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# CD40 Stimulates a “Feed-Forward” NF- $\kappa$ B–Driven Molecular Pathway That Regulates IFN- $\beta$ Expression in Carcinoma Cells

Aristides Moschonas, Marina Ioannou, and Aristides G. Eliopoulos

IFN- $\beta$  and the CD40L (CD154) share important roles in the antiviral and antitumor immune responses. In this study, we show that CD40 receptor occupancy results in IFN- $\beta$  upregulation through an unconventional “feed-forward” mechanism, which is orchestrated by canonical NF- $\kappa$ B and involves the sequential de novo synthesis of IFN regulatory factor (IRF)1 and Viperin (RSAD2), an IRF1 target. RelA (p65) NF- $\kappa$ B, IRF1, and Viperin-dependent IRF7 binding to the IFN- $\beta$  promoter largely controls its activity. However, full activation of IFN- $\beta$  also requires the parallel engagement of noncanonical NF- $\kappa$ B2 signaling leading to p52 recruitment to the IFN- $\beta$  promoter. These data define a novel link between CD40 signaling and IFN- $\beta$  expression and provide a telling example of how signal propagation can be exploited to ensure efficient regulation of gene expression. *The Journal of Immunology*, 2012, 188: 5521–5527.

Initially defined by their antiviral activity, the type I IFNs IFN- $\alpha$  and IFN- $\beta$  are now known to have a broader role in shaping the host immune response to diverse xenogeneic or autologous nucleic acids and inflammatory mediators (1). Endogenous type I IFNs produced by immune (2, 3) or malignant (4) cells have been recognized as potent regulators of dendritic and NK cell function (5), preventing the growth of carcinogen-induced and transplantable tumors (6).

Intriguingly, the spectrum of type I IFN functions displays similarities to that of CD40, a TNFR family member with a pivotal role in the generation of adaptive and innate immune responses (reviewed in Ref. 7). Humans with mutations in the CD40/CD40L genes develop a rare immune disorder called X-linked hyper-IgM syndrome, which is characterized by impaired macrophage, dendritic, and B cell function, and is clinically manifested by recurrent viral and bacterial infections (8, 9) and, interestingly, increased susceptibility to malignancy (10). The antiviral activity of CD40L has also been documented in the mouse (11), and increasing experimental and clinical evidence suggests that activation of the CD40 pathway could be exploited for cancer therapy (12, 13). Indeed, the stimulation of tumor cells with CD40L induces the expression of components of the Ag presentation machinery re-

quired for the engagement of antitumor immune responses (14) through the sequential activation of NF- $\kappa$ B and its target, IRF1 (15). Interestingly, IRF1 participates in an autoregulatory loop in the context of type I IFN signaling in which IRF1 is both a target and a transcriptional activator of IFN- $\beta$  (16, 17).

The aforementioned functional similarities provided a theoretical link between CD40 and IFN- $\beta$  and prompted us to evaluate the hypothesis that IFN- $\beta$  is a physiological target of CD40 signaling. Data presented in this study confirm this hypothesis and demonstrate that the IFN- $\beta$  gene is induced by CD40L in human carcinoma cells.

The mechanism of IFN- $\beta$  transactivation has mostly been studied in the context of virus infection and represents one of the best-characterized examples of inducible regulation of gene expression (reviewed in Ref. 18). Central to IFN- $\beta$  expression is a 50-bp sequence located between –105 and –55 bp upstream the transcription start site, which contains four positive regulatory domains (PRDs), PRDI–IV. Following virus infection, p65 (RelA) NF- $\kappa$ B binds the PRDs and “nucleates” the progressive assembly of a multiprotein complex termed the enhanceosome. In addition to p65, this complex comprises IFN regulatory factors (IRFs), cJun/ATF2 dimers, and high mobility group protein A1. The assembled enhanceosome recruits the coactivator protein PCAF, which stimulates histone acetylation in adjacent nucleosomes resulting in recruitment of RNA polymerase II and transcription activation.

Virus infection is the only known signal that can activate all of the IFN- $\beta$  transcriptional activators simultaneously (19, 20). In this paper, we describe a nonconventional mechanism of IFN- $\beta$  gene regulation that is orchestrated by NF- $\kappa$ B and involves the sequential de novo synthesis of IRF1 and viperin, a protein that participates in the IRF7-mediated induction of IFN- $\beta$  (21).

## Materials and Methods

### Cell culture, constructs, and reagents

The bladder carcinoma cell lines EJ and T24 and the immortalized keratinocytic cell line HaCaT were maintained in RPMI 1640 medium supplemented with 10% FCS. The human embryonic kidney (HEK) line 293 was cultured in DMEM supplemented with 10% FCS (Life Technologies). Wild-type and mutated CD40 constructs were established as described previously (22). The I $\kappa$ B kinase 2 (IKK2) kinase inhibitor was purchased from Calbiochem (SC-514) and dissolved in dimethyl sulfoxide prior

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A.M. conceived and performed the experiments, analyzed the data, and edited the manuscript; M.I. performed experiments; A.G.E. conceived and coordinated the project and wrote the manuscript.

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Abbreviations used in this article: ChiP, chromatin immunoprecipitation; HA, hemagglutinin; HEK, human embryonic kidney; IKK2, I $\kappa$ B kinase 2; IRF, IFN regulatory factor; PRD, positive regulatory domain; RNAi, RNA interference; RSAD2, radical S-adenosyl methionine domain-containing 2; siRNA, small interfering RNA; TRAF, TNFR-associated factor.

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to use. Recombinant soluble CD40L was purchased from Bender Med-Systems.

#### Abs and immunoblotting

The IRF1 (C-20), CD40 (C-20), p65 (C-20), and RNA polymerase II (N-20) Abs were purchased from Santa Cruz Biotechnology. The p52 Ab was purchased from Cell Signaling Technology, the IRF7 from Acris and the actin Ab from Sigma-Aldrich. Immunoblotting was performed as described previously (15).

#### RNA interference

For the delivery of small interfering RNAs (siRNAs),  $4 \times 10^4$  EJ or  $6 \times 10^4$  HEK293 cells were plated into each well of a 24-well plate (Costar), and the next day, the cells were transfected with siRNA duplexes by using the siMPORTEER transfection reagent, according to the instructions of the manufacturer (Upstate Biotechnology). IRF1, Viperin, and NF- $\kappa$ B2 were targeted with the SMARTpool siRNA duplexes M-011704, M-015423, and M-003918, respectively (Dharmacon). The control siRNA duplex comprised the sequence CGUACGCGGAUACUUCGAUU and the corresponding antisense strand. For the delivery of siRNAs, cells were cultured with the transfection mixture for 7–8 h in OptiMem medium (Invitrogen), and then, a volume of medium equal to that of the mixture was added. Twelve hours later, cells were replated into 24-well dishes. The next day, the cells were subjected to a second round of transfection to increase the siRNA-mediated gene suppression. Twenty-four hours later, cultures were stimulated with CD40L, before lysis, and further analysis.

#### Reporter assays

A reporter construct (pIFN- $\beta$ /Luc) in which the luciferase gene is driven by a 110-bp IFN- $\beta$  promoter segment containing the PRDs (PRDI-IV) in pGL2-Basic vector (Promega) was used to assess IFN- $\beta$  promoter activity. HEK 293 cells ( $1.5 \times 10^5$  cells/well in a 12-well plate) were transfected with 50 ng pIFN- $\beta$ /Luc and 50 ng of a *Renilla* plasmid and plasmids expressing CD40WT, CD40mT2/T3, CD40 mT2/T3/T6, or HA-p52 by using Lipofectamine (Invitrogen) in Opti-MEM medium (Invitrogen),

according to the manufacturer's instructions. Following a 10-h incubation with the transfection mixture, the medium was changed to DMEM supplemented with 10% FCS, and Luciferase and *Renilla* values were measured 24 h later as described previously (15).

#### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (15). The sequences of the primers used were as follows: Viperin forward, 5'-CTC CAC CAG CCA ATC AGT CT-3'; Viperin reverse, 5'-GGA GCA GGA CAC ACC TTC TT-3'; IFN- $\beta$  forward, 5'-CAT TTC CGG ATG GAG GAT AA-3'; and IFN- $\beta$  reverse, 5'-ACT CGG CTC TTC CAC TCA AA-3'.

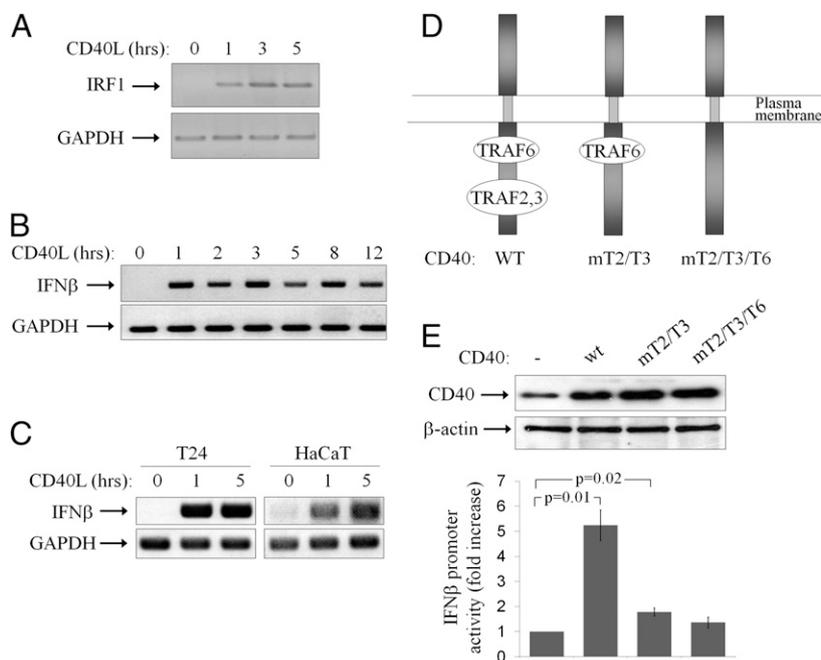
#### RT-PCR

RNA was isolated using TRIzol according to the instructions of the manufacturer (Invitrogen). Two micrograms of RNA was then used for cDNA synthesis with avian myeloblastosis virus reverse transcriptase and a reverse transcription system from Promega. PCRs were performed using 1/10 of the cDNA reaction mixture. The sequences of the primers used were as follows: Viperin forward, 5'-AGT GGC TCA CGC CTG TAA TC-3'; Viperin reverse, 5'-GTGTCCTTGGGCTTTCACAT-3'; IFN- $\beta$  forward, 5'-GTCACCTGTGCCTGGACCATA-3'; and IFN- $\beta$  reverse, 5'-TGCAG-TACATTAGCCATCAGTCA-3'.

## Results

### CD40 ligation upregulates IFN- $\beta$ gene expression

Our previous work has shown that CD40 signaling in carcinoma cells results in induction of NF- $\kappa$ B activity, which mediates rapid transactivation of IRF1 (Fig. 1A) (15). As both transcription factors have been implicated in the regulation of IFN- $\beta$ , we asked whether CD40 ligation also stimulates the induction of IFN- $\beta$  gene expression. Exposure of EJ bladder carcinoma cells to recombinant soluble CD40L led to rapid and sustained upregulation



**FIGURE 1.** CD40 activation results in IFN- $\beta$  gene expression. **(A)** Stimulation of EJ cells with 0.5  $\mu$ g/ml recombinant soluble CD40L results in upregulation of IRF1 mRNA expression levels, as determined by RT-PCR. **(B, C)** Stimulation of EJ (B) and T24 and HaCaT (C) cell lines with CD40L results in upregulation of IFN- $\beta$  mRNA expression levels, as determined by RT-PCR. **(D)** Graphical representation of CD40 and its TRAF binding domains. TRAF2 and TRAF3 associate with a membrane-distal domain at the cytoplasmic C terminus of CD40, whereas TRAF6 interacts with a membrane-proximal region. A T<sup>254</sup>→A mutation yielding CD40mT2/T3 (mT2/T3) inhibits TRAF2 and TRAF3 binding but leaves TRAF6-CD40 interactions intact, whereas a triple Q<sup>234</sup>E<sup>235</sup>T<sup>254</sup>→AAA mutation, yielding CD40mT2/T3/T6, perturbs the binding of all TRAFs. **(E)** The TRAF2/TRAF3-interacting domain of CD40 mediates IFN- $\beta$  promoter activation signals. HEK 293 cells were cotransfected with 50 ng of a reporter construct containing the *Luciferase* gene under the control of the IFN- $\beta$  promoter (PRDI-IV), a *Renilla* reporter to control for transfection efficiency, and 100 ng of the wild-type or mutated CD40 sequences described in (D). The mean relative Luciferase values (ratio of *Luciferase* to *Renilla* activities  $\pm$  SD) from four independent experiments were determined and expressed as fold increase in IFN- $\beta$  promoter activity. The *p* values, determined by Student *t* test, are shown. Immunoblotting with anti-CD40 Ab confirmed similar levels of transfected CD40 (upper panel).

of IFN- $\beta$  mRNA (Fig. 1B). Similar results were obtained in CD40-stimulated HaCaT, an immortalized aneuploid human keratinocyte cell line, and T24 bladder carcinoma cells (Fig. 1C), highlighting the generality of the CD40 effect.

CD40 signal transduction depends on TNFR-associated factor (TRAF) proteins (reviewed in Refs. 23–25). Specifically, a membrane-distal domain of the cytoplasmic C terminus of CD40 recruits TRAF2 and TRAF3, whereas a membrane-proximal region binds to TRAF6 (Fig. 1D). Our previous work in carcinoma cells has demonstrated that the TRAF2/TRAF3-interacting domain of CD40 is primarily responsible for the engagement of NF- $\kappa$ B, JNK, and p38 cascades and IRF-1 upregulation, whereas the TRAF6-binding region contributes to NF- $\kappa$ B signaling (15, 22). To address the impact of CD40–TRAF interactions on CD40L-induced IFN- $\beta$  induction, HEK 293 cells were transfected with a reporter construct (pIFN- $\beta$ /Luc) in which the luciferase gene is driven by an IFN- $\beta$  promoter segment containing the PRDs (PRDI–IV). Wild-type or mutated CD40 sequences, which are unable to directly associate with TRAF2/TRAF3 (CD40mT2/mT3), or all TRAFs (CD40mT2/T3/T6) (Fig. 1D) (22), were cotransfected with pIFN- $\beta$ /Luc. As previously described (26, 27), the overexpression of CD40 in 293 cells results in signal activation through the transient formation of receptor multimers and the recruitment of TRAFs in a ligand-independent manner. Consistent with these observations, ectopic expression of wild-type CD40 resulted in an ~5-fold increase in IFN- $\beta$  promoter activity relative to control vector, which was attenuated in either CD40mT2/mT3 or CD40mT2/T3/T6-transfected cells (Fig. 1E). We conclude that the TRAF2/TRAF3-interacting domain of CD40 is largely responsible for IFN- $\beta$  gene transactivation.

#### CD40 ligation stimulates the recruitment of NF- $\kappa$ B and IRFs to the IFN- $\beta$ promoter

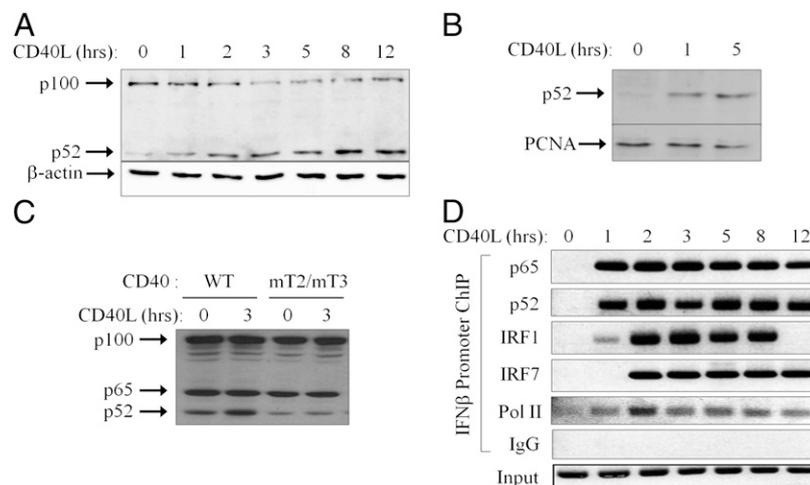
RelA (p65) NF- $\kappa$ B and IRFs such as IRF1, IRF3, and IRF7 have been implicated in IFN- $\beta$  gene regulation following virus infection.

CD40 engagement activates both canonical and noncanonical NF- $\kappa$ B pathways, resulting in nuclear translocation of p65 and p52 NF- $\kappa$ B, respectively (28). In EJ cells treated with CD40L, p100 processing was observed within 1 h of stimulation and increased over time (Fig. 2A), leading to elevated nuclear p52 levels (Fig. 2B). In line with a previous report (28), we found that NF- $\kappa$ B2 signaling critically depends on the TRAF2/TRAF3-interacting domain of CD40. Thus, unlike wild-type CD40, stimulation of CD40mT2/mT3-transfected HEK 293 cells with CD40L failed to promote NF- $\kappa$ B2 processing (Fig. 2C). Nuclear p65 and IRF1 also increase following stimulation of EJ cells with CD40L (15). On the basis of these observations, we proceeded to evaluate recruitment of NF- $\kappa$ B and IRFs to the IFN- $\beta$  promoter by ChIP assays.

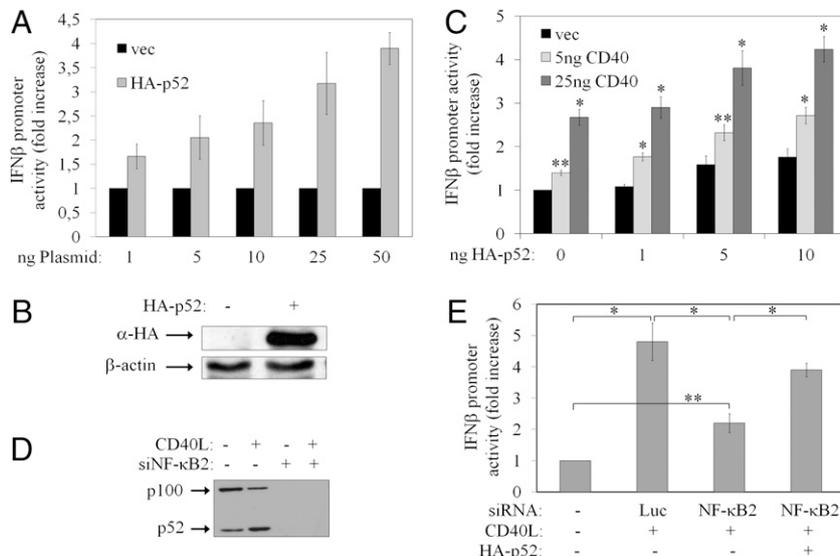
Chromatin from EJ cells treated with CD40L for various time intervals or from untreated controls was used for immunoprecipitation with anti-p65 or anti-p100/p52 Ab, and precipitated DNA encompassing the IFN- $\beta$  gene promoter was then assayed by PCR. The results (Fig. 2D) showed that the recruitment of both p65 and p52 increased within 1 h of CD40 stimulation and was maintained for the duration of the treatment, concomitant to IFN- $\beta$  mRNA expression levels (Fig. 1B). ChIP assays using an anti-IRF1 Ab showed small amounts of IRF1 associating with the IFN- $\beta$  promoter at 1 h of CD40L treatment, which increased progressively to reach a peak at 3 h, and returned to background levels at 12 h poststimulation (Fig. 2D). In contrast, binding of IRF7 occurred with somewhat slower kinetics than IRF1 but was sustained thereafter. As control (29), p52 and p65 but not IRF1 or IRF7 were found to bind the *skp2* promoter (data not shown).

#### Involvement of the noncanonical p100/p52 pathway in IFN- $\beta$ promoter regulation

Whereas RelA (p65) NF- $\kappa$ B is induced upon virus infection and is the first protein to bind the IFN- $\beta$  promoter (30) through interchromosomal interactions with specialized Alu elements (31, 32), it is not essential for virus-stimulated IFN- $\beta$  gene expression (33).



**FIGURE 2.** RelA, p52, and IRFs are recruited to the IFN- $\beta$  promoter following CD40 ligation. **(A)** CD40 stimulation induces NF- $\kappa$ B2 processing in carcinoma cells. EJ cell cultures were treated with 0.5  $\mu$ g/ml recombinant soluble CD40L for various time intervals, and lysates were resolved on SDS-PAGE and immunoblotted with anti-NF- $\kappa$ B2 Ab. The p100 NF- $\kappa$ B2 and its processed product p52 are indicated. **(B)** CD40 ligation results in p52 translocation to the nucleus. Nuclear extracts from EJ cells treated as described in **(A)** were immunoblotted with anti-NF- $\kappa$ B2 Ab. The nuclear proliferating cell nuclear Ag (PCNA) was used as loading control. **(C)** The TRAF2/TRAF3-interacting domain of CD40 mediates NF- $\kappa$ B2 signaling. HEK 293 cells were transfected with wild-type CD40 or CD40mT2/mT3, and 24 h later, cells were either left untreated or stimulated with CD40L as indicated. Lysates were analyzed for p100 NF- $\kappa$ B2/p52 levels by immunoblot. Reprobing the same membrane with anti-p65 Ab confirmed equal protein loading and transfer efficiency. **(D)** ChIP assays showing the kinetics of the *in vivo* recruitment of NF- $\kappa$ B subunits and IRFs to the IFN- $\beta$  promoter upon CD40 stimulation. ChIP assays were performed as described in detail in *Materials and Methods*. One-tenth of the volume of the chromatin obtained was used for PCR as input, and the remaining volume was immunoprecipitated with anti-IRF1, IRF7, RelA (p65), NF- $\kappa$ B2 (p52), or RNA polymerase II (Pol II) Ab or with control Ig (IgG). Precipitated DNA encompassing the IFN- $\beta$  promoter was then assayed by PCR. Data are representative of four independent experiments.



**FIGURE 3.** NF- $\kappa$ B2/p52 contributes to IFN- $\beta$  promoter activation. **(A)** Overexpression of p52 suffices for IFN- $\beta$  promoter activation. HEK 293 cells were transfected with 50 ng each pIFN- $\beta$ /Luc and *Renilla* reporter plasmids together with increasing amounts of HA-tagged p52 or control vector. The fold increase in promoter activity from four independent experiments is shown. **(B)** Representative immunoblot showing expression of HA-p52 in transfected HEK 293 cells. **(C)** Expression of p52 augments CD40-mediated IFN- $\beta$  transactivation. HEK 293 cells were transfected with 100 ng each pIFN- $\beta$ /Luc and *Renilla* reporter plasmids and increasing amounts of HA-p52 and/or CD40 as indicated. Reporter activities were assessed 48 h posttransfection and the fold increase ( $\pm$  SD) in promoter activation relative to control vector-transfected cells is shown. **(D)** Immunoblot showing the levels of p100 NF- $\kappa$ B2 and p52 following NF- $\kappa$ B2 knockdown in EJ cells. As control, NF- $\kappa$ B2 or control siRNA-transfected cells were treated with CD40L for 3 h, as indicated. **(E)** Endogenous p52 is required for CD40-mediated IFN- $\beta$  transactivation. EJ cells were transfected with siRNA targeting NF- $\kappa$ B2 together with the reporter constructs described in **(B)**. Following a 6-h treatment with CD40L, the fold increase in IFN- $\beta$  promoter activity (mean  $\pm$  SD from three independent experiments) was assessed and shown. Overexpression of HA-p52 was found to restore promoter activity in NF- $\kappa$ B2 siRNA-transfected cells; \* $p$  < 0.01, \*\* $p$  < 0.02, CD40 versus control vector-transfected cells, determined by Student *t* test.

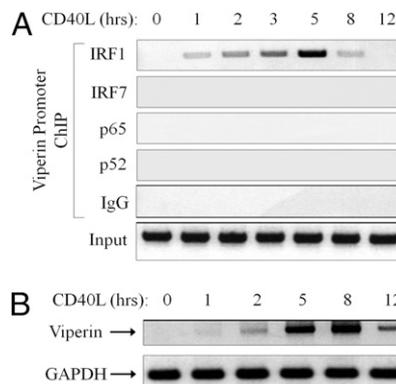
Instead, RelA has been proposed to maintain autocrine IFN- $\beta$  signaling and contribute to the early stages of gene expression (34). The influence of the noncanonical p100/p52 NF- $\kappa$ B2 pathway on IFN- $\beta$  remains unexplored. On the basis of the ChIP data shown in Fig. 2D, we examined the effect of p52 overexpression on IFN- $\beta$  promoter activity using reporter assays. HEK 293 cells were transfected with pIFN- $\beta$ /Luc and a *Renilla* plasmid to correct for transfection efficiency in the presence of increasing amounts of a hemagglutinin (HA)-tagged p52 expression vector. As shown in Fig. 3A, the overexpression of p52 stimulated IFN- $\beta$  promoter activity in a concentration-dependent manner. Moreover, the coexpression of CD40 and HA-p52 led to higher levels of IFN- $\beta$  transactivation compared with each vector alone (Fig. 3C), suggesting cooperation of NF- $\kappa$ B2 and other CD40-activated signaling pathways in IFN- $\beta$  gene regulation. Conversely, the RNA interference (RNAi)-mediated knockdown of p100/p52 (Fig. 3D) resulted in attenuation of CD40L-induced IFN- $\beta$  promoter activity (Fig. 3E). These data underscore a novel role for the noncanonical NF- $\kappa$ B2 pathway in IFN- $\beta$  gene regulation.

#### Viperin (RSAD2) is upregulated in CD40-stimulated cells

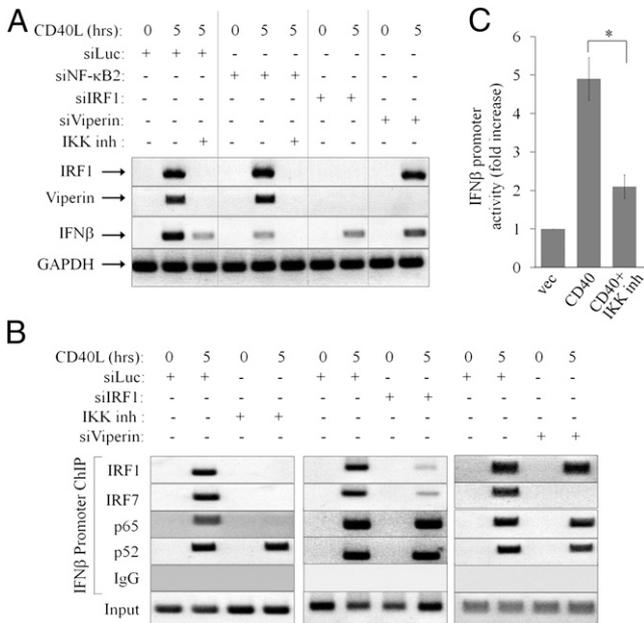
Viperin (virus inhibitory protein, endoplasmic reticulum associated, or IFN inducible), also known as radical S-adenosyl methionine domain-containing 2 (RSAD2), is an antiviral protein that participates in the IRF7-mediated induction of IFN- $\beta$  (21). Recent studies have demonstrated that IRF1 directly binds the viperin promoter and controls its activity (35). We have found that treatment of EJ cells with CD40L stimulates the recruitment of IRF1 but not of p65 or p52 to the viperin promoter (Fig. 4A) and rapidly induces viperin mRNA expression (Fig. 4B). The slight difference in the kinetics of IRF1 dissociation from the viperin promoter versus mRNA levels indicates the operation of additional mechanisms which may influence viperin expression, such as mRNA stability.

#### A “feed-forward” mechanism of IFN- $\beta$ gene regulation

The relative contribution of NF- $\kappa$ B subunits and IRFs to CD40-mediated IFN- $\beta$  regulation was further validated using a combination of RNAi and treatment with a chemical inhibitor of IKK2 (36). Suppression of canonical NF- $\kappa$ B signaling using the IKK2 kinase inhibitor (15) or of the noncanonical NF- $\kappa$ B2 using RNAi (Fig. 3D) impaired but did not abolish CD40L-induced IFN- $\beta$  expression (Fig. 5A). However, inhibition of both arms of NF- $\kappa$ B signaling had a deleterious effect on IFN- $\beta$  mRNA levels, sug-



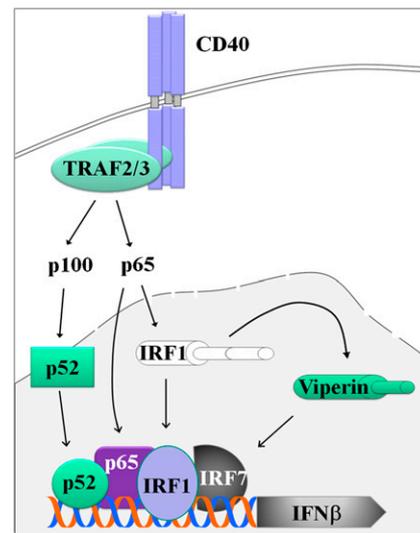
**FIGURE 4.** CD40 stimulation induces Viperin upregulation. **(A)** Representative ChIP assay showing the kinetics of the *in vivo* recruitment of IRF1 to the *Viperin* promoter upon CD40 stimulation. One-tenth of the volume of the chromatin obtained was used for PCR as input, and the remaining volume was immunoprecipitated with anti-IRF1 Ab or with control Ig (IgG). Precipitated DNA encompassing the *Viperin* promoter was then assayed by PCR. Precipitation with anti-p52, anti-IRF7, or anti-p65 Abs did not yield a PCR product. **(B)** CD40 ligation results in induction of *Viperin* mRNA expression levels, as determined by RT-PCR.



**FIGURE 5.** CD40 ligation stimulates IFN-β gene expression via a feed-forward molecular pathway. **(A)** Contribution of NF-κB subunits, IRF1, and Viperin to IFN-β gene expression. EJ cells were transfected with siRNAs targeting NF-κB2, IRF1, or Viperin or control siRNA against the Luciferase (Luc) gene prior to stimulation with CD40L for 5 h. Alternatively, cells were pretreated with the IKK2 inhibitor SC-514 (10 μM) for 30 min and then stimulated with CD40L before RNA was isolated. cDNA was synthesized and used as a template for semiquantitative PCR with primers specific for IRF1, Viperin, IFN-β, or the *GAPDH* housekeeping gene, which served as loading and amplification control. The results shown are representative of results from three independent experiments. **(B)** ChIP assays showing the impact of NF-κB2, IRF1, or Viperin knockdown or canonical pathway inhibition on the recruitment of transcription factors to the IFN-β promoter. Chromatin was obtained following a 5 h stimulation with 0.5 μg/ml CD40L and immunoprecipitated with anti-IRF1, IRF7, RelA (p65), NF-κB2 (p52) Ab, or with control Ig (IgG), as indicated. Precipitated DNA encompassing the IFN-β promoter was then assayed by PCR. Data are representative of three independent experiments. **(C)** IKK2 inhibition suppresses CD40-mediated IFN-β transactivation. HEK 293 cells were transfected with the reporter constructs described in Fig. 1E together with a CD40 expression vector. The IKK2 inhibitor was added 24 h prior to assessment of IFN-β promoter activity. \**p* < 0.02, reporter activities of IKK2-treated versus untreated cells transfected with CD40, by Student *t* test (*n* = 4).

gesting that whereas canonical and noncanonical NF-κB operate independently, they converge to coregulate the IFN-β gene promoter. In line with this observation, treatment of CD40L-stimulated EJ cells with the IKK2 inhibitor diminished the binding of p65/RelA, but not of p52 NF-κB2, to the PRDI-IV region (Fig. 5B) and decreased the activity of the IFN-β promoter (Fig. 5C). This treatment also resulted in dramatic reduction in the expression of IRF1 and its target Viperin (Fig. 5A) and impaired both IRF1 and IRF7 binding to the IFN-β promoter (Fig. 5B). Therefore, p65 NF-κB functions as key regulator of CD40-mediated IFN-β gene transcription through both direct and indirect mechanisms (Fig. 6).

To better characterize the p65 NF-κB axis that leads to IFN-β gene expression, IRF1 or Viperin was knocked down in EJ cells, and the effects of CD40 ligation on IFN-β mRNA levels and transcription factor recruitment to the IFN-β promoter were assessed. As shown in Fig. 5A and 5B, the siRNA-mediated knockdown of IRF1 attenuated both the upregulation of IFN-β mRNA and the binding of IRF1 and IRF7 to the IFN-β promoter, whereas



**FIGURE 6.** Proposed model of CD40-mediated IFN-β gene transactivation. The data presented in this study demonstrate that stimulated CD40, through its association with TRAFs, triggers the parallel engagement of canonical and noncanonical NF-κB pathways leading to nuclear translocation of RelA (p65) and p52, respectively. The recruitment of p65 to the IRF1 promoter results in rapid IRF1 synthesis, which in turn controls the expression of Viperin. RelA NF-κB, de novo synthesized IRF1, and Viperin-controlled IRF7 converge to regulate IFN-β gene expression. Noncanonical pathway-generated p52 also binds to the IFN-β promoter and contributes to its activity.

recruitment of p65 or p52 remained unaffected. In contrast, the knockdown of *Viperin* partially reduced the CD40L-mediated upregulation of IFN-β and abolished the recruitment of IRF7 but not of other transcription factors to the IFN-β promoter.

### Discussion

CD40L and IFN-β share important roles in the antiviral and anti-tumor immune response. Thus, both CD40L and IFN-β confer negative effects on carcinoma growth by improving tumor cell immunogenicity (4, 14, 15) and triggering direct antiproliferative and proapoptotic properties (37–41). Both CD40L and type I IFNs also act on immune cells to augment Th1 cell responses, enhance expression of MHC class I molecules, and generate NK and T cell-mediated cytotoxicity (reviewed in Refs. 1 and 12). The aforementioned functional similarities provided a theoretical link between CD40 and IFN-β and prompted us to evaluate the hypothesis that IFN-β is a physiological target of the CD40 signaling pathway. Data presented in this study confirm this hypothesis and demonstrate that the IFN-β gene is acutely induced by CD40L in human carcinoma and immortalized epithelial cells.

The established view of IFN-β gene regulation comes from studies predominantly performed in virus-infected cells and suggests that parallel activation and cooperative binding of RelA NF-κB, IRF3, IRF7, and ATF-2/c-Jun to the nucleosome-free PRD regions is responsible for IFN-β promoter activity (18). Whereas RelA (p65) NF-κB is induced upon virus infection and is the first protein to bind the IFN-β promoter (30), reportedly through interchromosomal interactions with specialized Alu elements (31, 32), it is not essential for virus-stimulated IFN-β gene expression (33). Instead, RelA has recently been proposed to maintain auto-crine IFN-β signaling and contribute to the early stages of gene expression in virus-infected cells (34). IFN-β transcription is also induced in macrophages and myeloid dendritic cells exposed to the TLR9 ligand CpG, but unlike virus infection, it requires IRF1 and IRF7 and is independent of IRF3 (42, 43). These reports highlight

differences in the regulatory mechanisms that govern IFN- $\beta$  gene expression in a stimulus and/or cell type-dependent manner.

Data presented in this study demonstrate that CD40 receptor occupancy results in IFN- $\beta$  upregulation in tumor cells via a novel mechanism that requires integration of canonical and noncanonical NF- $\kappa$ B signaling pathways. We show that the binding of RelA (p65) and p52 NF- $\kappa$ B subunits to the IFN- $\beta$  promoter directly contributes to gene expression and that CD40-mediated IFN- $\beta$  transactivation ceases only upon impairment of both pathways (Fig. 5A). These findings suggest that whereas canonical and noncanonical NF- $\kappa$ B operate independently, they converge to coregulate IFN- $\beta$  gene expression (Fig. 6). In line with this observation, overexpression of p52 alone is capable of inducing IFN- $\beta$  promoter activity (Fig. 3A), and blockade of the canonical NF- $\kappa$ B pathway does not interfere with p52 binding to the IFN- $\beta$  promoter (Fig. 5B). Importantly, however, the rapid, RelA-dependent de novo synthesis of IRF1 and Viperin also allows for the recruitment of IRFs, providing a tremendous enhancement of IFN- $\beta$  gene induction (Fig. 6).

The CD40 pathway holds promise for the immunotherapy of various types of cancer, including non-Hodgkin's lymphoma (44), chronic lymphocytic leukemia (45), and carcinoma (46, 47), and CD40 agonists have recently entered clinical trials, with promising results (38, 48). The aforementioned role of RelA as a master regulator of IFN- $\beta$ , coupled with its previously described involvement in the CD40-mediated Ag transporter and immunoproteasome gene expression (15), suggests that NF- $\kappa$ B may make a positive contribution to CD40L therapy by controlling antitumor immunity. This observation highlights the dual nature of NF- $\kappa$ B, which can either support or antagonize cancer in a stimulus- or tissue-dependent manner (49). Moreover, as Viperin limits replication of viruses such as CMV (50), hepatitis C virus (51), and vesicular stomatitis virus-pseudotyped HIV (52), it would be interesting to determine the contribution of the RelA-Viperin axis to the reported direct antiviral effects of CD40 ligation (11).

Investigations into the signal transduction pathways triggered by CD40 ligation have typically focused on direct (i.e., one-step) events that control gene expression. The data presented in this report reveal a novel mechanism by which CD40 signals can be propagated (Fig. 6), involving the sequential mobilization of NF- $\kappa$ B and de novo-synthesis of proteins which act in concert to ensure enhanced transactivation of IFN- $\beta$ . This coordinated mechanism of transcription factor activation represents a telling example of the significance of feed-forward cascades in the regulation of gene expression.

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## Disclosures

The authors have no financial conflicts of interest.

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