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TLR and RLR Signaling Are Reprogrammed in Opposite Directions after Detection of Viral Infection

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Innate immune recognition of RNA is key for the initiation of immunity in response to viral infection. Although the factors controlling the detection of viral RNA by innate immune receptors in host cells are increasingly well understood, little is known about the dynamic changes in signaling after the initial triggering of these receptors. In this study, we report that preconditioning with the synthetic dsRNA polyinosinic-polycytidylic acid [poly(I:C)], a mimetic of viral RNA, rapidly reprograms murine APCs by simultaneously augmenting sensitivity of endosomal TLRs and inhibiting activation of RIG-I–like receptors (RLRs) in an IFN-β–dependent manner. These changes in receptor sensitivity were also seen in vivo after treatment of mice with poly(I:C). Mechanistically, the increased sensitivity of the TLR pathway was associated with elevated MAPK and NF-κB activity. The RLR response was inhibited downstream of TANK-binding kinase-1, resulting in decreased IFN regulatory factor 3 phosphorylation. Reprogramming of pattern-recognition receptor signaling also occurred after viral infection, because infection of host cells with Sendai virus or their exposure to supernatant from virus-infected cells induced the same changes in TLR and RLR sensitivity as poly(I:C). Thus, innate recognition of viral infection critically modifies responses to pattern-recognition receptor stimulation. These dynamic adaptations to infection may reinforce antiviral immunity and at the same time serve to limit pathological inflammation. ***The Journal of Immunology*****, 2015, 195: 4387–4395.**

The innate immune system is equipped with germline-encoded pattern-recognition receptors (PRRs) that rapidly sense microbial infection and elicit protective responses (1). Upon viral infection, two receptor families, the RIG-I–like receptors (RLRs) and the TLRs, play a dominant role in first-line defense and in the induction of subsequent adaptive immunity (2, 3). The RLR family includes the cytoplasmic sensors RIG-I and MDA-5 that are widely expressed in many cell types. RIG-I and MDA-5 selectively recognize cytoplasmic RNA associated with viral infection and interact with the adaptor protein MAVS to induce the transcription factors IFN regulatory factor (IRF) 3, IRF7, and NF-κB (3). These transcription factors coordinately regulate the expression of type I IFNs, which are essential for antiviral defense.

In contrast to the ubiquitous RLRs, TLRs are expressed mainly on APCs such as dendritic cells (DCs), and TLR ligands trigger the development of adaptive immunity such as B cell and T cell responses (4). The nucleic acid–sensing TLRs consist of TLR3, TLR7, and TLR9, which recognize dsRNA, ssRNA, and CpG-containing DNA, respectively (5). These membrane-bound TLRs are localized to intracellular vesicles and shuttle to endosomes where they detect nucleic acids of microbial origin (6). The signaling pathways of endosomal TLRs, especially TLR7 and -9, differ markedly from RLR pathways: whereas TLR3 induces IRF3 and NF-κB via the adaptor protein Toll/IL-1R domain–containing adapter inducing IFN-β (TRIF), leading to the production of both IFN-β and proinflammatory cytokines, TLR7 and TLR9 employ the adaptor MyD88 to activate mainly the NF-κB pathway and the secretion of proinflammatory cytokines (4). In a specialized subset of DCs only, the plasmacytoid DCs (pDCs), TLR7 and -9 stimulation leads to induction of IFN-α, as in this cell type, MyD88 interacts with the transcription factor IRF7 (7).

A single infectious agent may thus harbor multiple PRR agonists and trigger the activation of different sets of receptors that must result in a coordinated innate response (8, 9). Earlier in vitro studies have shown that sequential triggering of TLR3, -4, -7, and -9 with synthetic ligands could lead to synergy, priming, or tolerance depending on the interval between stimulations and on the use of the adaptor proteins MyD88 or TRIF (10–15). In particular, it was shown that the synthetic dsRNA polyinosinic-polycytidylic acid [poly(I:C)] sensitizes for subsequent stimulation of MyD88-dependent TLR ligation (10, 13) but tolerizes toward further TLR3 stimulation (10). Very few reports analyzed non-TLR receptors in this respect: one study demonstrated

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C.H. and C.B. designed and guided the study, analyzed and interpreted results, and prepared the manuscript. C.H., L.C.R., T. Huber, A.S., A.O., M.T., and T. Herbst designed and performed the experiments and analyzed data. S.H. and S.E. gave conceptual advice and edited the manuscript.

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Abbreviations used in this article: DC, dendritic cell; IKK, IκB kinase; IRF, IFN regulatory factor; pDC, plasmacytoid DC; poly(I:C), polyinosinic-polycytidylic acid; 3P-RNA, 3′-triphosphate-RNA; PRR, pattern-recognition receptor; RLR, RIG-I–like receptor; SeV, Sendai virus; TBK-1, TANK-binding kinase-1; TRIF, Toll/IL-1R domain–containing adapter inducing IFN-β.

enhanced nucleotide-binding oligomerization domain 1/2 responses after poly(I:C) pretreatment or viral infection (16), and one study observed increased IFN-β release in response to TLR and RLR stimulation after preconditioning with TLR2 ligands (17). It is, however, unknown whether viral infection or poly(I:C) preconditioning influences subsequent RLR signaling.

We report in this study that poly(I:C) exposure profoundly modified the sensitivity of TLR and RLR signaling pathways in opposite directions. Whereas TLR responses in APCs were potentiated, RLR-mediated IFN responses were practically abolished within 24 h. This reprogramming in favor of TLRs over RLRs was mediated by IFN-β signaling and involved intracellular signaling changes downstream of PRRs. Importantly, viral infection and exposure of cells to supernatant of infected cells reprogrammed PRR responses with mechanisms and kinetics similar to those observed with poly(I:C). We thus propose that PRR reprogramming is a host adaptation to coordinate multiple receptors and that innate immune detection is more dynamic than previously imagined.

Materials and Methods

Mice

C57BL/6 mice were purchased from Janvier (Le Genest-Saint Isle, France) or Harlan Laboratories (Boxmeer, the Netherlands). All experiments were performed with C57BL/6 mice unless indicated otherwise. TRIF$^{-/-}$, IFNaR$^{-/-}$ as well as 129/Sv control mice were kindly provided by H. J. Anders (Ludwig-Maximilians-Universität, Munich, Germany). MDA-5$^{-/-}$ mice were obtained from S. Rothenfusser (Ludwig-Maximilian-Universität, Munich, Germany). This study was carried out in strict accordance to the guidelines of the German and Swiss animal protection laws. The protocol was approved by the responsible state offices.

Reagents for cell stimulation and ELISA

R848 and poly(I:C) low m.w., rOVA (EndoFit; endotoxin-free), and the inhibitor BX-795 were purchased from Invivogen (Toulouse, France), and phosphorothioate-modified CpG oligonucleotide 1826 (CpG, 5′-TCCATGACGTTCCTGACGTT-3′) was synthesized by Eurofins MWG Operon (Ebersberg, Germany). 5′−Triphosphate RNA (3P-RNA), 2.2 sense sequence (18)] was transcribed in vitro as previously published (19) or purchased from Invivogen. Poly(I:C) and 3P-RNA were complexed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) for cell-culture experiments and 3P-RNA with JetPEI (PolyPlus, Illkirch, France) for in vivo mouse experiments. ELISAs were purchased from BD Biosciences (Heidelberg, Germany) and BioLegend (San Diego, CA) and performed according to the manufacturer's protocol. Levels of IFNs were determined by self-established ELISAs as described earlier (14, 19). Recombinant type I IFNs (α and β), neutralizing polyclonal anti–IFN-α (rabbit pAb), anti–IFN-β (RMMb, mAb), and anti–IFN-β (rabbit pAb) Abs were obtained from PBL InterferonSource (Piscataway, NJ). The anti–IFNaR-1 Ab (MAR1-5A3) was from eBioscience (San Diego, CA). Riboshredder RNase was from Epicentre (Madison, WI). Working concentrations were: R848: 0.1 μg/ml; poly(I:C) (cell culture): 20 μg/ml (for J774) or 200 μg/ml (for DC); poly(I:C) (mouse injections): 100–250 μg; BX-795: 500 nM;

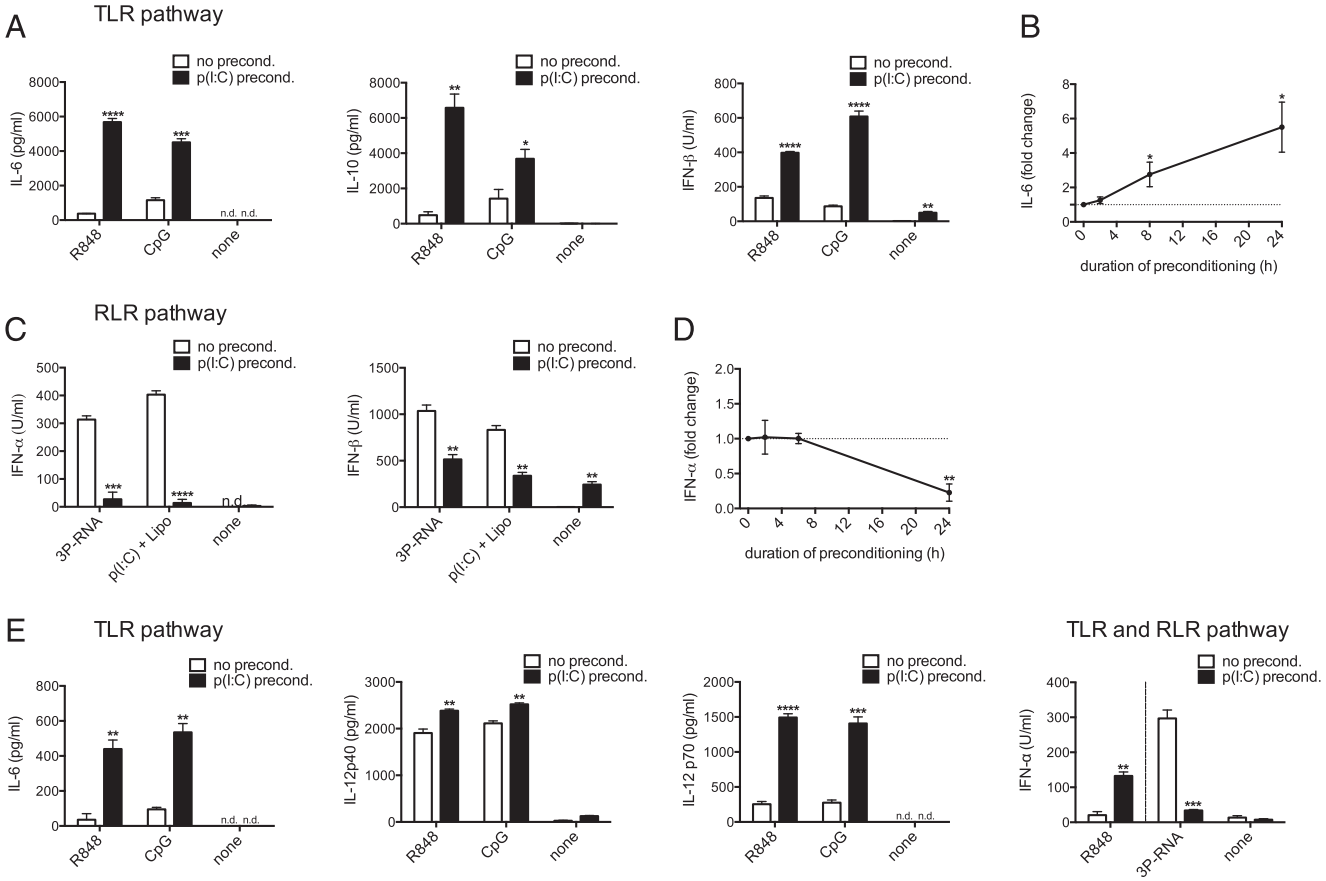

FIGURE 1. Poly(I:C) preconditioning changes TLR and RLR sensitivity. (**A** and **C**) IL-6, IL-10, IFN-α, and IFN-β in supernatants from J774 cells preconditioned for 24 h with naked poly(I:C) prior to stimulation for 24 h with CpG, R848, 3P-RNA complexed with Lipofectamine, or poly(I:C) complexed with Lipofectamine [p(I:C) + Lipo]. (**B** and **D**) IL-6 and IFN-α in supernatants from J774 preconditioned for different durations with naked poly (I:C) prior to stimulation for 24 h with R848 or complexed poly(I:C). Relative expression to the 0-h time point is shown. (**E**) IL-6, IL-12p40, IL-12p70, and IFN-α in supernatants of bone marrow cells treated and analyzed as in (A). (A, C, and E) Black bars versus white bars, Student *t* test. (B and D) Zero-h time point versus other conditions; one-way ANOVA, Dunnett multiple-comparison test. Data are representative of at least three independent experiments [mean + SEM of triplicates in (A), (C), and (E)]. Mean ± SEM of at least three independent experiments are shown in (B). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. n.d., cytokine levels below detection limit.

CpG: 3 μg/ml; 3P-RNA (cell culture): 1 μg/ml + 2.5 μl/ml Lipofectamine; 3P-RNA (mouse injections): 10 μg + 3 μl JetPEI (N/P ratio = 6); poly (I:C) + Lipofectamine: 2 μg/ml poly(I:C) + 2.5 μl/ml Lipofectamine (96-well) or 4 μg/ml poly(I:C) + 2 μl/ml Lipofectamine (24-well); IFN-β: 5000 U/ml; anti–IFN-α or -β: 5000 NU/ml; anti-IFNaR: 1.5 μg/ml; and Riboshredder: 5 U/ml.

Cell culture

J774 macrophages were cultivated in DMEM and 2 mmol L-glutamine (both from PAA Laboratories, Pasching, Austria) supplemented with 10% FBS (Life Technologies, Grand Island, NY) and ciprofloxacin (Bayer, Leverkusen, Germany). For cell stimulations, 96-well plates were used, and J774 cells were seeded at a density of 4×10^4 cells/well. CT26 cells were kept in RPMI 1640 containing 2 mmol L-glutamine, 25 μmol HEPES (all from PAA Laboratories), 10% FBS, and ciprofloxacin. Bone marrow cells and conventional DCs were harvested, differentiated, and cultivated as described previously (14). For the generation of pDCs, bone marrow cells were cultivated with Flt3 ligand (PeproTech or Tebu-bio, Offenbach, Germany) for 7 to 8 d, and $B220^+$ cells were isolated by BD iMAG (BD Biosciences). A total of 2×10^5 DCs or 4×10^5 primary bone marrow cells/well was seeded in 96-well plates.

Immunoblot analyses

J774 (5×10^5/well) were seeded in 24-well plates and stimulated in serum-free Opti-MEM (Life Technologies, Invitrogen). Cells were harvested and lysed in sample buffer (Tris-HCl [pH 6.8], 6% SDS, and 30% glycerol) and loaded onto 10% acrylamide gels, followed by electrophoresis and transfer onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, U.K.). Membranes were blocked in skimmed milk and primary Abs (all from Cell Signaling Technologies, Danvers, MA) were applied overnight and subsequently probed with secondary fluorescence-labeled Abs (anti-rabbit DyLight 680 and anti-mouse DyLight 800; Pierce Biotechnology, Rockford, IL) for 1 h. Membranes were analyzed in a LiCor fluorescence reader (LiCor, Lincoln, NE). The following primary Abs were used: anti–β-actin (8H10D10), anti–phospho-p38 (D3F9), anti-p38 (D13E1), anti–phospho-JNK (G9), anti-JNK (56G8), anti–phospho-p65 (93H1), anti-p65 (L8F6 or D14E12), anti–phospho-ERK1/2 (D13.14.4E), anti-ERK1/2 (3A7), anti–phospho-IRF3 (4D4G), anti-IRF3 (D83B9), anti–phospho–TANK-binding kinase-1 (TBK-1)/NAK (D52C2), anti–TBK-1/NAK (D1B4), anti–MDA-5 (D74E4), and anti–RIG-I (D14G6).

Quantitative real-time PCR

RNA isolation was performed with TRIzol reagent (Life Technologies, Invitrogen) following the manufacturer's instructions. A total of 500 ng RNA was converted to cDNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). Real-time PCR amplification was performed with the KAPA SYBR FAST qPCR Kit Master Mix (Kapa Biosystems, Boston, MA) on a StepOne Plus instrument (Applied Biosystems, Thermo Scientific). Relative gene expression is shown as a ratio of the expression level of the gene of interest to that of hypoxanthine phosphoribosyltransferase and *Gapdh* RNA determined in the same sample. The primers were obtained from Eurogentec (Seraing, Belgium). The following primers were used: hypoxanthine phosphoribosyltransferase: 5′-ATGAGCGCAAGTTGAATCTG-3′ (forward) and 5′-CAGATGGCCACAGGACTAGA-3′ (reverse); *Gapdh*: 5′-CAAAGTGGAGATTGTTGCCA-3′ (forward) and 5′-GCCTTGACTGTGCCGTTGAA-3′ (reverse); *Tlr7*: 5′-TTGCAACTGTGATGCTGTGT-3′ (forward) and 5′-TTTGTGTGCTCCTGGACCTA-3′ (reverse); *Ddx58*: 5′-ACCGCATACAGGTGAATGAA-3′ (forward) and 5′-GCACTTTCCACACAGCAGTT-3′ (reverse); and *Ifih1*: 5′-CCATGACGAGTGTCTCCACT-3′ (forward) and 5′-AAGAGTCCCTTCTCGAAGCA-3′ (reverse).

Viral infection

Sendai virus (SeV; Cantell strain) was purchased from Charles River Laboratories (Wilmington, DE). Cells were infected for 1 h with SeV (50 HA units/ml) in serum-free Opti-MEM. Fresh serum-containing medium was then added. To obtain supernatant from SeV-infected cells, CT26 cells (3×10^5/ml) were infected with SeV as described above. The next day, cell-free supernatant was collected and irradiated for 40 min under UV light to inactivate potential virus particles. Irradiated supernatants were freeze-thawed once prior to use.

Statistics

All statistical analyses were performed with GraphPad Prism software version 5.0d (GraphPad Software, San Diego, CA).

Results

Preconditioning with poly(I:C) alters TLR and RLR responses in opposite directions

To investigate how poly(I:C) exposure modulates TLR and RLR signaling, we preconditioned the J774 macrophage cell line with poly (I:C), a synthetic dsRNA that is a ligand for both MDA-5 (20) and TLR3 (21) when applied without transfection reagent. Cells were washed 24 h later and stimulated with synthetic ligands for TLR7 (R848) and TLR9 (CpG) or transfected with 3P-RNA or the double-stranded synthetic RNA poly(I:C) to stimulate the intracellular receptors RIG-I and MDA-5, respectively. In response to TLR7 or TLR9 stimulation in poly(I:C)-preconditioned cells, we observed a strongly enhanced secretion of IL-6, IL-10, and IFN-β (Fig. 1A). In contrast, secretion of IFN-α and IFN-β following stimulation of RLRs was entirely blocked in preconditioned cells (Fig. 1C). For further experiments, we chose to consistently measure IL-6 to monitor TLR activity and IFN-α to assess RLR activity, as these are signature cytokines for each of these pathways. Indeed, these cytokines are low or absent in the other pathway: we could not detect significant IFN-α secretion after TLR stimulation nor IL-6

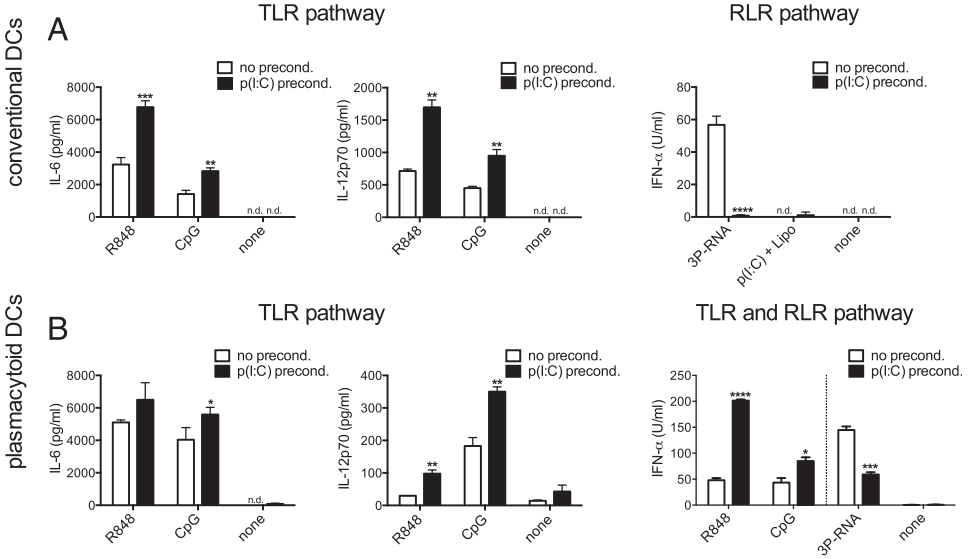

FIGURE 2. TLR and RLR sensitivity changes in DC subsets. Cytokine levels in supernatants from conventional DCs (**A**) and pDCs (**B**) treated and analyzed as in Fig. 1A. Black bars versus white bars, Student *t* test. Data are representative of at least three independent experiments (mean + SEM of triplicates). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. n.d., cytokine levels below detection limit.

release in response to RLR ligation in these cells (Supplemental Fig. 1A). To determine the kinetics of this phenotype, we preconditioned J774 cells with poly(I:C) for different times before stimulation with agonists for TLR7 or MDA-5. Both effects peaked at 24 h of preconditioning (Fig. 1B, 1D). Because of this delay in maximal modulation of subsequent PRR stimulation, we refer to the effects induced by poly(I:C) preconditioning as "PRR reprogramming," which differs from synergism. Taken together, these data show that in addition to priming of endosomal TLR pathways, poly (I:C) preconditioning led to a tolerance toward RLR stimulation, an observation that was to our knowledge not described before.

To examine whether PRR reprogramming affects IL-12 secretion, an essential cytokine for the development of adaptive immunity to viral infection, we selected primary bone marrow cells, as the J774 cell line does not secrete IL-12 isoforms upon TLR7/9 stimulation (not shown). Primary bone marrow cells were preconditioned with poly(I:C) and subsequently stimulated via TLRs and RLRs. In addition to IL-6, poly(I:C) preconditioning enhanced the TLR7- and TLR9-induced secretion of IL-12p40 and IL-12p70, whereas RIG-I–induced IFN-α was again inhibited (Fig. 1E). Interestingly, IFN-α secretion was observed in bone marrow cells after TLR7 stimulation and increased by poly(I:C) preconditioning, in sharp contrast to IFN-α secretion in response to RIG-I stimulation (Fig. 1E, *right*). Bone marrow cells contain a variety of immune cells, including the pDC subset that produces large amounts of IFN-α in response to TLR7 stimulation (22). We therefore then analyzed PRR reprogramming in different DC subsets.

TLR and RLR reprogramming occurs in DC subsets and in vivo

To examine the response of individual cell subsets, we differentiated conventional and pDC subsets from bone marrow. Cells were preconditioned with poly(I:C), followed by stimulation with ligands for TLRs and RLRs. In both cell types, we observed the same changes in sensitivity of TLR and RLR pathways as seen with primary bone marrow cells (Fig. 2A, 2B, Supplemental Fig. 1C). Importantly, IFN-α secretion was enhanced in pDCs in response to TLR7 activation, whereas the same cytokine was suppressed following RIG-I stimulation, clearly demonstrating that the direction of the reprogrammed response is dependent on the type of receptor activated (Fig. 2B, *right panel*). Transfected poly(I:C) did not induce detectable amounts of IFN-α in DCs (Fig. 2A, *right panel*). TLR7/9 stimulation did not induce IFN-β in cDCs (Supplemental Fig. 1C).

To assess whether PRR reprogramming can be detected in vivo, we injected mice with poly(I:C) and treated them 24 h later with either the TLR7 ligand R848 or with the RIG-I–activating 3P-RNA. Poly(I:C) pretreatment in vivo led to higher IL-6, TNF-α, IL-12p40, IFN-α, and IFN-β serum levels following TLR7 activation. In contrast, IL-6 and IFN-α serum levels were markedly decreased and IFN-β serum levels abolished in poly(I:C)-pretreated mice injected with 3P-RNA (Fig. 3A). IL-12p40 levels were generally very low in response to RIG-I stimulation, which is in accordance with the literature (23). To confirm that these in vivo effects were due to a reprogramming of immune cells, we isolated bone marrow cells from mice 24 h after injection with poly(I:C). These cells were stimulated with R848 and 3P-RNA ex vivo. Indeed, these cells showed augmented sensitivity for TLR7 ligation as well as a diminished response toward RIG-I stimulation (Fig. 3B, WT). PRR reprogramming thus represents a global phenomenon occurring similarly in different APC subsets as well as in vivo.

PRR reprogramming is a global phenomenon induced by MyD88-independent innate immune receptors

As naked poly(I:C) can trigger both TLR3 and MDA-5, we investigated which pathway was involved in PRR reprogramming in vivo.

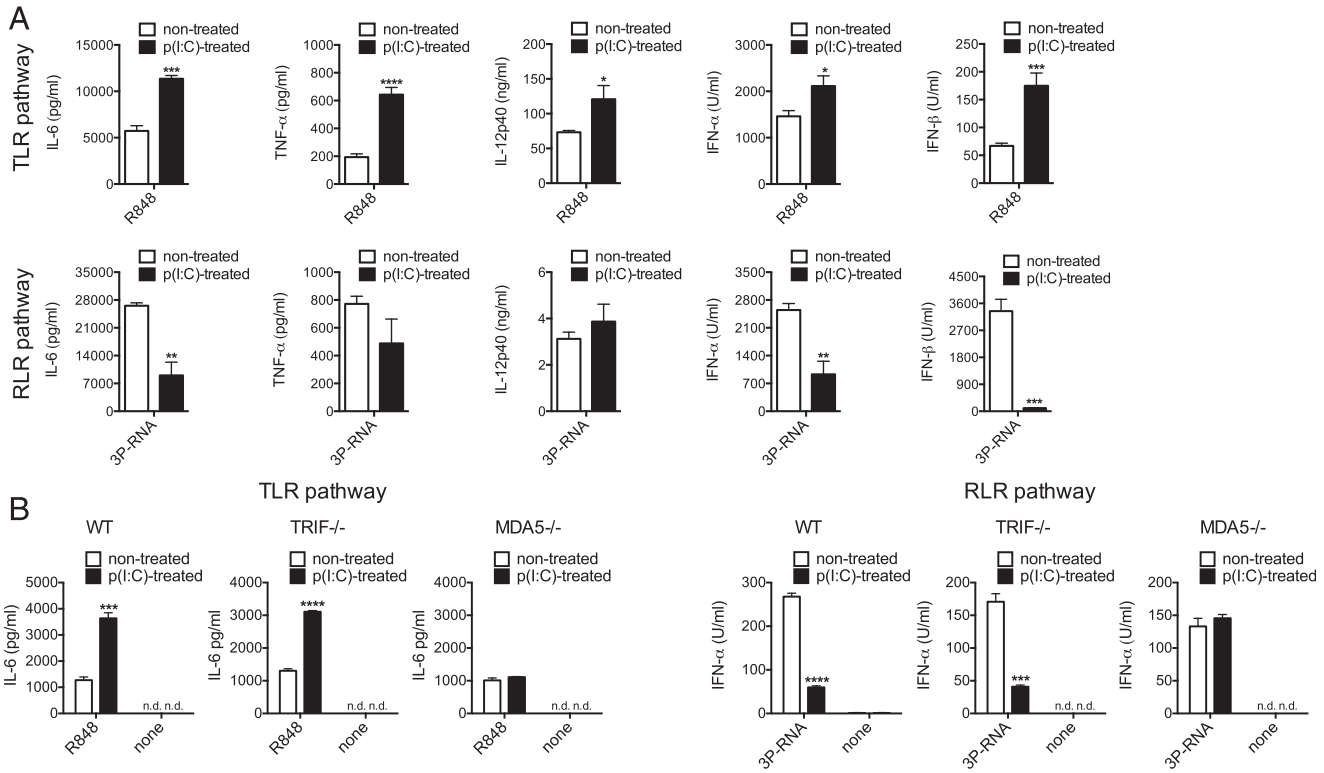

FIGURE 3. Changes in TLR and RLR sensitivity occur in vivo. (**A**) IL-6, TNF-α, IL-12p40, IFN-α, and IFN-β in serum of mice injected with naked poly (I:C) and treated 24 h later for 2 h with R848 or 3P-RNA complexed with JetPEI. (**B**) IL-6 and IFN-α in supernatants from bone marrow cells isolated after 24 h from poly(I:C)-treated wild-type (WT), TRIF$^{-/-}$, or MDA-5$^{-/-}$ mice and stimulated ex vivo with R848 or complexed 3P-RNA for an additional 24 h. (A) n = 4–6 mice/group (mean + SEM). Data are representative of three to six experiments per genotype [mean + SEM of triplicates in (B)]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, white versus black bars, Student t test. n.d., cytokine levels below detection limit.

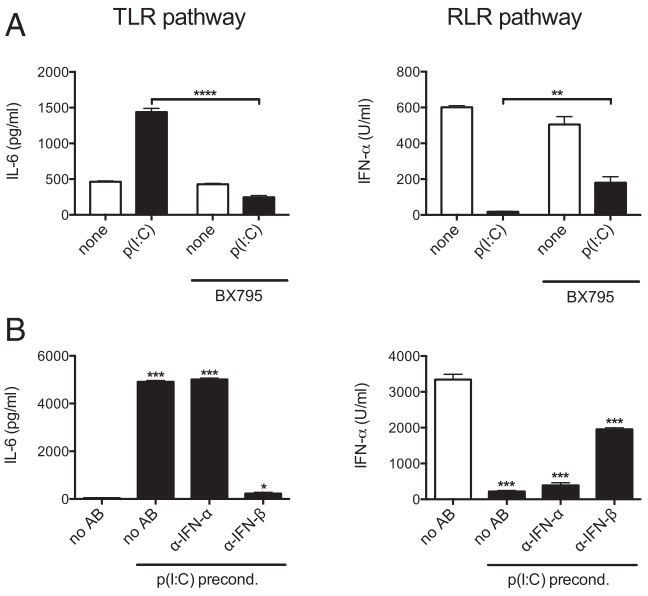

FIGURE 4. IFN-β is essential for poly(I:C)-induced modulation of TLR and RLR sensitivity. (**A** and **B**) IL-6 and IFN-α in supernatants from J774 cells preconditioned with naked poly(I:C) [p(I:C)] for 24 h prior to stimulation for 24 h with R848 (*left panels*) or complexed poly(I:C) (*right panels*). In some conditions, cells were treated with the TBK/IKK inhibitor BX-795 (A) or with Abs (AB) against IFN-α and β during the preconditioning phase (B). (A) Preconditioned versus preconditioned + inhibitor, Student *t* test. (B) Black bars versus white bar, one-way ANOVA, Dunnett multiple-comparison test. Data are representative of two to three independent experiments (mean + SEM of triplicates). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

We repeated the in vivo/ex vivo experiment described above with mice deficient for TRIF, the adaptor protein downstream of TLR3 (24), and with mice deficient for MDA-5. The change in receptor sensitivity was similar in cells derived from wild-type and TRIF$^{-/-}$ mice but abolished in cells from MDA-5$^{-/-}$ mice, demonstrating that MDA-5 activation is solely responsible for the sensitivity changes induced by poly(I:C) in vivo. Experiments with TRIF$^{-/-}$ and MDA-5$^{-/-}$ DC suggest that in contrast both pathways contribute to PRR reprogramming under in vitro conditions (Supplemental Fig. 2A). Preconditioning of J774 cells with LPS or with poly(I:C) applied with a transfection reagent, which activates mainly RLRs in contrast to naked poly(I:C) that also activates TLR3, induced similar sensitivity changes as naked poly(I:C) (Supplemental Fig. 2B). We hypothesized that induction of PRR reprogramming is a general phenomenon mediated by MyD88-independent signals.

IFN-β secretion is essential for TLR and RLR changes in sensitivity

In the next set of experiments, we examined the role of MyD88-independent signals in PRR reprogramming. We therefore blocked signaling of the known poly(I:C) receptors TLR3 and MDA-5 by pharmacologically inhibiting TBK/IκB kinase (IKK), a downstream kinase in both pathways, during the preconditioning phase. Treatment with BX-795 reverted the poly(I:C)-induced increased TLR7 sensitivity and partially restored responsiveness to MDA-5 stimulation (Fig. 4A). We obtained similar results when treating LPS-preconditioned cells with this inhibitor (not shown). We next investigated the mediators of PRR sensitivity changes in response to poly(I:C) preconditioning. As the role of type I IFNs in priming of TLR pathways in response to poly(I:C) preconditioning is controversial in literature (10, 12), we analyzed their contribution in TLR and RLR reprogramming. A neutralizing Ab against IFN-β, but not against IFN-α, inhibited changes in TLR7 as well as MDA-5 sensitivity induced by poly(I:C) (Fig. 4B). Similar results were obtained for RIG-I sensitivity and by blocking the

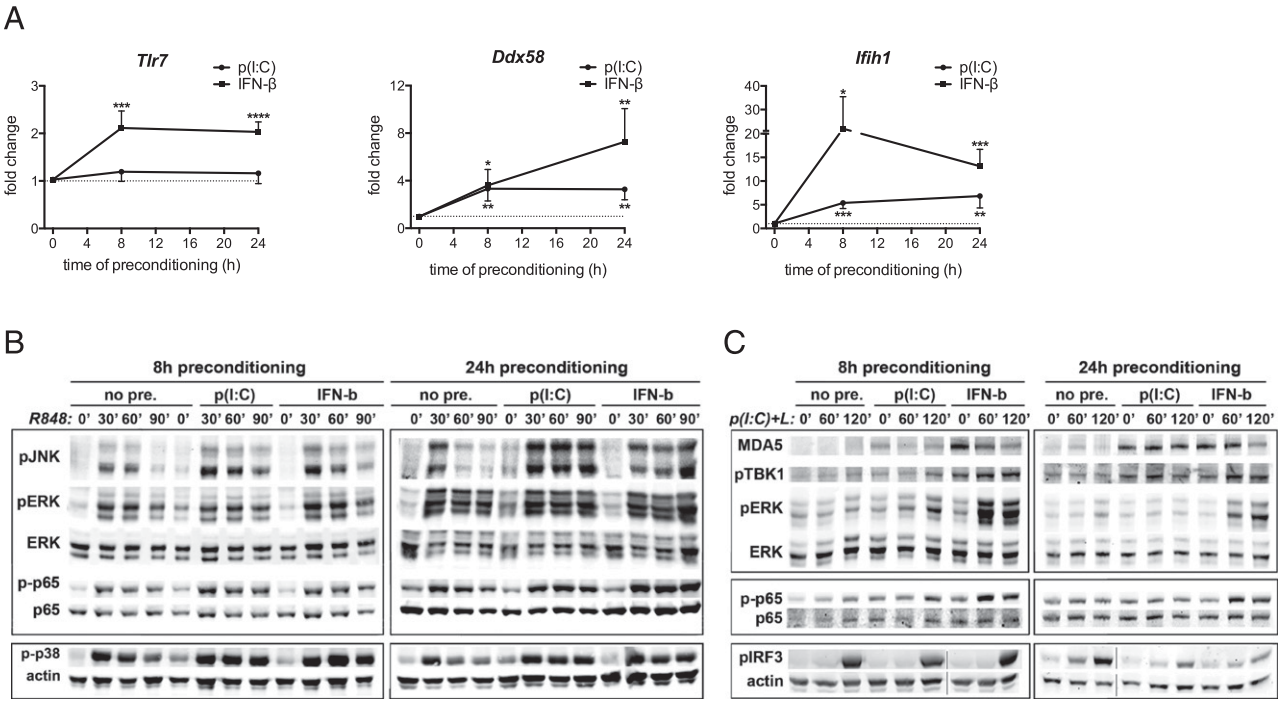

FIGURE 5. Molecular analysis of TLR and RLR sensitivity changes. (**A**) Quantitative RT-PCR analysis of *Tlr7*, *Ddx58* (RIG-I), and *Ifih1* (MDA-5) mRNA in J774 treated with naked poly(I:C) or rIFN-β for 8 and 24 h. Relative expression to the untreated control is shown. Immunoblot analysis of MAPK, NF-κB, TBK-1, and IRF3 phosphorylation in lysates of J774 preconditioned for 8 and 24 h with poly(I:C) or IFN-β prior to stimulation for 30, 60, and 90 min with R848 (**B**) or for 60 and 120 min with complexed poly(I:C) [*p(I:C)+L*] (**C**). Individual blots are depicted by rectangles. (A) Eight- and 24-h versus 0-h time point, Student *t* test. Mean ± SEM of three to four independent experiments are shown in (A). Data are representative of at least three independent experiments. Some blots were sliced for clarity as indicated by vertical black lines in (C). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

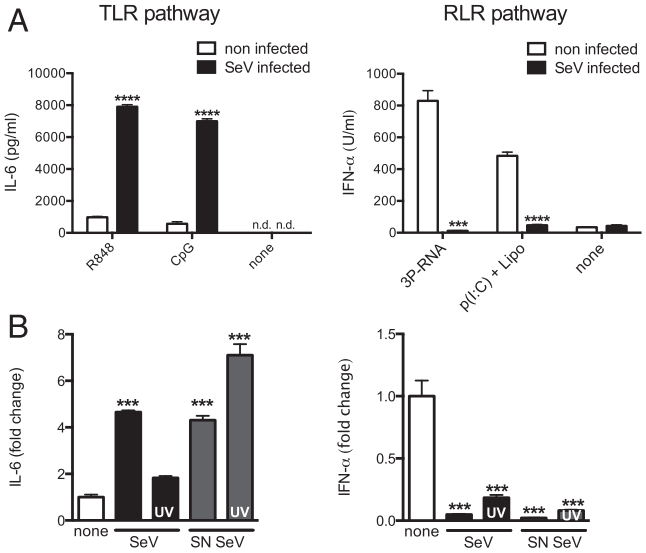

FIGURE 6. Viral infection changes TLR and RLR sensitivity in autocrine and paracrine fashion. (**A**) IL-6 and IFN-α in supernatants from J774 cells infected with SeV for 24 h prior to stimulation for 24 h with CpG, R848, 3P-RNA complexed with Lipofectamine, or poly(I:C) complexed with Lipofectamine [p(I:C) + Lipo]. (**B**) IL-6 and IFN-α in supernatants of J774 cells infected with SeV or preconditioned with supernatant of SeV-infected CT26 cells for 24 h prior to stimulation for 24 h with R848 or complexed poly(I:C). Relative expression to noninfected controls is shown. (A) Black bars versus white bars, Student *t* test. (B) White bar versus other conditions; one-way ANOVA, Dunnett multiple-comparison test. Data are representative of at least three independent experiments (mean + SEM of triplicates). $***p < 0.001$, $****p < 0.0001$. n.d., cytokine levels below detection limit; UV, UV light irradiation for 40 min.

type I IFN receptor in J774 cells (Supplemental Fig. 3A). Neutralizing Abs had no effect on stimulation with MDA-5 or TLR7 ligands when cells were not preconditioned (not shown). We then examined whether exposure to IFN-β was sufficient to elicit PRR reprogramming. TLR7 responses were increased following IFN-β treatment with a maximum after 8 h of preconditioning, albeit at a lower level than after preconditioning with poly(I:C) (Fig. 2C, Supplemental Fig. 3B). It is therefore probable that a second, unidentified factor secreted in response to poly(I:C) plays an additional role in the enhancement of TLR7 responses. MDA-5 repression was observed at 24 h after IFN-β treatment and comparable to poly(I:C) exposure (Supplemental Fig. 3B).

We further confirmed the essential role of type I IFNs for PRR reprogramming using IFNaR$^{-/-}$ DCs and Ab-mediated IFNaR blockade in those cells (Supplemental Fig. 3C, 3D). We conclude that type I IFN signaling is essential for the dynamic changes in PRR sensitivity mediated by poly(I:C) preconditioning.

Poly(I:C) changes signaling downstream of TLRs and RLRs

To investigate the molecular mechanisms for receptor reprogramming, we performed quantitative PCR for gene expression of TLRs and RLRs in J774 following exposure to poly(I:C) or IFN-β. We did not detect significant changes in *Tlr7* at 8 or 24 h postexposure to poly(I:C (Fig. 5). However, we did observe an ~2-fold upregulation of *Tlr7* following 8- and 24-h exposure to rIFN-β. In contrast to *Tlr7*, *Ddx58* (RIG-I gene) and *Ifih1* (MDA-5 gene) were upregulated in both conditions, which was confirmed at the protein level by immunoblotting (Fig. 5C). Thus, the changes in TLR- and RLR-induced cytokine secretion observed after poly(I:C) exposure could not be explained by changes in receptor expression.

We hypothesized that downstream signaling rather than receptor expression was affected by preconditioning. We exposed cells for 8 and 24 h to poly(I:C) or IFN-β and subsequently stimulated TLR7 or MDA-5. We analyzed the activation of kinases and transcription factors downstream of these receptors. Poly(I:C)-preconditioned cells showed higher TLR7-mediated NF-κB and MAPK activation (Fig. 5B), as shown previously for cells preconditioned with a TLR4 ligand and stimulated via TLR9 (11). We observed increased and prolonged phosphorylation of JNK, NF-κB (p65), and p38 but not ERK following stimulation with R848 in cells that were preconditioned for 24 h with poly(I:C). Importantly, IFN-β pretreatment also facilitated prolonged JNK, NF-κB, and p38 signaling, although JNK signaling was not as highly increased at 24 h by IFN-β as by poly(I:C) preconditioning.

In contrast to MAPK and p65, phosphorylation of IRF3, a transcription factor downstream of MDA-5, was decreased in MDA-5–stimulated J774 cells 24 h but not 8 h after preconditioning with poly(I:C) (Fig. 5C). MAPK and NF-κB signaling was generally low after stimulation of MDA-5, but was rather increased by IFN-β exposure and thus unlikely to have caused the observed reduction in IFN-α secretion (Fig. 5C). Interestingly, phosphorylation of TBK-1, an upstream kinase in the IRF3 signaling pathway (25), was not inhibited in preconditioned cells, unlike IRF3. Levels of unphosphorylated TBK-1 and IRF3 did not change significantly after poly(I:C) and IFN-β treatment (Supplemental Fig. 4). In summary, the phosphorylation patterns of IRF3 after MDA-5 stimulation and JNK after TLR7 stimulation matched the opposite changes and kinetics of PRR reprogramming.

Viral infection reprograms PRR pathways similarly to poly(I:C)

After having shown that the viral mimetic poly(I:C) reprograms PRR signaling, we investigated whether viral infection itself leads to similar dynamic and differential changes in receptor sensitivity. In a first attempt to analyze PRR sensitivity in virus-conditioned cells, we infected cells with encephalomyocarditis virus, which activates MDA-5 (26). Unfortunately, stimulation with synthetic ligands 24 h later was perturbed by the ongoing antiviral immune response (not shown). We then switched to SeV (Cantell strain), a ligand for both MDA-5 and RIG-I (26, 27). Virus-infected J774 cells showed the same PRR-reprogrammed phenotype as poly(I:C)-preconditioned cells (Fig. 6A). Interestingly, PRR reprogramming was also detected when J774 cells were not directly infected but exposed to supernatant from SeV-infected epithelial cells: TLR responses were increased and RLR responses decreased, even when viral particles were inactivated by 40-min UV irradiation of the supernatant (Fig. 6B). This indicated that: 1) direct infection was not necessary for modulation of TLR and RLR responses; and 2) receptor modulation could be induced by soluble factors produced by infected cells. In the last set of experiments, we therefore assessed the soluble factors responsible for virus-induced PRR reprogramming.

Paracrine IFN-β is essential for virus-induced PRR reprogramming

One possible mechanism is that virus-encoded RNA is released from infected cells and triggers an innate immune activation similar to poly(I:C). However, we observed that modulation of TLR7 responses induced by supernatant from SeV-infected cells was not sensitive to RNase treatment, in contrast to poly(I:C)-induced receptor modulation (data not shown). Further, treatment with the TBK/IKK kinase inhibitor BX-795 during the

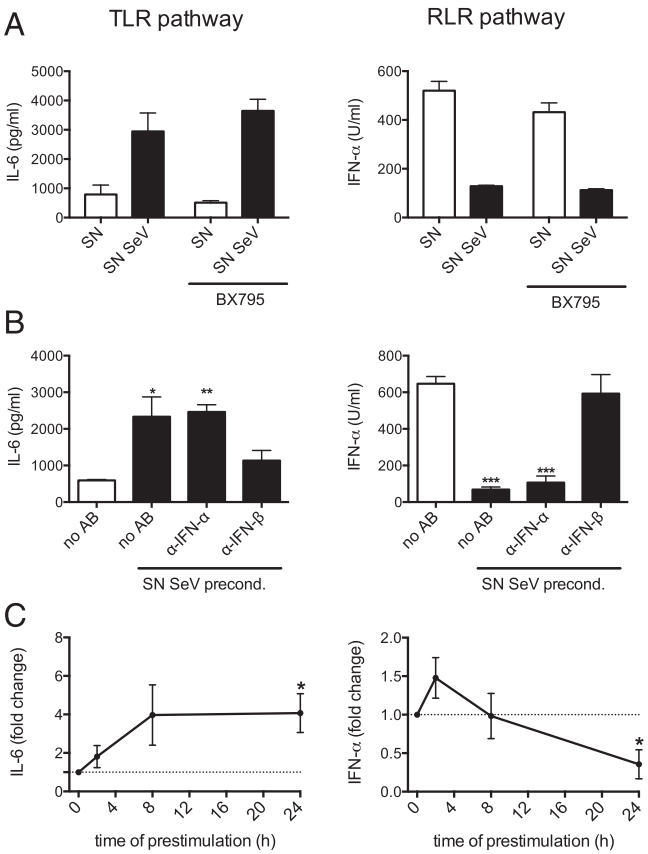

FIGURE 7. IFN-β is essential for viral modulation of TLR and RLR sensitivity. (**A** and **B**) IL-6 and IFN-α in supernatants (SN) from J774 cells preconditioned with supernatant from SeV-infected CT26 cells for 24 h prior to stimulation for 24 h with R848 (*left panels*) or complexed poly(I:C) (*right panels*). In some conditions, cells were treated with the TBK/IKK inhibitor BX-795 (A) or with Abs (AB) against IFN-α and β during the preconditioning phase (B). (**C**) IL-6 and IFN-α in supernatants from J774 preconditioned for different durations with supernatant from SeV-infected CT26 cells prior to stimulation for 24 h with R848 (*left panel*) or complexed poly(I:C) (*right panel*). Relative expression to the 0-h time point is shown. (A) Preconditioned versus preconditioned + inhibitor, Student *t* test. (B and C) Black bars versus white bar or 0-h time point versus other conditions, one-way ANOVA, Dunnett multiple-comparison test. Data are representative of two to three independent experiments [mean + SEM of triplicates in (A) and (B)]. Mean ± SEM of at least three independent experiments are shown in (C). $*p < 0.05$, $**p < 0.01$.

preconditioning phase did not interfere with PRR modulation by SeV supernatant (Fig. 7A) in contrast to preconditioning with poly(I:C) (Fig. 4A). We speculated that type I IFNs directly released by infected cells were involved in PRR reprogramming by viral supernatant. Similarly to poly(I:C), an anti–IFN-β Ab completely reverted the changes in PRR sensitivity, in contrast to the blockade of IFN-α (Fig 7B). We further analyzed kinetics of viral supernatant-induced changes in PRR signaling. Maximum TLR7 sensitivity was observed after 8–24 h, and maximal repression of MDA-5–induced IFN-α secretion was reached after 24 h of preconditioning (Fig. 7C), which is similar to the kinetics of poly(I:C)-induced sensitivity changes. Finally, we analyzed virus-induced PRR reprogramming in conventional DCs. As seen with J774 cells, viral supernatant induced a sensitization of the TLR7 pathway and a desensitization of the RIG-I pathway in DCs, which were both prevented when IFN-β–neutralizing Abs were added (Fig. 8).

In conclusion, we have demonstrated that initial innate detection of viral infection leads to unexpected systemic changes in PRR sensitivity, which are facilitated by autocrine as well as paracrine IFN-β signaling.

Discussion

Plasticity of innate immune signaling is essential to generate appropriate immune responses by the integration of signals from multiple PRRs. We show in this study that infection with an ssRNA virus or exposure to poly(I:C) profoundly modified the responsiveness of anti-viral innate receptors. In conditioned cells, sensitivity of endosomal TLRs was strongly enhanced within 24 h, while at the same time, the induction of type I IFN by RLRs was practically abolished. This switch in the predominance of PRR responses was mediated by immune recognition of virus or dsRNA and the release of type I IFN and thus represents a host adaptation mechanism and not a viral strategy of immune evasion. We suggest expanding the term "TLR reprogramming" (17, 28) to "PRR reprogramming" to refer to changes in PRR signaling different from synergy and involving multiple classes of innate receptors.

Poly(I:C) was described as ligand for both TLR3 and MDA-5 (20). We have observed that a combination of both signaling pathways is probably involved in PRR reprogramming in vitro, whereas MDA-5 was sufficient in vivo. In addition to MDA-5 (27), RIG-I is implicated in SeV-induced IFN secretion (26), which is, as we have shown, essential for PRR reprogramming. We have further demonstrated that the TRIF-dependent ligands polyadenylic-polyuridylic acid (data not shown) and LPS induced changes in receptor sensitivity similar to those seen with poly(I:C). We thus propose that PRR reprogramming is a widespread phenomenon following prior exposure to MyD88-independent ligands. Others have described priming of TLR responses by preconditioning with TLR3 and TLR4 ligands (10, 11, 13); however, they did not study RLR responses. Interestingly, preconditioning with TLR2 ligands, which are MyD88-dependent and do not induce type I IFN, led to an enhanced type I IFN secretion following secondary RLR stimulation (17). This finding is complementary to our observation in which MyD88-independent type I IFN inducers confer RLR tolerance. Our study completes the picture of dynamic sensitivity of PRRs in response to an initial innate stimulation. Altogether, these results support the concept that sensitization and desensitization of defined receptor pathways is a general adaptation of the innate immune system to integrate repetitive PRR signaling.

We showed that activation of the transcription factor IRF3 in response to MDA-5 stimulation was decreased in preconditioned cells. As phosphorylation of TBK-1, an IRF3 kinase, was not altered, the block in RLR signaling probably occurred at the level of IRF3 and not further upstream. Interestingly, a novel mechanism to limit IFN production has been recently described: rather than degradation of IRF3, the authors found dephosphorylation of this factor by PP2A to be responsible for terminating IRF3 signaling (29). As levels of unphosphorylated IRF3 remained unchanged during our experiments, PP2A could play a role in the observed RLR reprogramming. Although it was proposed earlier that type I IFN could modulate RLR signaling by inducing degradation of RIG-I (30), we did not observe a decrease in MDA-5 protein levels after exposure to poly(I:C), indicating a different mechanism.

Only few reports have observed changes in sensitivity of innate receptors after viral infection, and systematic studies analyzing different sets of PRRs are lacking. In superinfection models, changes in PRR sensitivity were reported to have detrimental effects on the immune response toward secondary bacterial challenge. Influenza infection induced long-lasting desensitization of antibacterial TLRs, correlating with higher bacterial load in sec-

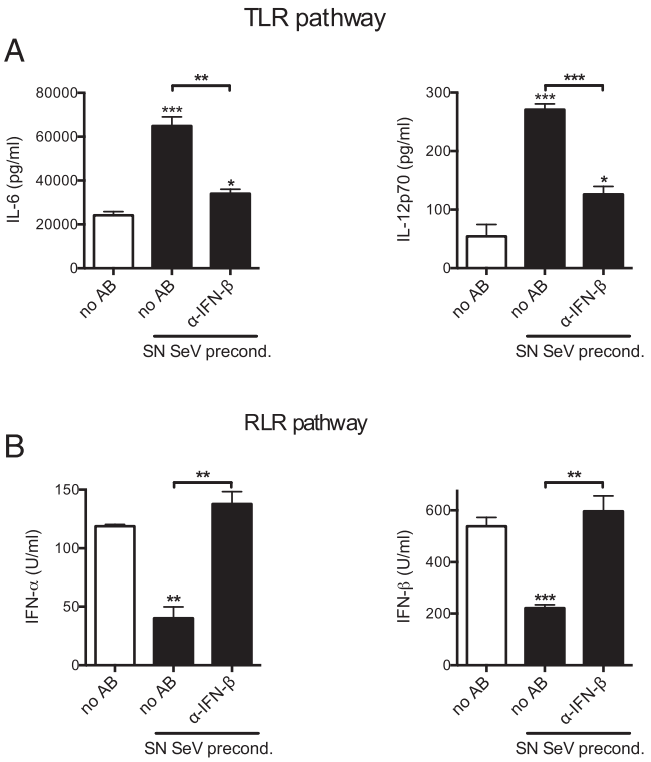

FIGURE 8. Virus-derived supernatant (SN) reprograms DCs. IL-6, IL-12p70, IFN-α, and IFN-β in supernatants from DCs preconditioned with supernatant from SeV-infected CT26 cells for 24 h prior to stimulation for 24 h with R848 (**A**) or 3P-RNA (**B**). In some conditions, cells were treated with an anti–IFN-β Ab (AB) during the preconditioning phase. Black bars versus white bar or as indicated. Data are representative of two independent experiments (mean + SEM of triplicates). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

ondary respiratory infection (31). At earlier times after viral encounter, increased TLR4 (32) as well as nucleotide-binding oligomerization domain 1/2 sensitivity (16) was reported. This increased sensitivity was the driver for pathological cytokine secretion in response to secondary bacterial challenge (16). Because corrupted immunity to secondary infection poses a strong counterselective pressure toward PRR modulation, this phenomenon must therefore come with a remarkable advantage. We propose that PRR reprogramming in the course of a single viral infection might potentiate antiviral immunity by increased availability of bioactive IL-12, the main cytokine for the development of Th1-type immunity (33) and an essential factor for NK cell activation (34). Thus, first-line infected cells could license nearby and remote DCs via IFN-β to increase TLR7-induced IL-12 production and Th1 cell differentiation as well as NK cell activation. Whether PRR reprogramming helps to resolve viral infection in vivo is still unclear. Mice preconditioned with poly(I:C) have decreased viral titers and reduced mortality when challenged with influenza virus (35). Although much of this effect may be attributed to the well-described "antiviral state" established by the action of type I IFNs (36, 37), we suggest that PRR reprogramming and in particular enhanced TLR7 signaling and reinforced Th1 responses may also contribute to viral clearance.

At first glance, the decrease in RLR responses after initial viral infection seems counterintuitive. However, type I IFN production can be maintained through specialized pDCs by the increased sensitivity of endosomal TLRs. Additionally, excessive or prolonged type I IFN release and RLR stimulation can have immunopathological (38–41) or immunosuppressive (42, 43) effects, underscoring the need for tight control of this cytokine. Interestingly, type I IFN was also shown to interfere with the induction of IL-12 and IFN-γ (44). Further, RIG-I–induced IRF3 selectively suppressed *il-12b* expression and thus hindered Th1 cell differentiation (23). Therefore, well-timed blockade of RLR signaling could prevent adverse events and at the same time support the development of Th1 immunity.

In summary, we propose that both the increase in TLR7 sensitivity and the decrease in RLR-induced IFN observed in this study favor increased IL-12 and IFN-γ signaling to enhance immunity during a viral infection. Our findings contribute to the understanding of how signals from different classes of antiviral innate receptors are integrated to program immune responses.

Disclosures

The authors have no financial conflicts of interest.

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