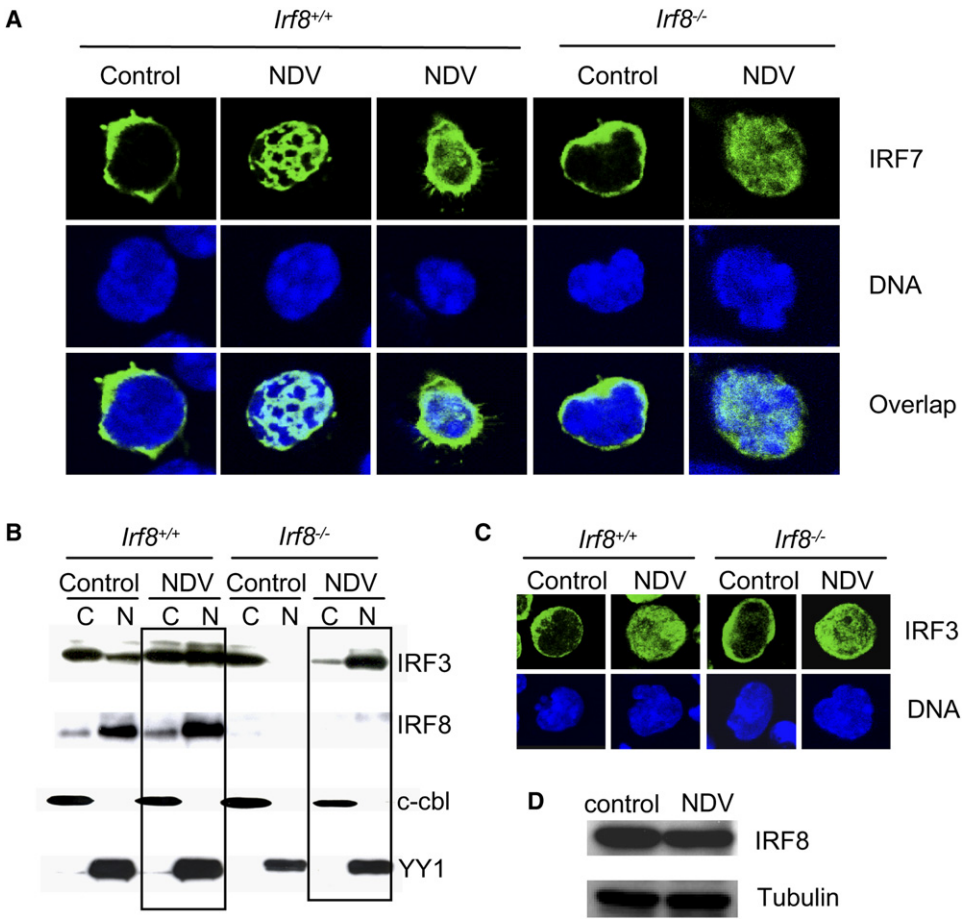

Figure 4. Nuclear Translocation of IRF7 and IRF3 in *Irf8*$^{-/-}$ DCs

(A) *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs were transduced with a vector for IRF7-GFP and stimulated without (control) or with NDV for 1 hr. Cells were fixed and stained with GFP antibody and counterstained with Hoechst 33342 for DNA. Images are representatives of at least 250 cells inspected for each sample. Similar results were observed in two to four independent experiments.

(B) *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs were infected with NDV for 1 hr as above, and nuclear and cytoplasmic fractions were tested for indicated proteins by immunoblot analysis. c-cbl and YY1 were used to monitor the quality of fractionation. Results are representative of two assays.

(C) *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs were stimulated with or without NDV and immunostained with antibody for endogenous IRF3 as in (A).

(D) *Irf8*$^{+/+}$ DCs stimulated with NDV for 7 hr, and whole-cell extracts were tested for IRF8 by immunoblot.

In contrast, cells transduced with IRF8 generated a second *Ifna* peak of much greater magnitude, although the magnitude of the first peak was not affected by IRF8. IRF8 also led to a dramatic amplification of the second peak for *Ifnb* transcripts. *Ifnb* induction in control cells was largely limited to the first phase, yielding a negligible second peak under these conditions. We noted that *Ifnb* transcription was higher than that of all *Ifna* transcripts in NIH 3T3 cells, suggesting a greater significance of this IFN subtype in fibroblasts. Together, in NIH 3T3 cells, type I IFN induction followed slower kinetics with a reduced second peak compared to those in DCs, and IRF8 introduction enhanced the second peak without altering the first peak.

To assess domains within IRF8 required for enhancing IFN induction, a series of IRF8 deletions and mutants were tested for IFN protein induction in NIH 3T3 cells. Previous studies have shown that both the DNA binding domain (DBD, gray in Figure 5C) and the IRF association domain in the C-terminal region (IAD, solid) are required for IRF8's transcriptional activity. Particularly, lysine (K) at position 79 and arginine (R) at 289 are essential for activity of the DBD and IAD, respectively (Tamura et al., 2005b; Tsujimura et al., 2003a). In Figure 5D, whereas full-length IRF8 and 1-390 gave a ~6-fold increase in the total amount of IFNA, other deletions, delN, 1-253, 1-305, and 1-356, gave only a background amounts of IFN. The mutants with substitutions in K79 and R289 also failed to enhance IFN production. These data are consistent with the idea that IRF8 enhances IFN induction at the point of transcription, not of IFN signaling.

IRF8 Binds to Type I IFN Promoters

Type I IFN transcription requires ordered binding of sequence specific factors such as IRF3 and IRF7 as well as cofactors that affect chromatin, leading to the recruitment of general transcription factors (Agalioti et al.,

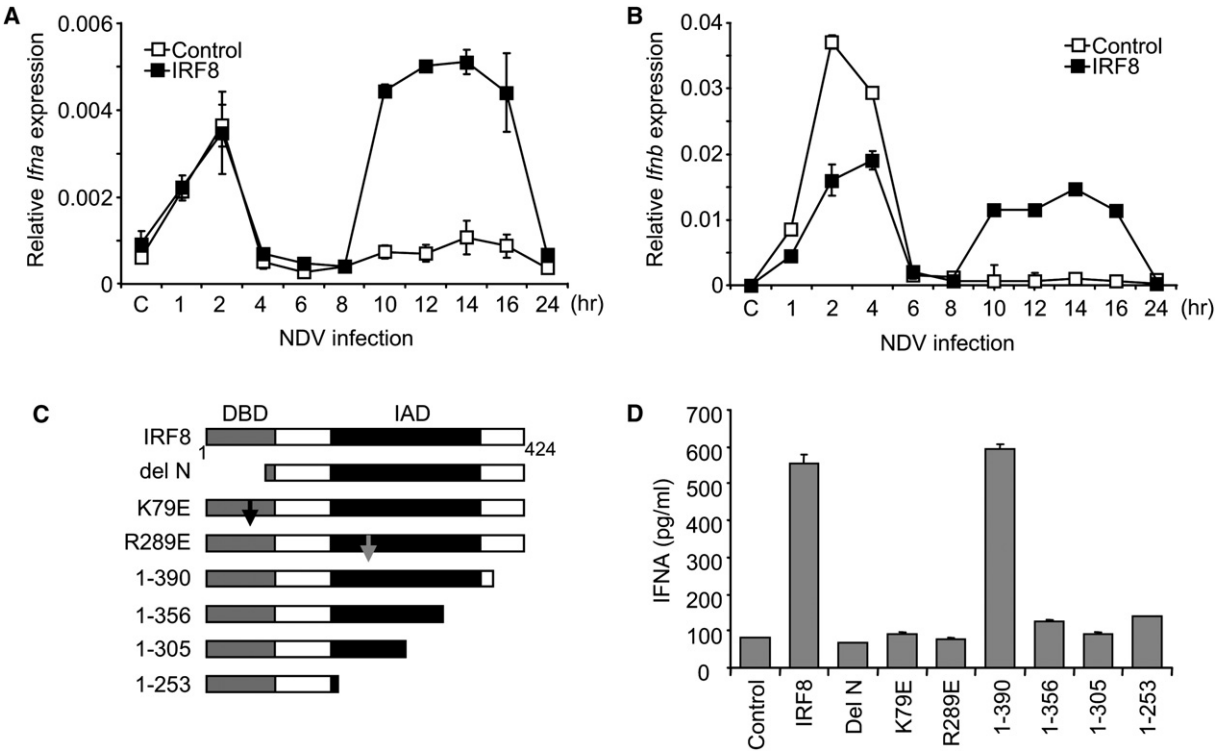

Figure 5. Amplification of the Second IFN Peak in Fibroblasts after IRF8 Introduction

(A and B) NIH 3T3 cells were transduced with control pMSCV vector or wild-type IRF8 vector and stimulated with NDV for 1 hr, and transcripts for *Ifna* and *Ifnb* were measured as in Figure 1A.

(C) Diagram of IRF8 mutants tested in (D).

(D) NIH 3T3 cells transduced with indicated IRF8 mutants were stimulated with NDV as above and production of IFNA was measured by ELISA. Values in all panels are the average of three samples. All data are representative of three assays.

2000; Maniatis et al., 1998; McWhirter et al., 2004). Ifna and Ifnb promoters carry IRF binding sites that are variously called the virus response element (VRE) and PRDI (Figure S5; Civas et al., 2006; Maniatis et al., 1998; Yang et al., 2003). To ascertain whether IRF8 participates in IFN transcription, we performed chromatin immunoprecipitation (ChIP) assays for several IFN promoters in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs. Figure 6A shows detailed kinetics of IRF8 binding to the *Ifna4* promoter after NDV addition. Whereas low constitutive IRF8 binding was seen before stimulation, the binding substantially increased after NDV stimulation, peaking at 4 hr with a slight decline at 5 and 6 hr. Afterwards, IRF8 binding increased slightly followed by a reduction again during the subsequent 11 hr period, overall showing a mild, oscillating pattern of recruitment, reminiscent of the patterns reported for other transcription factors (Reid et al., 2003). Specificity of IRF8 recruitment was substantiated by the lack of binding by control IgG to the promoter (Figure 6A) as well as by the lack of IRF8 antibody binding to the unrelated gene hypoxanthine guanine phosphoribosyl transferase, HPRT (Figure S5C). Further confirming specificity of ChIP results, IRF8 antibody did not bind to the promoter in *Irf8*$^{-/-}$ DCs (Figure 6B). Moreover, IRF8 was recruited to the *Ifna5* and *Ifnb* promoters as well, with a pattern similar to that for *Ifna4*, i.e., low constitutive binding prior to NDV stimulation, followed by increased recruitment after NDV stimulation (Figures 6B and 6C). We also performed ChIP assays after TLR signaling and found that IRF8 was recruited to the *Ifna4* promoter after CpG stimulation as well, supporting the view that IRF8 recruitment to the promoter is a general event that takes place during IFN induction (Figure S5B).

In Figure 6D, ChIP was performed in *Ifnar1*$^{-/-}$ DCs. Whereas IRF8 binding to the *Ifna4* promoter increased after NDV stimulation in *Ifnar1*$^{+/+}$ DCs, no increase in IRF8 binding was detected in *Ifnar1*$^{-/-}$ DCs. Similar results were seen for *Ifna5* and *Ifnb* promoter (not shown). These data indicate that increased IRF8 binding after NDV stimulation depends on IFN feedback signals.

ChIP assays were next performed to examine binding of IRF7 to the *Ifna4* promoter. To this end, we tested *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs expressing IRF7-Flag (Figure 6E). IRF7-Flag bound to the promoter to a low degree prior to NDV addition and the binding increased after NDV stimulation, reached a plateau at 1 or 3 hr and then declined at 6 hr both in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs. IRF7-Flag binding was slightly higher in *Irf8*$^{-/-}$ cells than *Irf8*$^{+/+}$ DCs, which may be partly attributed to higher ectopic expression of IRF7 noted in *Irf8*$^{-/-}$ compared to *Irf8*$^{+/+}$ cells. ChIP with

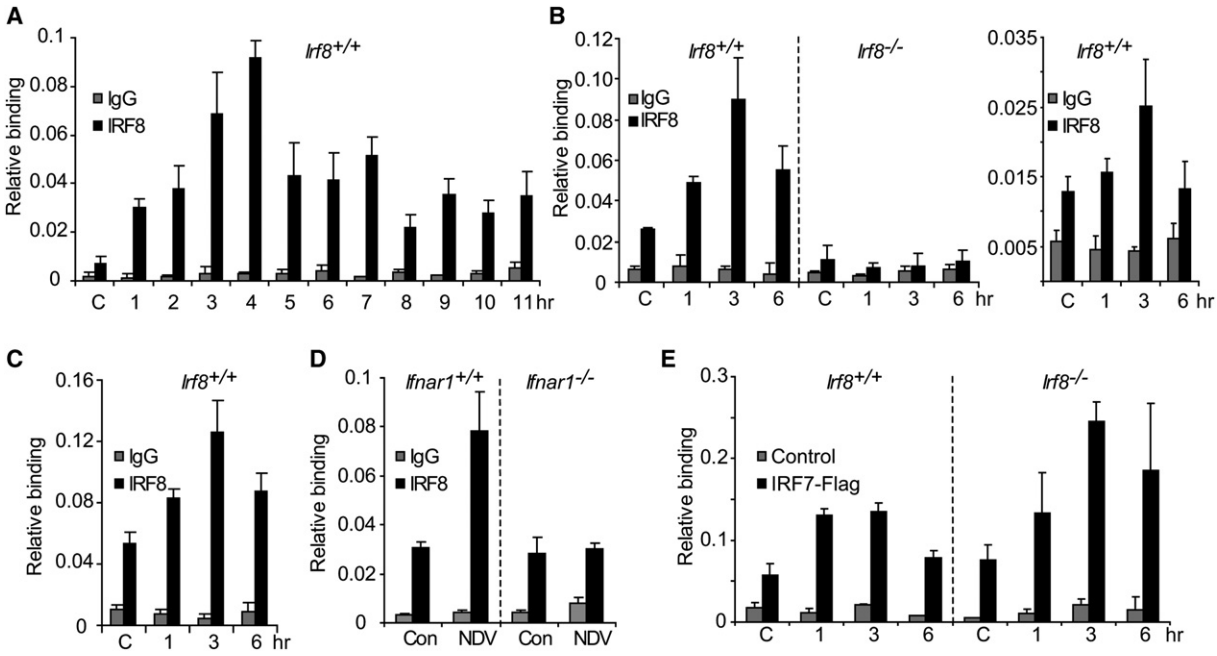

Figure 6. Recruitment of IRF8 to the IFN Promoters

(A) Kinetics of IRF8 recruitment to the *Ifna4* promoter. *Irf8*$^{+/+}$ DCs were stimulated with NDV, and ChIP analysis was performed at indicated times with anti-IRF8 or control IgG. Values in all graphs represent the average of three assays ± SD. Each experiment was reproduced two to three times.

(B) IRF8 ChIP analysis was performed with *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs for the *Ifna4* (left) and the *Ifna5* promoters (right).

(C) IRF8 ChIP analysis for the *Ifnb* promoter with *Irf8*$^{+/+}$ DCs.

(D) ChIP analysis was performed 3 hr after NDV stimulation for the *Ifna4* promoter with *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ DCs.

(E) *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs were transduced with pMSCV-IRF7-GFP-Flag and stimulated with NDV. ChIP analysis was performed for *Ifna4* promoter with anti-Flag.

anti-IRF7 in above cells also showed IRF7 binding both in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ cells (Figures S5D and S5E). These results show that binding of IRF7 to the IFN promoters does not require IRF8.

IRF8 Supports Sustained RNA Polymerase II Recruitment to the IFN Promoters

Transcription initiation begins after the assembly of basal transcription machinery at the promoter (Roeder, 2005; Sims et al., 2004). RNA polymerase II (RNAPII) is the central molecule in the machinery that catalyzes RNA synthesis. To address the mechanism by which IRF8 binding impacts on type I IFN transcription, we performed ChIP assays for RNAPII. Data in Figure 7A show kinetics of RNAPII recruitment to the *Ifna4* promoter in *Irf8*$^{+/+}$ DCs. RNAPII was not bound before stimulation, but strong binding was seen at 1 hr after NDV stimulation followed by a slight reduction at 2 hr. RNAPII recruitment rose again at 3 hr, after which RNAPII binding remained steady for the subsequent 6 hr period. In Figures 7B–7D, RNAPII recruitment to the *Ifna4, Ifna5, Ifnb* promoters was compared between *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs. RNAPII was recruited 1 hr after NDV stimulation both in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ cells on all promoters. In *Irf8*$^{+/+}$ cells, the recruitment continued for the remaining period. However, in *Irf8*$^{-/-}$ DCs, RNAPII recruitment rapidly declined after an initial rise; a decline was seen as early as at 3 hr with a further fall at 6 hr. Thus, although RNAPII was recruited to the IFN promoters in the early phase in both *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs, its subsequent recruitment was prematurely attenuated in the absence of IRF8. These data indicate that IRF8 facilitates sustained recruitment of RNAPII, most likely responsible for amplification of IFN transcription in the second phase.

DISCUSSION

This investigation revealed two unique features associated with type I IFN induction in DCs. First, IFN induction is composed of two responses, and elicitation of the second response, mediated by IFN feedback response, requires IRF8. This conclusion is supported by the observations that (1) *Irf8*$^{-/-}$ cDCs did not elicit the second IFN mRNA peak and that (2) ectopic IRF8 conferred a large second peak upon fibroblasts. Amplification of the second peak is likely to be a major contributor of copious IFN production characteristic to DCs, as shown by the fact that *Irf8*$^{-/-}$ DCs, although they generated the first response, produced only a background amount of IFNs. Thus, IRF8 appears to have a mechanism to augment IFN gene transcription in DCs in response to both viral and TLR signals. Our results indicate that IRF8 functions in a postinduction phase to amplify IFN transcription and

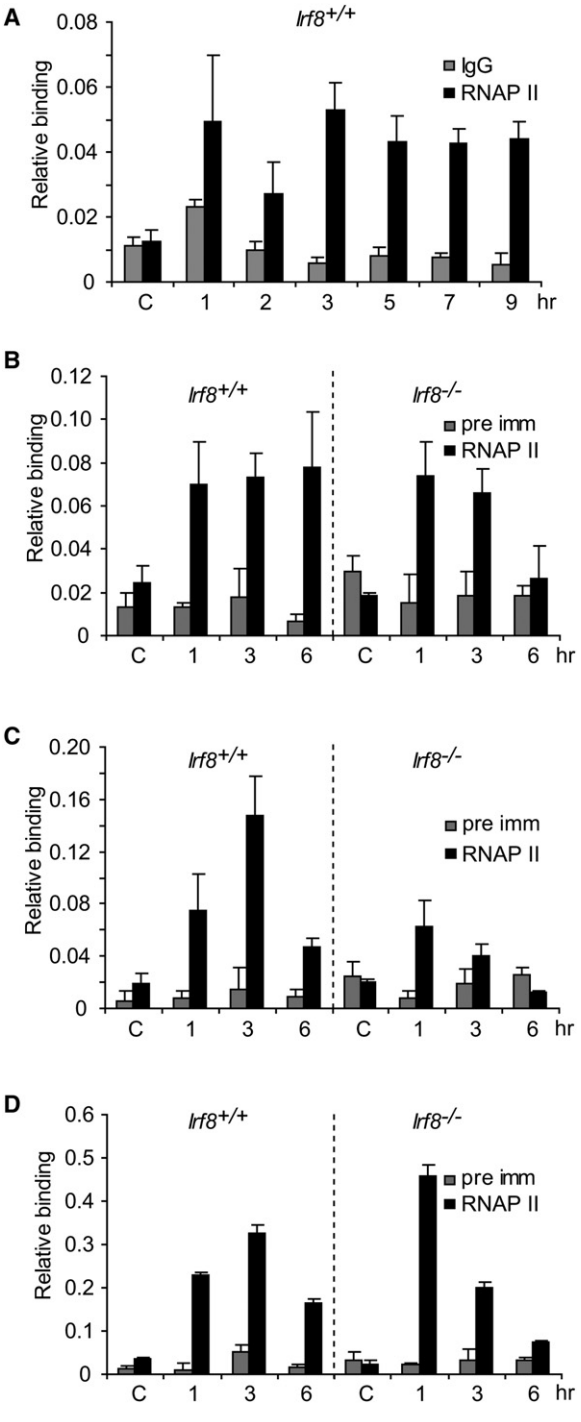

Figure 7. Recruitment of RNAPII to the IFN Promoters after NDV Stimulation

(A) Kinetics of RNAPII recruitment to the *Ifna4* promoter. $Irf8^{+/+}$ DCs were stimulated with NDV, and ChIP analysis was performed at indicated times with antibody for RNAPII. Values in these graphs represent the average of three determinations ± SD. Experiments were reproduced twice.

(B–D) ChIP for RNAPII was performed with $Irf8^{+/+}$ and $Irf8^{-/-}$ DCs for the *Ifna4* (B), *Ifna5* (C), and *Ifnb* (D) promoters.

protein production. The notion that IRF8 acts on a late phase of transcription is consistent with the previous reports that IRF8 augments expression of some IFN-responsive genes with a delayed time course in macrophages (Kanno et al., 2005).

The second aspect of DC-specific IFN induction unraveled in this work is that multiple *Ifna* subtypes are induced during the first phase in DCs, unlike in non-DCs where the initial induction is restricted to the *Ifnb* and *Ifna4* subtypes. Although the molecular basis of this difference is not fully clear at present, it is possible that the IFN gene cluster assumes an open chromatin configuration in DCs, allowing IRF3 and IRF7 to have immediate access to multiple *Ifna* promoters. Irrespective of the mechanism, early activation of multiple *Ifna* subtypes may have a large impact on the overall IFN production in DCs, because it would help provide a rapid onset and greater elicitation of second response. Supporting this view, the onset of the second peak was seen earlier and its magnitude was markedly greater in DCs than in NIH 3T3 fibroblasts. In view of DC-specific mode of gene expression revealed here, the IFN induction pathways previously outlined for non-DCs could be modified to accommodate the above characteristics (Figure S5F).

Our data clearly show that IRF8's main role is to stimulate IFN gene transcription, rather than to act in the upstream signaling events. In line with a role in transcription rather than signaling, IRF8 was not required for nuclear translocation of IRF3 and IRF7, nor was it required for establishment of an IFN feedback arm, as evidenced by induction of IFN-responsive genes in $Irf8^{-/-}$ DCs. The involvement of IRF8 in transcription was demonstrated by the recruitment of IRF8 to the *Ifna* and *Ifnb* promoters. In line with these data, Kawai et al. (2005) identified IRF8 as an activator of *Ifnb* promoter by screening a spleen cDNA library. Importantly, IRF8 recruitment was increased upon NDV addition, peaking at 4 hr well after induction of the first phase, consistent with its action in the second phase of transcription. It is clear that increased IRF8 recruitment was dependent on IFN feedback: $Ifnar1^{-/-}$ DCs revealed no increase in IRF8 recruitment after NDV. In these ChIP assays, a low amount of IRF8 appeared to be bound to the IFN promoters before and soon after NDV addition, suggesting that some fraction of IRF8 was recruited to the promoter as a functionally inactive state. It is of note here that binding of IRF8 (and that of IRF7-Flag) was not very robust in these experiments, although reproducibly observed. We attribute the relatively low binding to the fact that our ChIP assays were performed with primary DC cultures composed of heterogeneous cell populations, some of which may not express IFN genes.

What is the mechanism by which IFN feedback signals increase IRF8 recruitment? Given that IRF8 expression remained unchanged before and after NDV stimulation, it is likely that IFN signals increase the affinity of existing IRF8 for the promoters, presumably through a post-translational modification. Phosphorylation of IRF8 may be a major mechanism of increased binding, in light of

the fact that IFN signals activate kinases of the JAK and MAPK families (Darnell, 1996; Platanias, 2005). IFN signals may also stimulate IRF8 to interact with additional regulatory factors not associated before, allowing IRF8 to bind to the promoter more efficiently. Supporting this view, IRF8 is shown to be under control of tyrosine phosphorylation and dephosphorylation and to interact with a partner in an IFN signal-dependent manner (Huang et al., 2006; Kuwata et al., 2002; Sharf et al., 1997). Ubiquitination of IRF8, shown to occur through an IFN-inducible E3 ligase, may also play a role in modulating IRF8's transcriptional activity (Kong et al., 2007).

Specific transcription factors, when bound to the regulatory element, trigger assembly of many factors at or near the initiation site, culminating in the recruitment of the RNAPII complex and synthesis of new RNA chains (Roeder, 2005; Sims et al., 2004). RNAPII was not present on the IFN promoters prior to stimulation, but was recruited upon NDV stimulation. Our results are consistent with signal-dependent recruitment of RNAPII previously reported for the promoters of inducible proinflammatory genes (Saccani and Natoli, 2002). Once recruited, RNAPII continued to occupy the IFN promoters up to 9 hr in *Irf8*$^{+/+}$ DCs. However, in *Irf8*$^{-/-}$ cells, RNAPII recruitment fell prematurely after 1 hr in all three promoters. Our data indicate that IRF8 is equipped with the capacity to retain RNAPII on the IFN promoters for an extended period of time. This capacity is not shared by IRF7, as shown by the fact that IRF7 was bound to the promoter both in *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ DCs. It has been shown that activities of RNAPII are regulated at many steps including those of preinitiation, initiation, elongation, termination, and recycling (Sims et al., 2004). Given that IFN transcription in DCs was typified by prolonged RNAPII recruitment, recycling of RNAPII and transcriptional reinitiation may be an important mechanism that constitutes DC-specific IFN transcription. In light of our findings that IRF8 functions during the second phase of IFN transcription, it is conceivable that its main role is to support efficient transcriptional reinitiation by facilitating repeated recruitment of RNAPII to the promoters. Although mechanisms controlling RNAPII recycling are not fully understood, it has been shown that some DNA-specific transcription factors play a role in this process (Liu et al., 2001).

In summary, IRF8 plays a key role in amplifying the second phase of type I IFN gene transcription in DCs. It helps prolong RNAP II recruitment to the IFN promoters. Together, IRF8 is a critical contributor of rapid and abundant type I IFN production typified in DCs, thereby participating in the establishment of innate immunity.

EXPERIMENTAL PROCEDURES

Mice and Cell Cultures

Irf8$^{-/-}$ mice were maintained in the NICHD animal facility. *Ifnar1*$^{-/-}$ mice were obtained from J. Durbin (Ohio State University) through H. Young (NCI). All animal work conformed to the NICHD animal care and use committee guidelines. Bone marrow mononuclear cells were cultured in the presence of Flt3L to generate DCs (Tamura et al., 2005a; Tsujimura et al., 2003b). DC surface markers were examined by flow cytometry with anti-CD11b, anti-B220, and anti-CD11c (BD PharMingen), biotin-labeled anti-Siglec-H (Hycult Biotechnology, Netherlands), and FITC-labeled anti-Ly49Q (MBL International). Data were analyzed with FlowJo software (Tree Star San Carlos, CA). To prepare cDC and pDC-enriched DC populations, ~10^7 cells were incubated at 4°C for 15 min with FITC-conjugated anti-B220 (1:500 dilution) in 200 μl of PBS containing 0.5% BSA and 2 mM EDTA. Cells were washed and incubated with anti-FITC microbeads (10 μl) in total 100 μl buffer. Cells were then subjected to magnetic separation with MACS separation columns (Miltenyi Biotec, Germany). Cells that did not bind to column were collected as pDC-free cDCs, and the bound fraction was collected as a pDC-enriched population. For NDV viral stimulation, 10^6 DCs were infected with the Heartz stain of NDV at 640 hemagglutination units (Schiavoni et al., 2002) for 1 hr. Similarly, 10^6 DCs were infected with wild-type MCMV, derived from pSM3fr, a Bac clone of the Smith strain (Kulesza and Shenk, 2006) at a moi of 5 for 1 hr. Cells were washed twice and incubated with complete media and harvested at indicated intervals starting from initial exposure of virus. For stimulation with TLR ligands, DCs were incubated with 1 μg/ml of CpG 1826 (Lofstrand Labs), 100 μg/ml of polyIC (Amersham Pharmacia), or 100 nM of R848 (Alexis Biochemicals) for indicated periods of time. NIH 3T3 cells were maintained as in Laricchia-Robbio et al. (2005). 1 × 10^5 cells in a well of 6-well plate of control or IRF8-transduced NIH 3T3 cells were infected with NDV at 100 hemaglutination units/ml for 1 hr, and cells were washed twice and incubated in the media for 24 hr. Supernatants were analyzed for IFNA production by ELISA (Pestka Biological Laboratories). Total RNA was extracted with TRIzol reagent (Invitrogen), and cDNA was prepared with Superscript II enzyme (Invitrogen) according to manufacturer's protocol. For qPCR, amplification of sample cDNA was monitored with the fluorescent DNA-binding dye SYBR Green (SYBR Green PCR master kit; Applied Biosystems) in combination with the ABI Prism 7000 Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. Transcript levels were normalized by amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Primer sequences used for PCR are listed in Table S2. Semiquantitative RT-PCR was performed with primer sequence described in Marie et al. (1998).

Retroviral Transduction

pMSCV retroviral vectors for IRF8 and mutants were described (Tamura et al., 2005a; Tsujimura et al., 2003a). Construction of pMSCV vector for IRF7-Flag is described along with Table S2. DCs were transduced with the viral supernatants prepared in BOSC23 packaging cells by spinoculation (2400 rpm, 33°C, 1 hr) with 4 μg/ml polybrene on day 5 and selected by 2 μg/ml of puromycin for 48 hr (Tamura et al., 2005a). NIH 3T3 cells were transduced as above and selected with 4 μg/ml puromycin for 4 days (Laricchia-Robbio et al., 2005).

Immunofluorescent Staining

DCs were placed on cytospin slides and fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min, and blocked by 3% BSA for 1 hr. Cells were incubated with anti-GFP (Roche Applied Science) diluted at 1:200, or rabbit anti-IRF3 (Zymed) diluted at 1:100 followed by Biotin-conjugated anti-mouse or rabbit IgG (Amersham Biosciences) for 45 min and then incubated with Streptavidin Alexa-488 (Molecular Probes). Cells were counterstained with Hoechst 33342. Stained cells were viewed on a confocal microscope (Leica, Model TCS, SP2).

Immunoblot Analysis

Nuclear extracts and cytoplasmic fractions were prepared as follows. Ten million DCs were incubated in 0.3 ml of buffer containing 10 mM HEPES (pH 7.9), 2.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40 with protease inhibitor cocktail (Roche Applied Science) on ice for 15 min, and centrifuged. Supernatants were used as cytoplasmic fractions. Nuclear pellets were incubated with 20 mM HEPES (pH 7.9), 2.5 mM $MgCl_2$, 20 mM EDTA, 420 mM KCl, 25% glycerol supplemented with

protease inhibitor cocktail on ice with occasional stirring, and supernatants were collected by centrifugation. Proteins separated on 4%–12% SDS-PAGE were transferred onto PVDF membrane (Millipore) and reacted with indicated antibodies. Secondary detection was carried out with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Amersham Bioscience), followed by chemiluminescence development with ECL reagent (Pierce Biotechnology).

ChIP Assays

This assay was performed with the protocol recommended by Upstate with slight modifications (Tamura et al., 2005a). In brief, 2.5–10 × 10^6 cells were treated with 1% formaldehyde for 30 min at room temperature. Cells were lysed in 650 μl of lysis buffer and sonicated on ice with an XL2007 sonicator (Misonix) to shear DNA into 300 bp–2 kb long fragments. After centrifugation, 100 μl of supernatants were precleared with 25% of protein A/G agarose slurry (Santa Cruz Biotechnology) supplemented with 200 μg/ml of salmon sperm DNA for 1 hr at 4°C. Chromatin was then incubated with 2 μg of normal goat IgG or goat anti-IRF8 (C-19, Santa Cruz Biotechnology) overnight with rotation. Normal mouse IgG (2 μg), monoclonal anti-Flag antibody M2 (2 μg, Sigma), Ig control, and rabbit anti-RNAPII (8WG16; Covance) were used for ChIP assays for IRF7-Flag and RNAPII. This was followed by incubation with Protein A/G agarose slurry supplemented with salmon sperm DNA for additional 1 hr, and precipitated. Precipitates were washed twice with low salt buffer, twice with high salt buffer, then with a LiCl buffer and finally with Tris-EDTA buffer (pH 8.0). After elution, complexes were digested with RNase and proteinase K. DNA was purified by phenol: chloroform, precipitated in ethanol, and resuspended in 50 μl of buffer containing 5 mM Tris (pH 8.0). Five microliters of the final preparations were subjected to qPCR with primers in Table S2. Input DNA (1%) was used for normalization.

Supplemental Data

Five figures and two tables are available at http://www.immunity.com/cgi/content/full/27/2/228/DC1/.

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