The Journal of Immunology

Murine Dendritic Cell Type I IFN Production Induced by Human IgG-RNA Immune Complexes Is IFN Regulatory Factor (IRF)5 and IRF7 Dependent and Is Required for IL-6 Production

Kei Yasuda, Christophe Richez, Joseph W. Maciaszek, Neerja Agrawal, Shizuo Akira, Ann Marshak-Rothstein and Ian R. Rifkin

---

References This article **cites 65 articles**, 29 of which you can access for free at:
http://www.jimmunol.org/content/178/11/6876.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Murine Dendritic Cell Type I IFN Production Induced by Human IgG-RNA Immune Complexes Is IFN Regulatory Factor (IRF)5 and IRF7 Dependent and Is Required for IL-6 Production[1]

Kei Yasuda,* Christophe Richez,* Joseph W. Maciaszek,* Neerja Agrawal,* Shizuo Akira,‡ Ann Marshak-Rothstein,† and Ian R. Rifkin[2]*

Dendritic cell (DC) activation by nucleic acid-containing IgG complexes is implicated in systemic lupus erythematosus (SLE) pathogenesis. However, it has been difficult to definitively examine the receptors and signaling pathways by which this activation is mediated. Because mouse FcγRs recognize human IgG, we hypothesized that IgG from lupus patients might stimulate mouse DCs, thereby facilitating this analysis. In this study, we show that sera and purified IgG from lupus patients activate mouse DCs to produce IFN-α, IFN-β, and IL-6 and up-regulate costimulatory molecules in a FcγR-dependent manner. This activation is only seen in sera with reactivity against ribonucleoproteins and is completely dependent on TLR7 and the presence of RNA. As anticipated, IFN regulatory factor (IRF)7 is required for IFN-α and IFN-β production. Unexpectedly, however, IRF5 plays a critical role in IFN-α and IFN-β production induced not only by RNA-containing immune complexes but also by conventional TLR7 and TLR9 ligands. Moreover, DC production of IL-6 induced by these stimuli is dependent on a functional type I IFNR, indicating the need for a type I IFN-dependent feedback loop in the production of inflammatory cytokines. This system may also prove useful for the study of receptors and signaling pathways used by immune complexes in other human diseases.   *The Journal of Immunology,* 2007, 178: 6876–6885.

S ystemic lupus erythematosus (SLE)[3] is an autoimmune disease characterized by the loss of tolerance to diverse self-Ags and the consequent production of autoantibodies (1). Many of the more common autoantigens are components of either DNA/protein or RNA/protein multimolecular particles that are normally found in the cell nucleus, but which cluster and concentrate at the surface of cells undergoing apoptosis (2, 3). Although the underlying etiology remains incompletely understood and likely represents a complex interaction of genetic and environmental factors, a number of potential contributors to disease pathogenesis have been identified.

The multiple subtypes of IFN-α and the single IFN-β belong to the type I IFN family, and all signal through a single shared receptor, the IFN-α receptor (IFNAR) (4). Type I IFNs may play an important role in SLE pathogenesis, and activation of the type I IFN system is a prominent feature of early and active disease (5, 6). Plasmacytoid dendritic cells (DC) (pDCs, also called natural IFN-producing cells) are the major source of type I IFN during viral infections and are thought also to be the most likely source of type I IFN in SLE (7–9). Attention has focused recently on the possibility that immune complexes containing self-nucleic acid might be the major inducers of type I IFN production by pDCs in SLE.

Immune complexes or IgG obtained from the sera of certain patients with SLE induce normal human pDCs to produce IFN-α in vitro. This activation generally requires the addition of an exogenous source of autoantigen to the cultures to promote the formation of stimulatory immune complexes (10, 11), although for certain IgG, the addition of exogenous Ag is not needed (12, 13). The stimulatory ability of the immune complexes depends on the presence of either DNA or RNA within the complexes, as well as engagement of FcγRIIa on the pDC.

Studies using defined mAb-nucleic acid immune complexes and mouse DCs have helped determine the mechanism whereby nucleic acid-containing immune complexes activate DCs. In the case of immune complexes containing anti-nucleosome mAb bound to nucleosomes, myeloid DC activation involves internalization of the immune complex by a cell surface FcγR with subsequent engagement of TLR9 and possibly other chromatin-reactive receptor(s) intracellularly (14). TLR9 is an intracellular receptor originally identified as being critically involved in the recognition of unmethylated CpG motifs in viral or bacterial DNA (15), although subsequent studies have shown that it can also recognize self-DNA under conditions where self-DNA can gain access to TLR9 (16–18). In the case of immune complexes containing an anti-Smith Ag (Sm) mAb complexed to small nuclear ribonucleoproteins (RNPs) containing U1 RNA, a similar activation mechanism appears to

[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; DC, dendritic cell; cDC, conventional DC; FL, fms-like tyrosine kinase 3 ligand; FL-DC, DC obtained from bone marrow cells cultured in FL; IRF, IFN regulatory factor; pDC, plasmacytoid DC; poly(I:C), poly(deoxyinosinic-deoxycytidylic acid); RNP, ribonucleoprotein; Sm, Smith Ag; WT, wild type.

operate, only in this case DC activation is largely TLR7 dependent (19). TLR7 is an intracellular receptor that recognizes single-stranded U-rich RNA (20–22). However, although these studies using defined mAb-nucleic acid complexes offer valuable insights into potential mechanisms underlying pDC activation by immune complexes in SLE, they only represent a small fraction of the autoantigen specificities present in the polyclonal autoantibody response of lupus patients. It may be that the requirement for engagement of TLRs or of other non-TLR nucleic acid receptors varies depending on the precise specificities of the individual autoantibodies.

Human pDCs express both TLR9 and TLR7, and a number of studies have examined the role of these receptors in the activation of human pDC by IgG from lupus patients. Although such studies have the important advantage of being able to test effects of patient derived autoantibodies and/or immune complexes, it is much more difficult to definitively prove the involvement of specific receptors in any observed activation. Nevertheless, these studies show that human pDC activation induced either by DNA-containing immune complexes or by immune complexes consisting of anti-RNP Abs bound to an exogenously added source of RNP can be decreased by inhibitors of the TLR7/9 signaling pathway (13, 23, 24). However, it is worth noting that the mechanisms of actions of these inhibitors are incompletely understood and may involve pathways upstream of TLR7/9 rather than TLR7/9 antagonism per se (25).

Recent studies showing an association between IFN regulatory factor (IRF)5 polymorphisms and an increased risk of SLE in humans potentially links TLR signaling pathways to SLE pathogenesis (26, 27). The intracytoplasmic adaptor molecule MyD88 is required for signaling through TLR7 and TLR9. There are two major pathways downstream of MyD88, one leading to proinflammatory cytokine production and the other to type I IFN production. IRF5 and IRF7 are members of a family of transcription factors that controls inflammatory and immune responses, and both are involved in these downstream pathways (28). IRF7 is the master regulator of type I IFN-dependent immune responses and is required for type I IFN production (29). The role of IRF5 is not clearly established. Although it was originally identified as a regulator of type I IFN expression in human cell lines (30, 31), recent data in IRF5-deficient mice suggest that IRF5 is involved in the production of proinflammatory cytokines but not in the production of type I IFN (32). At present, the role of IRF5 in DC activation induced by nucleic acid-containing immune complexes is not known.

Mouse FcγRs are able to bind human IgG with specificities similar to that of human FcγRs (33). Therefore, we hypothesized that serum or IgG from lupus patients would activate mouse DCs and that this would facilitate the analysis of receptors and signaling pathways involved in DC activation. These studies demonstrate that this is the case and reveal a number of unanticipated activation pathways of relevance not only to lupus but also to TLR signaling in general.

Materials and Methods
Mice

C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory. FcR common γ-chain-deficient mice (B6.129P2-*Fcerg1^{tm1}*; backcrossed 12 generations to C57BL/6) were obtained from Taconic Farms. TLR7-deficient mice (backcrossed 7 generations to C57BL/6) have been described previously (34). IRF5-deficient mice (backcrossed 8 generations to C57BL/6) and IRF7-deficient mice (backcrossed three generations to C57BL/6) were provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan) and Dr. T. Mak (University of Toronto, Toronto, Canada) (29, 32). Type I IFNR-deficient mice backcrossed more than nine generations to C57BL/6 were provided by Dr. J. Sprent (Garvan Institute of Medical

Research, Sydney, Australia) (35). All mice were maintained at the Boston University School of Medicine Laboratory Animal Sciences Center or at Charles River Laboratories in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Reagents

CpG-A ODN 2336 (GGg gac gac gtc gtc gtg GGGGGG) were purchased from Coley Pharmaceutical Group. Capital letters show phosphorothioate backbone, and lowercase letters show phosphodiester backbone. Poly(deoxyinosinic-deoxycytidylic acid) (poly(I:C)), LPS, and R848 were purchased from InvivoGen. Pam3Cys-Ser-Lys4 was a gift from Prof. G. Jung (University of Tubingen, Tubingen, Germany).

Patient sera

Blood samples were obtained from the patient population at Boston Medical Center, all of whom met the American College of Rheumatology 1982 revised criteria for SLE (36). The study was approved by the Institutional Review Board, and written informed consent was obtained from all patients. Nonanticoagulated peripheral blood was obtained from the patients, placed at room temperature for 1 h, and centrifuged at $350 \times g$ for 5 min. The serum was collected, aliquot, and stored at $-80°C$. Serum autoantibody levels were measured in samples collected on the same day as the sera collected for the in vitro experiments. Anti-nuclear Ab titers were measured by indirect immunofluorescence on HEp2 cells in the Clinical Immunology Laboratory at Boston Medical Center. Other autoantibodies were measured in the RDL Reference Laboratory using the Farr assay for dsDNA and ELISA for Sm, RNP, Ro, and La.

IgG purification

Patient sera were diluted with $3\times$ volume of PBS and run over a protein G-Sepharose column (GE Healthcare Bio-Sciences). IgG was eluted from the column with glycine HCl (pH 2.8) and dialyzed three times against PBS at 4°C before aliquotting and freezing at $-80°C$. In some experiments, the purified IgG was treated with or without RNase A (USB) at a final concentration of 100 μg/ml in PBS for 24 h at 37°C and then again purified using a protein G-Sepharose column as above to remove the RNase.

Preparation of DCs

Bone marrow cells were seeded at 1.5×10^6 cells/ml in complete RPMI 1640 (10% FBS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin) supplemented with 7.5–10% conditioned medium from fms-like tyrosine kinase 3 ligand (Flt3-ligand or FL)-transfected B16 melanoma cells. The FL B16 cells were originally made by Dr. H. Chapman (University of California, San Francisco) (37) and provided by Dr. U. Von Andrian (Harvard Medical School, Boston, MA). The cells were used for experiments after 8 days, at which time >90% were CD11c positive, of which 15–40% displayed a pDC phenotype (CD11c^{pos}CD45RA^{high}B220^{high}CD11b^{low}) and the remainder displayed a conventional DC (cDC) phenotype (CD11c^{pos}CD45RA^{low}B220^{low}CD11b^{high}). Collectively, the cells obtained at 8 days containing both pDC and cDC populations are referred to as FL-DCs.

DC stimulation

Before setting up each assay, the FL-DCs were routinely checked by flow cytometry for the relative percentages of pDCs and cDCs and the respective DC activation status after staining with anti-CD11c-PE, biotinylated anti-CD45RA, followed by streptavidin-PE-Cy5, anti-B220-FITC, anti-CD11b-FITC, and anti-CD62L-FITC Abs (all from BD Biosciences). FL-DCs (3×10^5) were seeded in 96-well round-bottom plates and cultured in complete RPMI 1640 with the appropriate TLR ligands, patient sera, or patient IgG in a total well volume of 200 μl. In some experiments, RNase A (5 μg/ml; USB) was added to the cultures 1 h before addition of the ligands and patient IgG. After 24 h, the supernatants were collected for cytokine measurement. After collection of the supernatants, the FL-DCs were labeled with a combination of anti-B220-PE-Cy5, anti-CD11b-FITC, and anti-CD86-PE Abs (all from BD Biosciences), detected with a FACScan flow cytometer (BD Biosciences), and analyzed with FlowJo software (Tree Star). To obtain purified pDC (CD11c^{pos}B220^{high}CD11b^{low}) and cDC (CD11c^{pos}B220^{neg}CD11b^{high}) populations for certain experiments, FL-DCs from 8 day cultures were stained with anti-B220-PE-Cy5, anti-CD11c-PE, and anti-CD11b Abs (all from BD Biosciences), and the DC populations were separated using a MoFlo cell sorter (DakoCytomation).

FIGURE 1. Sera and IgG from lupus patients activate murine FL-DCs. Bone marrow-derived FL-DCs from WT C57BL/6 mice were cultured with sera (*A*) or IgG (*B*) from lupus patients (SLE1, SLE2, SLE6, SLE8, and SLE10), the CpG-A oligodeoxynucleotide (ODN) 2336 (100 nM in *A* and 50 nM in *B*), and LPS (25 ng/ml). IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data shown are the mean $\pm$ SD of one of two representative experiments (*A*) and one of three representative experiments (*B*).
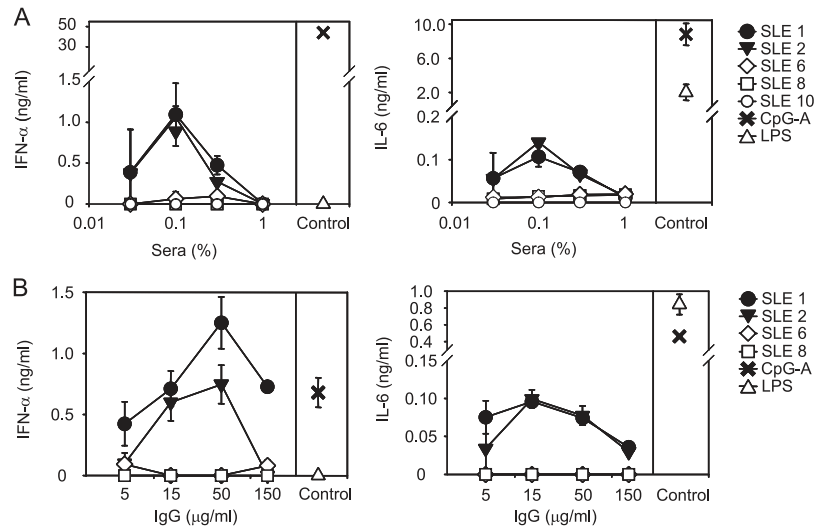
Cytokine measurement

Cytokine levels in the tissue culture supernatants were measured using in-house ELISAs developed using commercially available Abs. For the IL-6 ELISA, we used the rat anti-mouse IL-6 mAb (MP5-20F3; BD Pharmingen), the biotinylated rat anti-mouse IL-6 Ab (MP5-32C11; BD Pharmingen), and recombinant mouse IL-6 standard (554582; BD Pharmingen). The detection limit of the IL-6 ELISA is 16 pg/ml. For the IFN-α ELISA, we used the rat anti-mouse IFN-α mAb (22100-1; PBL Biomedical Laboratories), rabbit anti-mouse IFN-α polyclonal Ab (32100-1; PBL Biomedical Laboratories), HRP-conjugated donkey anti-rabbit IgG (711-036-152; Jackson ImmunoResearch Laboratories), and mouse rIFN-α standard (12100-1; PBL Biomedical Laboratories). The detection limit of the IFN-α ELISA is 80 pg/ml. For the IFN-β ELISA, we used the rat anti-mouse IFN-β mAb (17662-10A; US Biological), rabbit anti-mouse IFN-β polyclonal Ab (32400-1; PBL Biomedical Laboratories), HRP-conjugated donkey anti-rabbit IgG (711-036-152; Jackson ImmunoResearch Laboratories), and mouse rIFN-β standard (12400-1; PBL Biomedical Laboratories). The detection limit of the IFN-β ELISA is 130 pg/ml.

Results

Sera and IgG from lupus patients activate murine DCs

Mouse bone marrow cells cultured in vitro with FL develop into a mixed population of pDCs and cDCs, collectively referred to as FL-DCs. These pDCs and cDCs show equivalent properties to pDCs and cDCs isolated directly from mouse spleen (38). We began by testing whether sera from lupus patients could activate mouse FL-DCs. We found that certain sera induced the production of IFN-α and small amounts of IL-6 whereas others did not (Fig.

1*A*). The sera stimulated maximally at a concentration of 0.1%, much lower than the 10–20% concentration used in studies with human PBMCs (12, 13, 23, 24). The stimulatory sera in this initial screen contained autoantibodies reactive against dsDNA and the uridine-rich U RNA-complexed proteins Sm and RNP, whereas the nonstimulatory sera contained autoantibodies reactive against dsDNA and the uridine-rich hY RNA-complexed proteins Ro and La (Table I). To identify the stimulatory component of the sera, we purified IgG using protein G affinity chromatography and found that this IgG recapitulated the stimulatory ability of the sera (Fig. 1*B*). These experiments were performed without the addition of any exogenous Ag or other costimuli to the cultures. Thus, certain IgG, or immune complexes containing IgG, from lupus patients are able to activate mouse FL-DCs.

*FL-DC activation by lupus IgG correlates with Sm/RNP reactivity and is TLR7 dependent*

The initial stimulatory sera tested, SLE1 and SLE2, contained autoantibodies with reactivity against dsDNA, Sm, and RNP. As activation of human pDCs by protein G-purified IgG with dsDNA reactivity from lupus patients has been linked to TLR9 (13), we first compared the ability of SLE1 and SLE2 sera to activate WT and TLR9-deficient FL-DCs. However, these two sera activated WT and TLR9-deficient FL-DCs equally well (data not shown), suggesting either that TLR9 was not involved in activation by

Table I. *Sera reactivities and types of nephritis of lupus patients in this study*

Patients	Anti-nuclear Ab Titer	Autoantibody Reactivity					Nephritis
		Sm	RNP	Ro	La	dsDNA[a]	
SLE 1	1/2560	+++	+++	+++	+++	378	Class IV
SLE 2	1/2560	+++	+++	neg	neg	23	Unclassified
SLE 3	1/2560	+++	+++	neg	neg	<5	None
SLE 4	1/640	++	+	neg	neg	<5	Class III
SLE 5	1/640	neg[b]	+++	++	neg	20	None
SLE 6	1/640	neg	neg	+++	+++	190	Class III, V
SLE 7	1/2560	neg	neg	+++	++	31	Class IV
SLE 8	1/1280	neg	neg	+++	neg	<5	None
SLE 9	1/2560	neg	neg	++	neg	10701	Class III, V
SLE 10	1/2560	neg	neg	neg	neg	586	None
SLE 11	1/160	neg	neg	neg	neg	152	Class IV, V

[a] For Abs against dsDNA, values > 5 IU/ml are considered positive.
[b] Negative for indicated autoantibody reactivity.
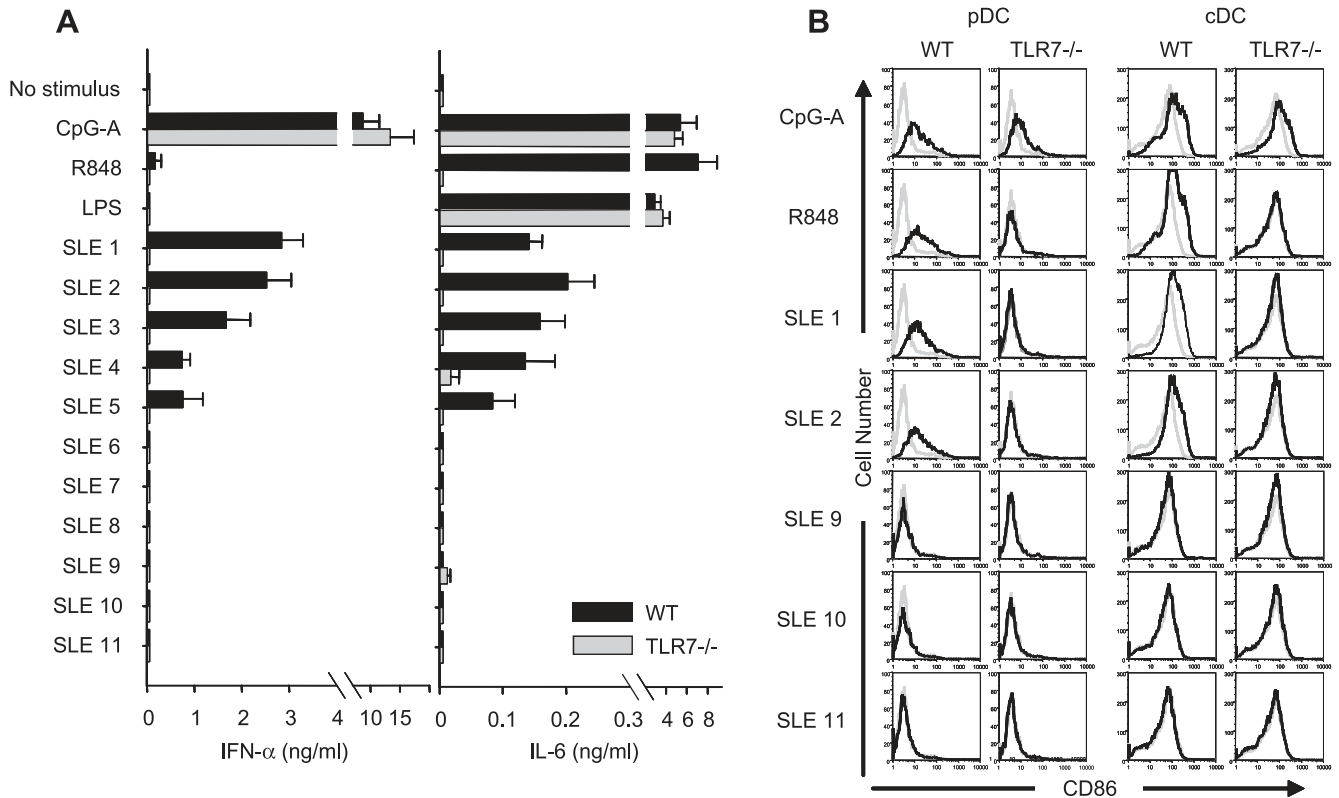
FIGURE 2. Activation of murine DCs by IgG from lupus patients is TLR7 dependent. Bone marrow-derived FL-DCs from WT C57BL/6 and TLR7-deficient (TLR7$^{-/-}$) mice were cultured with the CpG-A ODN 2336 (50 nM), R848 (15 nM), LPS (25 ng/ml), and IgG from lupus patients (50 μg/ml; SLE1–11). *A*, IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of 3–8 experiments (WT) and 3–10 experiments (TLR7 deficient). *B*, After removal of supernatants for cytokine analysis, the cells were stained with Abs against B220, CD11b, and CD86. pDCs (B220^{high} and CD11b^{neg}) and cDCs (B220^{neg} and CD11b^{high}) were analyzed separately. CD86 expression is shown with stimulus-induced staining intensity (■) compared with staining intensity of the nonstimulated cultures (▫). A representative example of the experiments in *A* is shown.

anti-dsDNA Abs or, alternatively, that the stimulatory ability of the sera was not due to the anti-dsDNA Abs.

To analyze this further, we prepared IgG from a more extensive panel of lupus sera (Table I). The ability of the IgG to activate FL-DCs correlated with specificity of the IgG for Sm or RNP but did not correlate with specificity for dsDNA, Ro, or La (Fig. 2*A*). Substantial amounts of IFN-α were induced by the stimulatory IgG, but only modest amounts of IL-6. In addition to cytokine production, the stimulatory IgG also induced up-regulation of the costimulatory molecule CD86 on the surface of both pDCs and cDCs to a similar degree as that induced by the conventional TLR9 ligand CpG-A and the TLR7 ligand R848 (Fig. 2*B*).

Because of the correlation of Sm/RNP reactivity with the stimulatory IgG, we next tested the possibility that the FL-DC activation observed was due to the presence of RNA-containing immune complexes in the cultures. If that was the case, it seemed likely that TLR7 would be involved in the activation (19, 23, 24, 39). Indeed, both cytokine production and CD86 up-regulation induced by the stimulatory IgG was found to be completely TLR7 dependent (Fig. 2, *A* and *B*). This was not due to an inability of the TLR7-deficient DCs to make cytokines because IFN-α and IL6 production induced by the TLR9 ligand CpG-A was equal in both WT and TLR7-deficient cells.

*FL-DC activation requires both FcγR engagement and the presence of RNA*

A current hypothesis for how RNA-containing immune complexes activate DCs is that the immune complex is internalized via a FcγR on the cell surface, and the RNA within the complex is then

delivered to TLR7 or other intracellular RNA-reactive receptors to induce cell activation (40). It was important to verify that this paradigm applied to our experimental system.

The FcRγ chain is required for the efficient assembly and cell surface expression of all the mouse activating FcγRs, FcγRI, FcγRIII, and FcγRIV (41, 42). To test whether FcR engagement was required for FL-DC activation by stimulatory IgG from lupus patients, we compared FL-DCs from WT and FcRγ chain-deficient mice. The stimulatory IgG failed to elicit cytokine production or CD86 up-regulation by FL-DCs from FcRγ chain-deficient mice, although cytokine production and CD86 up-regulation in response to conventional TLR ligands was the same as in WT FL-DC (Fig. 3 and data not shown).
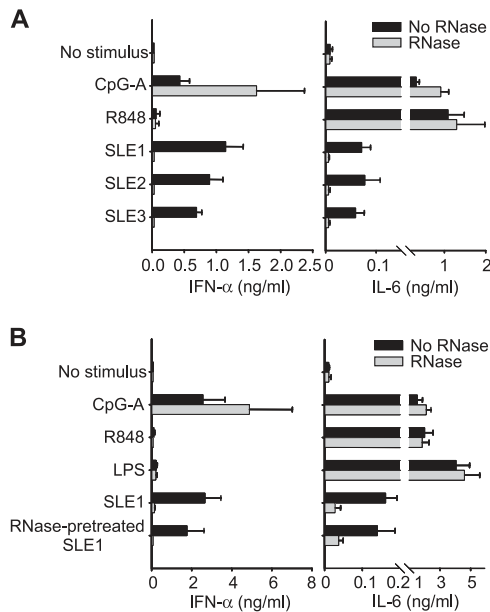
To determine whether RNA was required for the response, cultures were set up in the presence or absence of RNase. In the presence of RNase, almost no cytokine production was induced by the stimulatory IgG (Fig. 4*A*). Thus, FL-DC activation required both FcγR engagement and the presence of RNA, strongly suggesting that the stimulatory response was due to RNA-containing immune complexes in the cultures.

As no exogenous Ag was added to the cultures, the source of RNA must have been either RNA-containing immune complexes originally present in the patient sera or, alternatively, apoptotic or necrotic mouse cells in the FL-DC cultures. To distinguish between these possibilities, we pretreated the stimulatory IgG with a high concentration of RNase for 24 h to remove endogenous patient RNA bound to the IgG, purified the IgG by protein G purification to remove the RNase, and then added the IgG to the FL-DC cultures. We found that pretreatment of the stimulatory
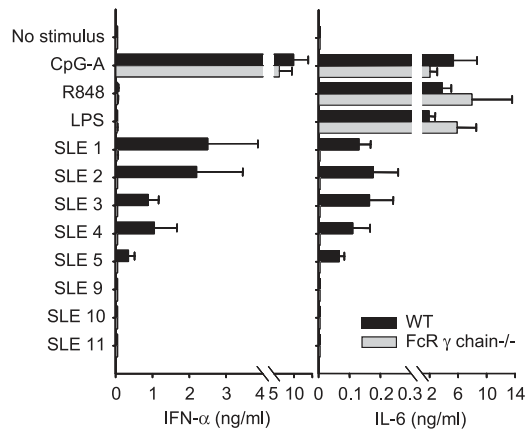
FIGURE 3. Activation of murine DCs by IgG from lupus patients is FcγR-dependent. Bone marrow-derived FL-DC from WT C57BL/6 and Fcγ chain-deficient (FcRγ-chain$^{-/-}$) mice were cultured with the CpG-A ODN 2336 (50 nM), R848 (15 nM), LPS (25 ng/ml), and IgG from lupus patients (50 μg/ml; SLE1–11). IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of three experiments.

Table II. *Cytokine production by sorted murine pDCs and cDCs activated with conventional TLR ligands or with IgG from patients with SLE*

	IFN-α (pg/ml)		IL-6 (pg/ml)	
	pDCs	cDCs	pDCs	cDCs
Media	<80	<80	<16	<16
CpG-A	42,350 ± 3,041	<80	295 ± 49	3,410 ± 1,485
R848	<80	<80	5,653 ± 1,595	570 ± 396
LPS	<80	<80	<16	1,310 ± 382
SLE 1	8,630 ± 11	<80	<16	<16
SLE 2	13,643 ± 2,106	<80	<16	<16
SLE 3	3,590 ± 53	<80	<16	<16
SLE 5	4,576 ± 3,928	<80	<16	<16
SLE 6	<80	<80	<16	<16
SLE 7	<80	<80	<16	<16
SLE 8	<80	<80	<16	<16
SLE 10	<80	<80	<16	<16
SLE 11	<80	<80	<16	<16

IgG with RNase did not substantially reduce the ability of the IgG to activate FL-DCs. However, the addition of RNase to the actual FL-DC cultures, even at low concentration, effectively prevented activation (Fig. 4B). This suggests that RNA from apoptotic or necrotic mouse cells in the FL-DC cultures is sufficient for immune complex-mediated in vitro activation.

pDCs are the source of IFN-α in lupus IgG-stimulated FL-DC cultures

Although pDCs are the major IFN-α-producing cells, several studies have shown that cDCs can produce appreciable levels of IFN-α under certain conditions, including viral infection or after internalization of class A CpG oligodeoxynucleotides by transfection (43, 44). In mice, both pDCs and cDCs express TLR7 and FcγRs and so were potential sources of IFN-α in the FL-DC cultures. To determine which cell type was responsible for IFN-α production,
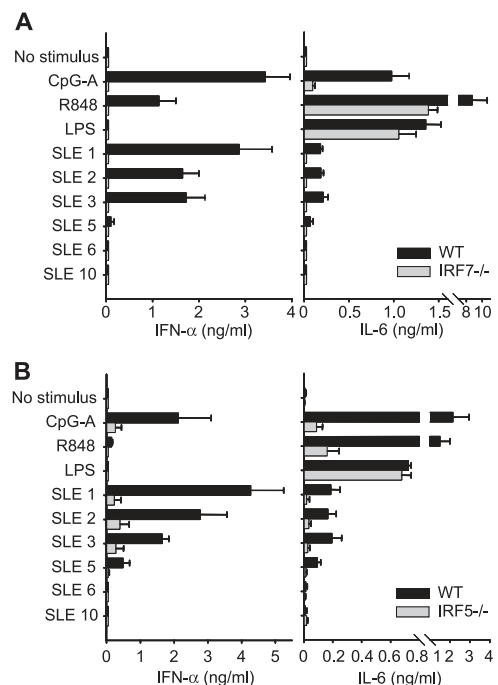

FIGURE 4. RNA is required for murine FL-DC activation by IgG from lupus patients. *A*, Bone marrow-derived FL-DCs from WT C57BL/6 mice were preincubated for 60 min with or without RNase A (5 μg/ml) and then cultured with the CpG-A ODN 2336 (50 nM), R848 (15 nM), and IgG from lupus patients SLE1, SLE 2, and SLE3 (50 μg/ml). IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of three experiments. *B*, IgG from lupus patient SLE1 was pretreated (RNase-pretreated SLE1) or not pretreated (SLE1) with RNase (100 μg/ml) for 24 h, protein G purified to remove the RNase, and then added to the FL-DC cultures in the presence or absence of RNase A (5 μg/ml). IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of three experiments.

FIGURE 5. IRF5 and IRF7 are both required for mouse FL-DC activation by IgG from lupus patients. Bone marrow-derived FL-DCs from WT C57BL/6 and IRF7-deficient (IRF7$^{-/-}$) mice (*A*) or WT C57BL/6 and IRF5-deficient (IRF5$^{-/-}$) mice (*B*) were cultured with the CpG-A ODN 2336 (50 nM), R848 (15 nM), LPS (25 ng/ml), and IgG from lupus patients (50 μg/ml; SLE1, SLE2, SLE3, SLE5, SLE6, and SLE10). IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of three experiments for *A* and five experiments for *B*.
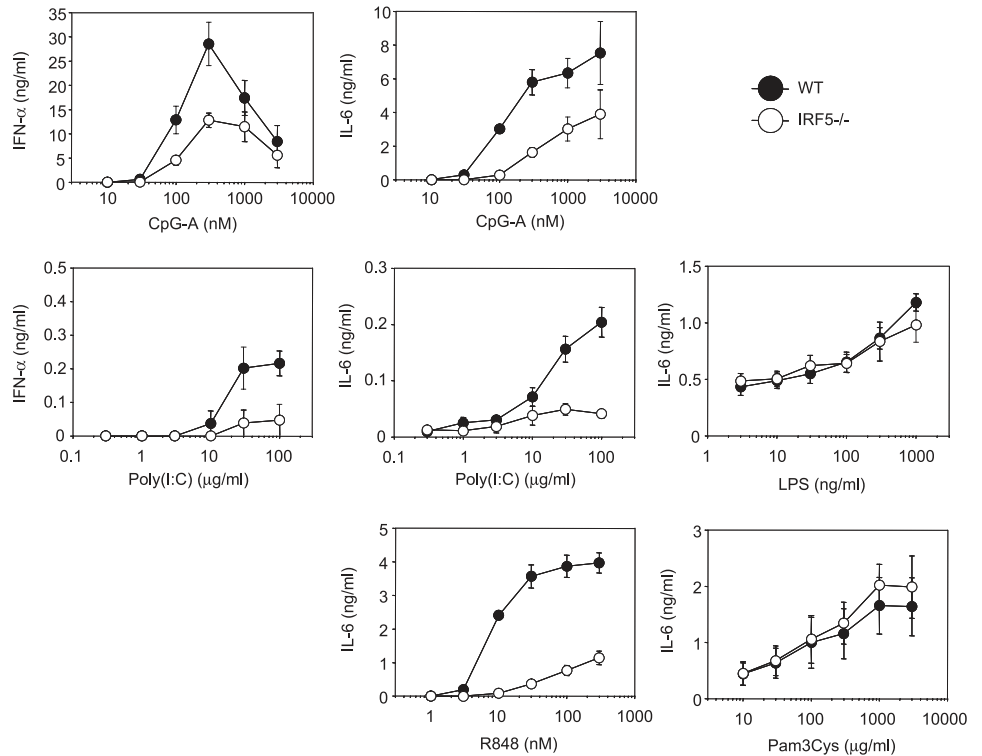
FIGURE 6. IRF5 is involved in FL-DCs IFN-α and IL-6 production induced by TLR3, TLR7, and TLR9 ligands. Bone marrow-derived FL-DCs from WT C57BL/6 and IRF5-deficient (IRF5$^{-/-}$) mice were cultured with indicated concentrations of the CpG-A ODN 2336, poly(I:C), R848, LPS, and Pam3Cys. IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of three experiments.

we separated FL-DCs into pDC and cDC populations by cell sorting. The pDCs produced high levels of IFN-α in response to stimulatory lupus IgG, whereas no IFN-α production was seen from cDCs (Table II). The TLR7/8 ligand R848 did not induce IFN-α production by pDCs, although it did induce high levels of IL-6 from pDCs. The reasons for this lack of IFN-α induction by R848 are not clear, but similar in vitro findings have been reported previously by other investigators (21). Intriguingly, the low level of IL-6 induced by the stimulatory lupus IgG in FL-DC cultures was no longer seen in cultures of purified pDCs and cDCs.

IRF7 and IRF5 are both critical mediators of FL-DC activation induced by stimulatory lupus IgG

IRF7 and IRF5 are transcription factors that contribute importantly to the specific gene expression programs induced by TLR7 and

TLR9 (28). However, the roles of IRF7 and IRF5 in DC IFN-α and proinflammatory cytokine production induced by nucleic acid immune complexes are unknown. To examine this, we compared lupus IgG activation of FL-DCs from WT, IRF7-deficient, and IRF5-deficient mice. We first confirmed that the percentages of pDCs and cDCs in the FL-DCs from the three different mouse strains were similar, so that any differences in cytokine production observed on activation could not simply be attributed to differences in DC subset number (data not shown). The stimulatory lupus IgG and the conventional TLR9 ligand CpG-A failed to induce IFN-α production by IRF7-deficient FL-DCs (Fig. 5A), which is consistent with the role of IRF7 as the master regulator of type I IFN production (29). Unexpectedly, however, we found a marked reduction in IL-6 production by the IRF7-deficient FL-DC in response to the stimulatory IgG, CpG-A, and R848. This was not due
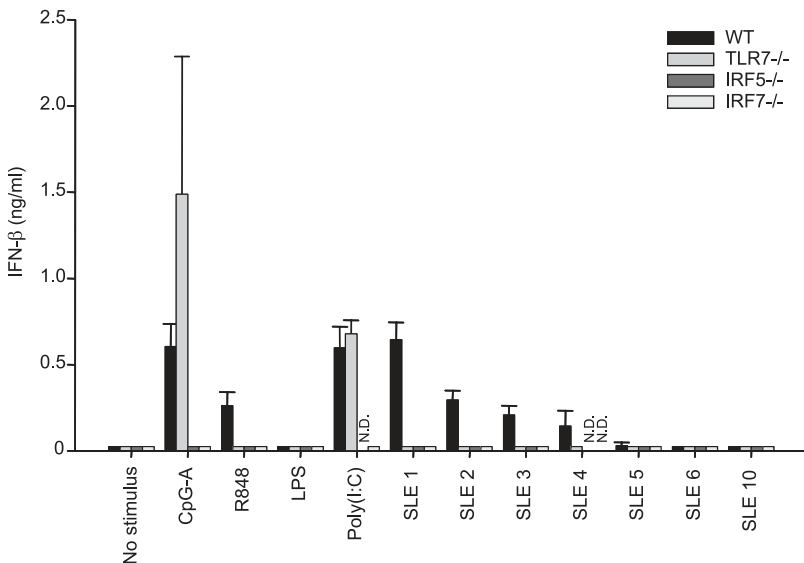
FIGURE 7. IFN-β production by FL-DCs stimulated with IgG from lupus patients is TLR7, IRF5, and IRF7 dependent. Bone marrow-derived FL-DCs from WT C57BL/6, TLR7-deficient (TLR7$^{-/-}$), IRF5-deficient (IRF5$^{-/-}$), and IRF7-deficient (IRF7$^{-/-}$) mice were cultured with the CpG-A ODN 2336 (50 nM), R848 (15 nM), LPS (25 ng/ml), and IgG from lupus patients (50 μg/ml; SLE1–6, 10). IFN-β concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of 13 experiments (WT), 4 experiments (TLR7$^{-/-}$), 4 experiments (IRF5$^{-/-}$), and 3 experiments (IRF7$^{-/-}$). N.D., Not done.
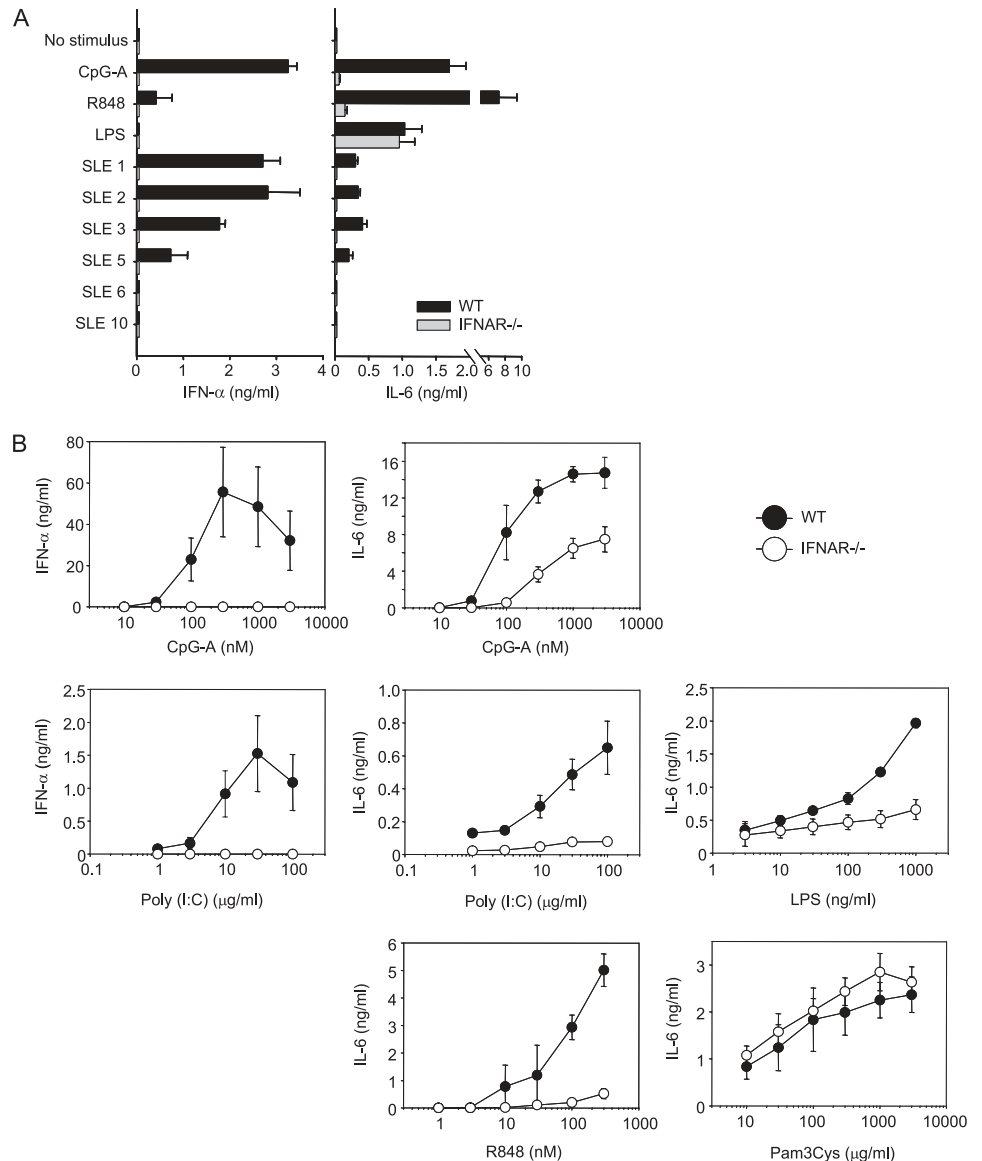
FIGURE 8. FL-DC IL-6 production induced by TLR3, TLR4, TLR7, and TLR9 ligands is dependent on signaling through the type I IFNR. Bone marrow-derived FL-DCs from WT C57BL/6 and type I IFNR-deficient (IFNAR$^{-/-}$) mice were cultured with the CpG-A ODN 2336 (50 nM), P848 (15 nM), LPS (25 ng/ml), and IgG from lupus patiens (50 μg/ml; SLE1, 2, 3, 5, 6, 10) (*A*) or with the indicated concentrations of the CpG-A ODN 2336, poly(I:C), R848, LPS, and Pam3Cys (*B*). IFN-α and IL-6 concentrations in supernatants collected after 24 h were measured by ELISA. Data represent mean ± SEM of three experiments for both *A* and *B*.

to an inability of IRF7-deficient FL-DCs to make IL-6 as LPS induced similar levels of IL6 in WT and IRF7-deficient FL-DCs.

In IRF5-deficient DCs, we found that IFN-α production induced by the stimulatory lupus IgG and CpG-A was substantially, but not completely, reduced as compared with WT DCs (Fig. 5*B*). This was a surprising result as other investigators have shown that pDC IFN-α production induced by CpG-A at 1 μM is IRF5 independent (32). Because we used a lower concentration of CpG-A (50 nM) in our experiments, we performed more extensive dose-response assays to determine whether IRF5 would still play a role in IFN-α production at higher CpG-A concentrations (Fig. 6). We found decreased IFN-α production in IRF5-deficient FL-DCs at a range of CpG-A concentrations, although this was less apparent at the highest concentrations. IFN-α production in response to the TLR3 ligand poly(I:C) was also partially IRF5 dependent. IL-6 production by IRF5-deficient DCs was reduced substantially in response to stimulatory lupus IgG and the conventional TLR3, TLR7, and TLR9 ligands but not in response to the TLR2 ligand Pam3Cys or the TLR4 ligand LPS.

Lupus IgG induces IFN-β secretion by FL-DCs

IFN-α and IFN-β share the same receptor and activate similar IFN-$\alpha\beta$-inducible gene programs (4). It is thus possible that IFN-β

could potentially contribute to the type I IFN effects in lupus generally attributed to the effects of IFN-α. However, it is not known whether RNA-containing immune complexes are able to induce IFN-β production or, more generally, whether IRF5 plays a role in TLR7-induced IFN-β production in DCs. We found that stimulatory lupus IgG, as well as conventional TLR7 and TLR9 ligands, induced appreciable IFN-β production by FL-DCs and that this was dependent on both IRF5 and IRF7 (Fig. 7). As for other readouts of activation induced by stimulatory lupus IgG, this was also TLR7 dependent.

FL-DC IL-6 production induced by TLR7 and TLR9 ligands is dependent on signaling through the type I IFNR

After TLR7 or TLR9 engagement, signaling pathways downstream of MyD88 bifurcate into NF-κB- and IRF7-activating pathways, with NF-κB being required for inflammatory cytokine gene expression and IRF7 being essential for type I IFN gene induction (45). Therefore, we were surprised by our observations that lupus IgG failed to induce IL-6 in purified pDC and cDC cultures (Table II) and also that IL-6 production was markedly reduced in IRF7-deficient FL-DC (Fig. 5*A*). This led us to hypothesize that IL-6 production induced in FL-DCs by the stimulatory lupus IgG might require a synergistic signal from IFN-α produced by the pDCs and,

thus, that these pathways might be interdependent. To test this, we compared FL-DCs from WT and type I IFN receptor (IFNAR)-deficient mice. Consistent with our hypothesis, IL-6 production was not seen in IFNAR-deficient FL-DCs activated with stimulatory lupus IgG. Remarkably, we also found the same requirement for type I IFN signaling in the production of IL-6 induced by low concentrations of the conventional TLR9 and TLR7 ligands but not in the production of IL-6 induced by a low concentration of the TLR4 ligand LPS (Fig. 8A). We further observed that IFN-α production in response to lupus IgG or CpG-A was abrogated in FL-DCs from IFNAR-deficient mice (Fig. 8A), possibly due in part to the absence of the type I IFN-dependent amplification loop previously reported to be required for the robust sustained production of IFN-α by pDCs following TLR9 engagement (46).

To determine whether IFNAR signaling was also required for IL-6 production at higher concentrations of TLR ligands, we performed more extensive dose-titration assays and also tested additional TLR ligands. These studies confirmed our initial findings, except that at higher CpG-A concentrations some IL-6 production by IFNAR-deficient FL-DC was observed, although substantially less than that produced by WT FL-DCs (Fig. 8B). IL-6 production was independent of the IFNAR at a low concentration of LPS (Fig. 8A). However, at higher LPS concentrations, an IFNAR-dependent effect was observed (Fig. 8B), presumably reflecting IFN-β production induced by LPS at the higher concentrations. IL-6 production induced by the TLR3 ligand poly(I:C) and the TLR7/8 ligand R848 was almost entirely dependent on IFNAR signaling at all ligand concentrations tested. In contrast, IL-6 production induced by the TLR2 ligand Pam3Cys was independent of the IFNAR. This result can be explained by the fact that, unlike TLR3, TLR4, TLR7, and TLR9 ligands, TLR2 ligands do not induce type I IFN (47–50). Thus, IL-6 production induced by TLR9 and TLR7 ligands, as well as by TLR3 and TLR4 ligands, is not simply a result of NF-κB activation downstream of these TLRs but also requires a signal through the IFNAR.

Discussion

Dysregulated activation of DCs by nucleic acid-containing immune complexes is implicated in the pathogenesis of SLE (6, 9). However, it has been difficult to definitively examine the receptors and signaling pathways involved in DC activation by human lupus immune complexes using human DCs. In this study, we show that immune complexes comprised of RNA and human IgG can effectively activate mouse DCs and that this system can be used to identify the receptors and signaling pathways involved in this activation.

Mouse FL-DC activation by the stimulatory lupus sera and purified IgG was FcγR dependent and TLR7 dependent and was inhibited by RNase, strongly suggesting that RNA-containing immune complexes were responsible for the activation. No exogenous Ag was added to the cultures, so the source of RNA must have been either RNA-containing immune complexes originally present in the patient sera or, alternatively, apoptotic or necrotic mouse cells in the FL-DC cultures. If the latter is the case, as is suggested by the IgG RNase pretreatment experiments (Fig. 4B), it may be that RNA-containing immune complexes in lupus are not present in the circulation but instead are formed locally at sites of increased apoptosis or impaired apoptotic cell clearance (9, 51). In either case, based on the autoantibody reactivity of the stimulatory sera, it is likely that RNA associated with Sm protein and/or RNP is an important component of the immune complex, although we cannot exclude the possibility that unidentified anti-RNA Abs in lupus sera are also playing a role. The uridine-rich hYRNAs that are associated with Ro or La proteins are also potential candidates

to activate pDCs (23), but we found no correlation between anti-Ro or anti-La reactivity and the stimulatory ability of the sera or purified IgG. This may reflect a lack of Ro/hYRNA and La/hYRNA in the cultures rather than an intrinsic lack of stimulatory ability of hYRNA per se, and it is possible that in certain in vivo or different in vitro situations sufficient Ro/hYRNA and La/hYRNA might be available to allow the formation of stimulatory immune complexes (52, 53).

Our finding that FL-DC activation by human IgG RNA-containing immune complexes was TLR7 dependent is consistent with a number of previous reports. In studies in which immune complexes were formed by combining IgG from lupus patients with an exogenous source of RNA, human pDC activation was blocked substantially by inhibitors of the TLR7 signaling pathway (23, 24). Furthermore, TLR7 was necessary for mouse pDC or B cell activation induced by immune complexes consisting of a mouse monoclonal anti-Sm Ab bound to RNA-protein Ag (19, 39). TLR7 was also required for the development of autoantibodies against RNA-related Ags in mouse models in vivo (54–57). Although we cannot exclude the possibility that other intracellular nucleic acid sensors such as the RNA helicases melanoma differentiation-associated gene 5 and retinoic acid-inducible gene I (58, 59) may also play a role in detecting RNA containing immune complexes, recent studies demonstrating specificity of retinoic acid-inducible gene I for viral RNA make this less likely (60, 61).

Immune complexes containing mammalian DNA can activate DCs and B cells, at least in part through TLR9 (12–14, 16). However, we did not observe DC activation in response to sera or IgG from patients with reactivity to dsDNA, even though the samples were obtained from patients with active disease and most were of high titer. It may be that not all patients with dsDNA reactivity have circulating DNA-containing immune complexes or that the human DNA, if present, is not able to effectively engage mouse TLR9. Alternatively, it is possible that the stimulatory DNA-containing immune complexes are not stable after acid-elution from protein G as was found in one study (12), although these complexes were reported to be stable after acid-elution in another study (13). However, if instability was the reason, we might still have expected to find DC activation with the dsDNA-reactive sera before IgG purification, which we did not. The specificity of human autoantibodies for mouse autoantigens also needs to be considered. It is possible that human autoantibodies may not bind to mouse and human autoantigens equally because of limited autoantigen cross-reactivity. Thus, even if potentially immunostimulatory DNA is released by the mouse DCs in vitro, it may not form effective immune complexes with the human anti-DNA autoantibodies. As the focus of our study was on RNA-containing immune complexes, these issues were not addressed in detail, but it will be important to re-examine them in future work.

A previous study has linked IRF5 with TLR-induced proinflammatory cytokine production but not with the production of type I IFN (32). Therefore, we were surprised by our finding that IFN-α and IFN-β production induced by RNA-containing immune complexes, as well as by CpG-A and poly(I:C), was mostly IRF5 dependent. There are a number of possible explanations for this difference. The other investigators used 1 μM of the TLR9 ligand CpG-A to induce IFN-α by splenic pDCs and found no difference in the production of IFN-α by WT and IRF5-deficient cells. Production of type I IFN induced by TLR7 activation was not evaluated in these studies. In contrast, we used a lower dose of CpG-A (50 nM) in our initial studies, and, in subsequent dosage studies, we found that differences in CpG-A-induced IFN-α production between WT and IRF5-deficient FL-DCs were more pronounced at lower CpG-A concentrations than at higher CpG-A concentrations,

although differences were present at a range of concentrations. The issue of dosage may be especially relevant in terms of lupus pathogenesis where the strength of activation of TLR7 or TLR9 by nucleic acid-containing immune complexes is very likely less than that elicited by maximal doses of microbial and synthetic TLR7 and TLR9 ligands. It is also possible that pDCs derived from spleen differ from pDCs in FL-DCs in terms of the requirement for IRF5 in CpG-A induced IFN-α production. There is additional in vitro evidence in human cell lines supporting a role for IRF5 in type I IFN production. For example, IRF5 was originally identified in cell lines as a regulator of type I IFN gene expression induced by infection with certain viruses (30, 31), and IRF5 was shown to be a central mediator of type I IFN production induced following TLR7 activation by R848 in HEK293 cells (62). The role of IRF5 in DC type I IFN production induced by nucleic acid immune complexes may be especially relevant in SLE, given the potential contribution of IFN-α to disease pathogenesis (5, 6) and recent studies showing an association between IRF5 polymorphisms and an increased risk of SLE in humans (26, 27).

We did not see a difference in FL-DC IL-6 production between WT and IRF5-deficient FL-DCs activated with the TLR4 ligand LPS or the TLR2 ligand Pam3Cys. Our findings are consistent with those in WT and IRF5-deficient bone marrow-derived macrophages pretreated with IFN-γ in which equivalent production of IL-6 was seen in response to LPS (63). However, they differ from the decreased production of IL-6 observed with LPS activation of IRF5-deficient mouse splenic macrophages reported by others (32). This could be due to differences in the activation state of the different macrophage populations in the published studies or, in our studies, to cell-specific differences between macrophages and DCs in the requirement for IRF5 in IL-6 production. Certainly, cell-specific differences are found in humans, where there are multiple IRF5 isoforms with distinct cell type-specific expression, cellular localization, differential regulation, and dissimilar functions in virus-mediated type I IFN gene induction (64).

TLR7 and TLR9 induce both inflammatory cytokine and type I IFN production, with the intracytoplasmic adaptor molecule MyD88 being essential in this process (65). The signaling pathways downstream of MyD88 leading to production of these cytokines are generally regarded as distinct and separate, with the NF-κB pathway being required for inflammatory cytokine gene expression and the IRF7 pathway being required for type I IFN gene induction (45). However, we found that production of the inflammatory cytokine IL-6 induced by RNA-containing immune complexes or by conventional TLR7 and TLR9 ligands, as well as by TLR3 and TLR4 ligands, was dependent on signaling through the IFNAR. This was not due simply to the direct induction of IL-6 by type I IFN because only very low levels of IL-6 were induced in FL-DC cultures by rIFN-α or IFN-β alone, even at high concentrations (data not shown). In contrast, IL-6 production induced by the TLR2 ligand Pam3Cys was independent of the IFNAR. This result can be explained by the fact that, unlike TLR3, TLR4, TLR7, and TLR9 ligands, TLR2 ligands do not induce type I IFN (47–50). Thus, signaling through the IFNAR presumably augments IL-6 production induced via the NFκB pathway following activation of those TLRs able to induce type I IFN. A similar requirement for type I IFN signaling in the production by mouse bone marrow-derived myeloid DCs of IL-12p70 induced either by TLR7/TLR4 or TLR7/TLR3 coengagement has been reported previously (48). The marked decrease in IL-6 production we observed in IRF7-deficient DCs in response to TLR7 and TLR9 ligands likely reflects a requirement for IFN signaling in IL-6 production as discussed above rather than a direct role for IRF7 in the NF-κB pathway. It will be important to determine the extent to which

IFNAR signaling contributes to the production of other proinflammatory cytokines induced by TLR7 and TLR9 engagement.

It is necessary to interpret the data presented here recognizing that there may be inherent differences between mouse and human DCs both in signaling pathways and receptor expression and that findings will ultimately need to be confirmed in human cells. Nevertheless, this system using human IgG and mouse DCs may provide important information that would be difficult to obtain in other ways and may also prove generally applicable to the study of other human immune complex-mediated diseases and other cell types.

Disclosures

Ian Rifkin and Ann Marshak-Rothstein have financial interests in that US patent application 10/487,885 entitled Method and Composition for Treating Immune Complex Associated Disorders and corresponding foreign applications have been licensed and provide royalty income.

References

1. Kotzin, B. L. 1996. Systemic lupus erythematosus. *Cell* 85: 303–306.
2. Tan, E. M. 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv. Immunol.* 44: 93–151.
3. Hall, J., C. L. Casciola-Rosen, and A. Rosen. 2004. Altered structure of autoantigens during apoptosis. *Rheum. Dis. Clin. North Am.* 30: 455–471.
4. Theofilopoulos, A. N., R. Baccala, B. Beutler, and D. H. Kono. 2005. Type I interferons (α/β) in immunity and autoimmunity. *Annu. Rev. Immunol.* 23: 307–336.
5. Baechler, E. C., P. K. Gregersen, and T. W. Behrens. 2004. The emerging role of interferon in human systemic lupus erythematosus. *Curr. Opin. Immunol.* 16: 801–807.
6. Banchereau, J., and V. Pascual. 2006. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity* 25: 383–392.
7. Nakano, H., M. Yanagita, and M. D. Gunn. 2001. CD11c$^+$B220$^+$Gr-1$^+$ cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J. Exp. Med.* 194: 1171–1178.
8. Liu, Y. J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* 23: 275–306.
9. Ronnblom, L., M. L. Eloranta, and G. V. Alm. 2006. The type I interferon system in systemic lupus erythematosus. *Arthritis Rheum.* 54: 408–420.
10. Bave, U., G. V. Alm, and L. Ronnblom. 2000. The combination of apoptotic U937 cells and lupus IgG is a potent IFN-α inducer. *J. Immunol.* 165: 3519–3526.
11. Lovgren, T., M. L. Eloranta, U. Bave, G. V. Alm, and L. Ronnblom. 2004. Induction of interferon α production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum.* 50: 1861–1872.
12. Vallin, H., S. Blomberg, G. V. Alm, B. Cederblad, and L. Ronnblom. 1999. Patients with systemic lupus erythematosus (SLE) have a circulating inducer of interferon α (IFN-α) production acting on leucocytes resembling immature dendritic cells. *Clin. Exp. Immunol.* 115: 196–202.
13. Means, T. K., E. Latz, F. Hayashi, M. R. Murali, D. T. Golenbock, and A. D. Luster. 2005. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J. Clin. Invest.* 115: 407–417.
14. Boule, M. W., C. Broughton, F. Mackay, S. Akira, A. Marshak-Rothstein, and I. R. Rifkin. 2004. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J. Exp. Med.* 199: 1631–1640.
15. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745.
16. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607.
17. Yasuda, K., P. Yu, C. J. Kirschning, B. Schlatter, F. Schmitz, A. Heit, S. Bauer, H. Hochrein, and H. Wagner. 2005. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. *J. Immunol.* 174: 6129–6136.
18. Barton, G. M., J. C. Kagan, and R. Medzhitov. 2006. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat. Immunol.* 7: 49–56.
19. Savarese, E., O. W. Chae, S. Trowitzsch, G. Weber, B. Kastner, S. Akira, H. Wagner, R. M. Schmid, S. Bauer, and A. Krug. 2006. U1 small nuclear ribonucleoprotein immune complexes induce type I interferon in plasmacytoid dendritic cells through TLR7. *Blood* 107: 3229–3234.

20. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529–1531.

21. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* 303: 1526–1529.

22. Lund, J. M., L. Alexopoulou, A. Sato, M. Karow, N. C. Adams, N. W. Gale, A. Iwasaki, and R. A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 101: 5598–5603.

23. Vollmer, J., S. Tluk, C. Schmitz, S. Hamm, M. Jurk, A. Forsbach, S. Akira, K. M. Kelly, W. H. Reeves, S. Bauer, and A. M. Krieg. 2005. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. *J. Exp. Med.* 202: 1575–1585.

24. Barrat, F. J., T. Meeker, J. Gregorio, J. H. Chan, S. Uematsu, S. Akira, B. Chang, O. Duramad, and R. L. Coffman. 2005. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J. Exp. Med.* 202: 1131–1139.

25. Lenert, P. 2005. Inhibitory oligodeoxynucleotides: therapeutic promise for systemic autoimmune diseases. *Clin. Exp. Immunol.* 140: 1–10.

26. Sigurdsson, S., G. Nordmark, H. H. Goring, K. Lindroos, A. C. Wiman, G. Sturfelt, A. Jonsen, S. Rantapaa-Dahlqvist, B. Moller, J. Kere, et al. 2005. Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. *Am. J. Hum. Genet.* 76: 528–537.

27. Graham, R. R., S. V. Kozyrev, E. C. Baechler, M. V. Reddy, R. M. Plenge, J. W. Bauer, W. A. Ortmann, T. Koeuth, M. F. Gonzalez Escribano, B. Pons-Estel, et al. 2006. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nat. Genet.* 38: 550–555.

28. Honda, K., and T. Taniguchi. 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat. Rev. Immunol.* 6: 644–658.

29. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434: 772–777.

30. Barnes, B. J., P. A. Moore, and P. M. Pitha. 2001. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon α genes. *J. Biol. Chem.* 276: 23382–23390.

31. Barnes, B. J., A. E. Field, and P. M. Pitha-Rowe. 2003. Virus-induced heterodimer formation between IRF-5 and IRF-7 modulates assembly of the IFNA enhanceosome in vivo and transcriptional activity of IFNA genes. *J. Biol. Chem.* 278: 16630–16641.

32. Takaoka, A., H. Yanai, S. Kondo, G. Duncan, H. Negishi, T. Mizutani, S. Kano, K. Honda, Y. Ohba, T. W. Mak, and T. Taniguchi. 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 434: 243–249.

33. Hulett, M. D., and P. M. Hogarth. 1994. Molecular basis of Fc receptor function. *Adv. Immunol.* 57: 1–127.

34. Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3: 196–200.

35. Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264: 1918–1921.

36. Tan, E. M., A. S. Cohen, J. F. Fries, A. T. Masi, D. J. McShane, N. F. Rothfield, J. G. Schaller, N. Talal, and R. J. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 25: 1271–1277.

37. Shi, G. P., J. A. Villadangos, G. Dranoff, C. Small, L. Gu, K. J. Haley, R. Riese, H. L. Ploegh, and H. A. Chapman. 1999. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity* 10: 197–206.

38. Naik, S. H., A. I. Proietto, N. S. Wilson, A. Dakic, P. Schnorrer, M. Fuchsberger, M. H. Lahoud, M. O'Keeffe, Q. X. Shao, W. F. Chen, et al. 2005. Cutting edge: generation of splenic CD8$^+$ and CD8$^-$ dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* 174: 6592–6597.

39. Lau, C. M., C. Broughton, A. S. Tabor, S. Akira, R. A. Flavell, M. J. Mamula, S. R. Christensen, M. J. Shlomchik, G. A. Viglianti, I. R. Rifkin, and A. Marshak-Rothstein. 2005. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J. Exp. Med.* 202: 1171–1177.

40. Martin, D. A., and K. B. Elkon. 2005. Autoantibodies make a U-turn: the Toll hypothesis for autoantibody specificity. *J. Exp. Med.* 202: 1465–1469.

41. Takai, T. 2005. Fc receptors and their role in immune regulation and autoimmunity. *J. Clin. Immunol.* 25: 1–18.

42. Nimmerjahn, F., and J. V. Ravetch. 2006. Fcγ receptors: old friends and new family members. *Immunity* 24: 19–28.

43. Diebold, S. S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L. E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow, and C. Reis e Sousa. 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 424: 324–328.

44. Honda, K., Y. Ohba, H. Yanai, H. Negishi, T. Mizutani, A. Takaoka, C. Taya, and T. Taniguchi. 2005. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 434: 1035–1040.

45. Kaisho, T., and S. Akira. 2006. Toll-like receptor function and signaling. *J. Allergy Clin. Immunol.* 117: 979–987.

46. Kerkmann, M., S. Rothenfusser, V. Hornung, A. Towarowski, M. Wagner, A. Sarris, T. Giese, S. Endres, and G. Hartmann. 2003. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. *J. Immunol.* 170: 4465–4474.

47. Toshchakov, V., B. W. Jones, P. Y. Perera, K. Thomas, M. J. Cody, S. Zhang, B. R. Williams, J. Major, T. A. Hamilton, M. J. Fenton, and S. N. Vogel. 2002. TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. *Nat. Immunol.* 3: 392–398.

48. Gautier, G., M. Humbert, F. Deauvieau, M. Scuiller, J. Hiscott, E. E. Bates, G. Trinchieri, C. Caux, and P. Garrone. 2005. A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J. Exp. Med.* 201: 1435–1446.

49. Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 7: 179–190.

50. Bagchi, A., E. A. Herrup, H. S. Warren, J. Trigilio, H. S. Shin, C. Valentine, and J. Hellman. 2007. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J. Immunol.* 178: 1164–1171.

51. Clancy, R. M., and J. P. Buyon. 2004. More to death than dying: apoptosis in the pathogenesis of SSA/Ro-SSB/La-associated congenital heart block. *Rheum. Dis. Clin. North Am.* 30: 589–602.

52. Gottenberg, J. E., N. Cagnard, C. Lucchesi, F. Letourneur, S. Mistou, T. Lazure, S. Jacques, N. Ba, M. Ittah, C. Lepajolec, et al. 2006. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren's syndrome. *Proc. Natl. Acad. Sci. USA* 103: 2770–2775.

53. Lovgren, T., M. L. Eloranta, B. Kastner, M. Wahren-Herlenius, G. V. Alm, and L. Ronnblom. 2006. Induction of interferon α by immune complexes or liposomes containing systemic lupus erythematosus autoantigen- and Sjogren's syndrome autoantigen-associated RNA. *Arthritis Rheum.* 54: 1917–1927.

54. Pisitkun, P., J. A. Deane, M. J. Difilippantonio, T. Tarasenko, A. B. Satterthwaite, and S. Bolland. 2006. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* 312: 1669–1672.

55. Subramanian, S., K. Tus, Q. Z. Li, A. Wang, X. H. Tian, J. Zhou, C. Liang, G. Bartov, L. D. McDaniel, X. J. Zhou, et al. 2006. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc. Natl. Acad. Sci. USA* 103: 9970–9975.

56. Christensen, S. R., J. Shupe, K. Nickerson, M. Kashgarian, R. A. Flavell, and M. J. Shlomchik. 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* 25: 417–428.

57. Berland, R., L. Fernandez, E. Kari, J. H. Han, I. Lomakin, S. Akira, H. H. Wortis, J. F. Kearney, A. A. Ucci, and T. Imanishi-Kari. 2006. Toll-like receptor 7-dependent loss of B cell tolerance in pathogenic autoantibody knockin mice. *Immunity* 25: 429–440.

58. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5: 730–737.

59. Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y. M. Loo, M. Gale, Jr., S. Akira, et al. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* 175: 2851–2858.

60. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* 314: 997–1001.

61. Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, et al. 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science* 314: 994–997.

62. Schoenemeyer, A., B. J. Barnes, M. E. Mancl, E. Latz, N. Goutagny, P. M. Pitha, K. A. Fitzgerald, and D. T. Golenbock. 2005. The interferon regulatory factor, IRF5, is a central mediator of Toll-like receptor 7 signaling. *J. Biol. Chem.* 280: 17005–17012.

63. Negishi, H., Y. Ohba, H. Yanai, A. Takaoka, K. Honma, K. Yui, T. Matsuyama, T. Taniguchi, and K. Honda. 2005. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc. Natl. Acad. Sci. USA* 102: 15989–15994.

64. Mancl, M. E., G. Hu, N. Sangster-Guity, S. L. Olshalsky, K. Hoops, P. Fitzgerald-Bocarsly, P. M. Pitha, K. Pinder, and B. J. Barnes. 2005. Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms: multiple isoforms with distinct cell type-specific expression, localization, regulation, and function. *J. Biol. Chem.* 280: 21078–21090.

65. Hemmi, H., T. Kaisho, K. Takeda, and S. Akira. 2003. The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. *J. Immunol.* 170: 3059–3064.