TLR9-Signaling Pathways Are Involved in Kilham Rat Virus-Induced Autoimmune Diabetes in the Biobreeding Diabetes-Resistant Rat

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Viral infections are associated epidemiologically with the expression of type 1 diabetes in humans, but the mechanisms underlying this putative association are unknown. To investigate the role of viruses in diabetes, we used a model of viral induction of autoimmune diabetes in genetically susceptible biobreeding diabetes-resistant (BBDR) rats. BBDR rats do not develop diabetes in viral-Ab-free environments, but ~25% of animals infected with the parvovirus Kilham rat virus (KRV) develop autoimmune diabetes via a mechanism that does not involve β cell infection. Using this model, we recently documented that TLR agonists synergize with KRV infection and increase disease penetrance. We now report that KRV itself activates innate immunity through TLR ligation. We show that KRV infection strongly stimulates BBDR splenocytes to produce the proinflammatory cytokines IL-6 and IL-12p40 but not TNF-α. KRV infection induces high levels of IL-12p40 by splenic B cells and Flt-3-ligand-induced bone marrow-derived dendritic cells (DCs) but only low levels of IL-12p40 production by thioglycolate-elicited peritoneal macrophages or GM-CSF plus IL-4-induced bone marrow-derived DCs. KRV-induced cytokine production is blocked by pharmacological inhibitors of protein kinase R and NF-κB. Genomic KRV DNA also induces BBDR splenocytes and Flt-3L-induced DCs from wild-type but not TLR9-deficient mice to produce IL-12p40; KRV-induced up-regulation of B lymphocytes can be blocked by TLR9 antagonists including inhibitory CpG and chloroquine. Administration of chloroquine to virus-infected BBDR rats decreases the incidence of diabetes and decreases blood levels of IL-12p40. Our data implicate the TLR9-signaling pathway in KRV-induced innate immune activation and autoimmune diabetes in the BBDR rat. The Journal of Immunology, 2007, 178: 693–701.

Environmental perturbants, in particular viral infections, are hypothesized to play a role in triggering type 1 diabetes (TID)1 in genetically susceptible individuals (1–3). Epidemiological studies (4, 5) and a relatively low rate of disease concordance in monozygotic twins support this hypothesis (6–8). There is, however, little direct evidence of a cause-effect relationship between viral infections and diabetes and the mechanisms underlying such a relationship are unknown.

To investigate this problem, we used a model of virus-induced diabetes in genetically susceptible, but phenotypically normal biobreeding diabetes-resistant (BBDR) rats (9, 10). Diabetes does not occur in viral-Ab-free BBDR rats (11). Naturally occurring infection with Kilham rat virus (KRV) induces diabetes in ~1% of animals and deliberate infection by parenteral injection induces diabetes in ~25–40% of treated rats (10–15). KRV is an ssDNA parvovirus (16), encoding three overlapping structural proteins, VP1, VP2, and VP3, and two overlapping nonstructural proteins, NS1 and NS2 (16). KRV is one of the three autonomous rat parvoviruses (i.e., parvoviruses that replicate without a helper virus); H-1 and rat parvovirus-1 (formerly designated “orphan parvovirus”) are the other two (16).

The susceptibility of BBDR rats to diabetes induced by KRV infection appears to be under genetic control (17), but the immunological mechanisms underlying the induction of autoimmunity in BBDR rats following KRV infection are only partially understood. The mechanism is not cytolysis of β cells, as KRV does not infect islets (18). There are also data to suggest that molecular mimicry is probably not the mechanism (19).

We (10, 13, 14) and others (2, 19) have focused on adaptive immune responses and hypothesized that one component of the mechanism involves alteration of the balance between regulatory T cells (Treg) and autoreactive effector cells. This hypothesis is supported by the observation that infection of BBDR rats with KRV leads to a reduction in the frequency of CD4+CD25+ and CD4+CD45RClow Treg (10, 19, 20) via a mechanism that could involve the interaction of T cells with APCs (21).

An alternative mechanism involves activation of innate immunity. The innate immune system senses the presence of pathogens via TLRs that recognize pathogen-associated molecular patterns expressed by microbes (22–24). The binding of pathogen-associated molecular patterns to TLRs expressed on B lymphocytes,
dendritic cells (DC), and macrophages triggers a maturation program that includes up-regulation of MHC molecules and costimulatory molecules on the cell surface and expression of proinflammatory cytokines and chemokines, including TNF-α, IL-1, IL-6, IL-12, and IFN-γ-inducible protein 10 (25, 26, 26).

We recently reported that TLR activation synergizes with KRV infection to increase both the speed and frequency with which diabetes is induced in BBDR rats. This was achieved using both synthetic TLR ligands and natural TLR agonists including heat-killed *Escherichia coli* and *Staphylococcus aureus* (9). Pretreatment with these TLR ligands can precipitate disease in BBDR rats infected with very low viral titers that are nondiabetogenic when given alone (9). In addition, infection of BBDR rats with KRV induces the up-regulation of cytokines associated with the innate immune response in pancreatic lymph nodes (9), leading us to hypothesize that innate immune activation plays a key role in the induction of diabetes in KRV-infected BBDR rats (9).

In the present study, we investigated the specific immune responses and cell subsets activated by KRV infection or by genomic KRV DNA in vitro. We present evidence suggesting that KRV activates innate immunity directly through the TLR9-signaling pathway and that this pathway is critical for the induction of diabetes by KRV infection.

### Materials and Methods

**Animals, viruses, and cell lines**

Viral-Ab-free BBDR/Wor rats of either sex were obtained from BRM. MHC-compatible diabetes-resistant WF rats were obtained from Harlan Sprague Dawley. Animals from these vendors are certified to be free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H-1, GD7, Reo-3, *Mykoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and *Encephalitozoon cuniculi*. Wild-type and TLR9-knockout C57BL6 mice were obtained from colonies maintained at the University of Massachusetts Medical School. All animals were housed in a viral-Ab-free facility until used and maintained in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

**KRV propagation**

KRV UMass isolate and the normal rat kidney (NRK) cell line were obtained from stocks maintained in our laboratories (9, 10). NRK cells were cultured in high-glucose complete DMEM (cDMEM) containing 10% heat-inactivated FBS, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μM 2-ME (Invitrogen Life Technologies). KRV at a multiplicity of infection of 1 was used to infect NRK cells; virus was harvested from supernatant of infected cultures 2 days after infection and kept at −70°C until use. Viruses were titered using a plaque assay as previously described (10).

**In vitro activation of splenocytes by KRV, TLR ligands, and KRV DNA**

For in vitro stimulation assays, BBDR or WF spleen cells were added to 96-well round-bottom microtiter plates at a concentration of 2×10^6/ml in a total volume of 100 μl. For induction of IL-12p40, KRV was added at a final concentration of 2×10^6 PFU/ml.

Polynosinic-polycytidylic acid (poly(I:C)) was purchased from Sigma-Aldrich. Contaminating endotoxin concentration was <50 endotoxin units/mg (Charles River Endosafe). Ultra-purified LPS (O111:B4) was purchased from Invivogen. R848 was purchased from GL Synthesis. All were dissolved in Dulbecco’s PBS (1 mg/ml), and stored at −20°C until used. The sequence of the CpG oligonucleotide used in this study was 5′-TCGGTTGGTTGCCTTTCTGTTT-3′ (27) and was synthesized by Tri-Link BioTechnologies. The sequence of inhibitory CpG (iCpG) used was 5′-TTCCCGAGGCGGTGT-3′ (28) and was synthesized by Invivogen Life Technologies. The doses used for cell activation were 50 μg/ml for poly(I:C), 1 μg/ml for LPS, and 1 μg/ml for CpG DNA.

Genomic KRV DNA and control DNA derived from NRK cells and used for in vitro spleen activation were isolated from culture supernatants by using the QIAamp DNA minikit according to the manufacturer’s instructions. The concentration of contaminating endotoxin in these agents was determined commercially (Charles River Endosafe) and was <10 endotoxin units/mg. A final concentration of 1.25 and 0.125 μg/ml genomic DNA was added to 2×10^6/ml BBDR spleen cells in a 96-well round-bottom microtiter plate in a total volume of 100 μl. The iCpG oligonucleotide and the endosomal inhibitor, chloroquine (28), were added at various concentrations as indicated in the text. The NF-κB inhibitor, pyrroline dithiocarbamate (PDTC), and the protein kinase, 2-aminopurine (2AP), were purchased from Sigma-Aldrich and added at various concentrations as shown in the text.

**Induction and diagnosis of diabetes**

BBDR rats of either sex at 22–25 days of age were injected i.p. on 3 consecutive days with poly(I:C) (1 μg/g body weight) and then injected i.p. with 1×10^6 PFU of KRV in a volume of 1 ml of PBS on the following day, as described (9–11, 29). We have previously documented that this dose of poly(I:C) by itself is incapable of inducing diabetes in the BBDR rat (9). Animals were screened three times weekly for glycosuria (Tes-Tape Eli Lilly). Diabetes was diagnosed on the basis of a plasma glucose concentration >11.1 mM (OneTouch Ultra Glucometer; LifeScan).

To determine the effect of chloroquine on diabetes induction, BBDR rats 22–25 days of age were treated on days 0, 1, and 2 with 1 μg/g body weight poly(I:C) and on day 3 with 1×10^6 PFU of KRV and then randomized into two groups. Group 1 was treated with a daily i.p. injection of chloroquine (100 μg/g body weight) on days 3–24. Group 2 was injected with PBS on the same schedule.

To determine whether chloroquine activated the induction of innate immunity in BBDR rats infected with poly(I:C) and infected with virus, animals were treated with poly(I:C) on days 0, 1, and 2 and 1×10^6 PFU of KRV on day 3. One group of animals was injected with 100 μg/g body weight of chloroquine beginning on the day of infection for the BBDR rat (9). A second group was injected with PBS for 4 days. Four days after viral infection, serum was recovered and the level of IL-12p40 was determined by ELISA as described below.

**Spleen cell isolation and B cell purification and activation**

Spleens were obtained from 3- to 6-wk-old BBDR rats. Single spleen cell suspensions were prepared and erythrocytes were lysed with a hypotonic NH4Cl solution. Cells were washed twice with PBS and suspended in cDMEM. Splenic B lymphocytes were purified using two experimental protocols. In one protocol, B cells were sorted on a FACS digital Vantage SE (DiVa) cell sorter (BD Biosciences) using a PE-conjugated anti-CD235 mAb (clone HIS24, mouse IgG2b; obtained from BD Biosciences). In a second protocol, B cells were enriched using the MACS magnetic cell separation system (Miltenyi Biotech). Briefly, spleen cells were depleted of T cells using purified anti-Thy1 mAb (clone R73, mouse IgG1, obtained from BD Biosciences). B cells were positively selected using PE-conjugated anti-CD235 mAb. The purity of the purified cells obtained using either B cell purification protocol was >99%. Activation of spleen cells or purified spleen B cells was performed by incubating 2×10^6 cells/ml for 18 h in 96-well round-bottom microtiter plates in a total volume of 100 μl in the absence or in the presence of KRV or the purified TLR ligands LPS (1 μg/ml), CpG (1 μg/ml), or poly(I:C) (50 μg/ml).

**Peritoneal macrophages, GM-CSF plus IL-4-induced-, and Flt-3L-induced bone-marrow-derived DC from rats and mice**

Peritoneal macrophages were recovered from BBDR rats 4 days after an i.p. injection of 3 ml of 3% thioglycolate (BD Biosciences). Recovered macrophages were incubated at a concentration of 2×10^6 cells/ml for 18 h in the presence or absence of KRV or TLR ligands at the concentrations indicated in the text.

GM-CSF plus IL-4-induced bone-marrow-derived DC (hereafter referred to as GM-CSF-induced), i.e., immature myeloid DCs, were obtained by culturing bone marrow cells in the presence of 10 ng/ml rGM-CSF plus 10 ng/ml rIL-4 (both from BD Pharmingen) (30). Flt-3L-induced DCs, i.e., CD11c+ plasmacytoid DCs (31) from BBDR rats, wild-type and TLR9 knockout mice were obtained by incubating 2×10^6/ml bone marrow-derived cells in cDMEM in the presence of 25 ng/ml human recombinant Flt-3L (PeproTech). Fresh medium containing GM-CSF plus IL-4 or human Flt-3L was added to the cultures every 2–3 days. Activation of GM-CSF-induced and Flt-3L-induced bone marrow-derived DCs from rats or mice were performed on days 5 and 7, respectively, by culturing 2×10^6 cells/ml in the presence or absence of 2×10^6 PFU KRV. TLR ligands, DNA extracted from KRV, or control DNA extracted from NRK cells for 18 h.
that no other paired comparisons were statistically significant. Used for detection at a concentration of 0.5 were added to the plates in duplicate and biotinylated anti-IL-12p40 was blocked with 2.5% nonfat dry milk (Bio-Rad). The standards and samples sandwich ELISA (R&D Systems). Levels of mouse IL-12 p40 in tissue culture supernatants were measured by an optimized to the manufacturer’s instructions (BD Pharmingen). Levels of mouse IL-12p40, IL-6, and TNF-α in tissue culture supernatants were measured by ELISA according to the manufacturer’s instructions. Briefly, 96-well, flat-bottom microtiter plates (MaxiSorp Immuno microwell plates; Nunc) were coated overnight at 4°C containing (at final concentration) either LPS (1 μg/ml), CpG DNA (1 μg/ml), poly(I:C) (50 μg/ml), or KRV (2 × 10^7 PFU/ml). The concentration of IL-12p40, IL-6, and TNF-α in culture supernatant was then measured by ELISA as described in Materials and Methods. Each bar represents the mean ± SD of three or four independent measurements. *p < 0.01 vs control and LPS. **p < 0.001 vs LPS. ***p < 0.001 vs control. ANOVA revealed that no other paired comparisons were statistically significant.

ELISA
Levels of rat IL-12p40 in tissue culture supernatants were measured using an optimized sandwich ELISA (BioSource International), according to manufacturer’s instructions. Briefly, 96-well, flat-bottom microtiter plates (MaxiSorp Immuno microwell plates; Nunc) were coated overnight at 4°C with 2.5 μg/ml anti-IL-12p40/p70 capture mAb in PBS. Plates were blocked with 2.5% nonfat dry milk (Bio-Rad). The standards and samples were added to the plates in duplicate and biotinylated anti-IL-12p40 was used for detection at a concentration of 0.5 μg/ml. Ab binding was visualized using streptavidin-HRP. Levels of IL-12p40 in serum were measured by an ELISA kit (BioSource International). Levels of IL-6 and TNF-α in tissue culture supernatants were measured by ELISA according to the manufacturer’s instructions (BD Pharmingen). Levels of mouse IL-12 p40 in tissue culture supernatants were measured by an optimized sandwich ELISA (R&D Systems).

Statistical analysis
Parametric data are reported as the arithmetic mean ± SD. The average duration of diabetes-free survival is presented as the median. Diabetes-free survival times among groups were compared by life-table analysis using the method of Kaplan and Meier; the equality of diabetes-free distributions of KRV for 18 h, and the concentration of IL-12p40 in the culture supernatant was determined as described in Materials and Methods. There were no statistically significant differences in the concentration of IL-12p40 measured at any time point. Data represent the mean ± SD of three independent measurements at each time point. A spleen cells from BBDR or WF rats were incubated in the presence of varying concentrations of KRV for 18 h, and the concentration of IL-12p40 in the culture supernatant was then measured by ELISA. Each data point represents the mean ± SD of three to five independent measurements. *p < 0.01 by unpaired t test.

FIGURE 1. Production of IL-12p40, IL-6, and TNF-α by spleen cells cultured in the presence of KRV or TLR ligands. A, Spleen cells from BBDR or WF rats were incubated in the presence of varying concentrations of KRV for 18 h, and the concentration of IL-12p40 in the culture supernatant was determined as described in Materials and Methods. There were no statistically significant differences in the concentration of IL-12p40 measured at any time point. Data represent the mean ± SD of three independent measurements at each time point. B, Spleen cells from BBDR rats were incubated for 18 h in culture medium alone (“control”) or in medium containing (at final concentration) either LPS (1 μg/ml), CpG DNA (1 μg/ml), poly(I:C) (50 μg/ml), or KRV (2 × 10^7 PFU/ml). The concentration of IL-12p40, IL-6, and TNF-α in culture supernatant was then measured by ELISA as described in Materials and Methods. Each bar represents the mean ± SD of three or four independent measurements. *p < 0.01 vs control and LPS. **p < 0.001 vs LPS. ***p < 0.001 vs control. ANOVA revealed that no other paired comparisons were statistically significant.

Results
Spleen cells cultured in vitro in the presence of KRV secrete IL-12p40 and IL-6 but not TNF-α
To understand the immune response to KRV infection as it relates to diabetes pathogenesis, we first measured the secretion of IL-12p40 by BBDR and WF spleen cells cultured in vitro with KRV at varying concentrations. WF rats share the RT1b MHC haplotype of BBDR rats but are diabetes resistant. We observed that both BBDR and WF spleen cells are stimulated to secrete IL-12p40 in the presence of KRV and that the dose-response curves for both BBDR and WF spleen cells are statistically similar (Fig. 1A).

Having validated this in vitro approach to the assessment of the KRV immune response, we next measured the secretion of IL-12p40, IL-6, and TNF-α by BBDR spleen cells cultured in the presence of medium alone, KRV, or various purified TLR ligands. In comparison with control cultures, KRV at a concentration as-
CD45R<sup>+</sup> B cells than by CD45R<sup>-</sup> non-B cells (Fig. 2, p < 0.01). This pattern was not observed in cultures of cells incubated with either poly(I:C) or LPS. In those cases, secretion of IL-12p40 into the medium was low and statistically similar (p = N.S.) for both cell types.

**KRV stimulates Flt-3L-induced but not GM-CSF plus IL-4-induced bone marrow-derived DCs to produce high levels of IL-12p40 in vitro**

We next assessed the ability of KRV to stimulate IL-12p40 production by Flt-3L-induced and GM-CSF-induced bone marrow-derived DCs. We observed that exposure to KRV induced a statistically significant increase in IL-12p40 production by DCs generated using either protocol. However, the absolute amount of IL-12p40 produced by KRV-stimulated Flt-3L-induced bone marrow-derived DCs was two orders of magnitude greater than that produced by KRV-stimulated GM-CSF-induced bone marrow-derived DCs (Table I).

**Macrophages cultured in the presence of KRV secrete relatively little IL-12p40, IL-6, or TNF-α**

We next assessed the ability of KRV to stimulate cytokine production in vitro by macrophages (26, 33). For these experiments, thioglycolate-elicited peritoneal macrophages from BBDR rats were cultured in the presence of medium alone, KRV, or various purified TLR ligands. In comparison with control cultures, KRV induced modest but detectable secretion of IL-12p40 (Fig. 3). KRV also stimulated BBDR macrophages to secrete modest amounts of IL-6 and TNF-α (Fig. 3). Interestingly, ligation of TLR9 with CpG DNA induced the same pattern of cytokine production by macrophages: small increases in IL-12p40, IL-6, and TNF-α. In contrast, and as expected (9), LPS and poly(I:C) were up to two orders of magnitude more potent as robust inducers of these cytokines in macrophages (Fig. 3). This difference is consistent with previous reports showing that different cell types can respond differently to the same TLR ligand, and that B lymphocytes express low levels of TLR3 and are known to be poor responders to poly(I:C) (34–36).

**Genomic DNA isolated from KRV induces the production of IL-12p40 in spleen cells from BBDR rats**

Recent studies have indicated that ssDNA can directly bind and activate TLR9 (37, 38). KRV is an ssDNA virus and we hypothesized that the actual TLR ligand responsible for cytokine induction following infection is genomic KRV DNA. To test this hypothesis, we isolated DNA from tissue culture supernatants of KRV-infected NRK cells. We used genomic DNA isolated from uninfected NRK cells that are used to propagate KRV to exclude the possibility that spleen cell activation is mediated via DNA that is potentially released from NRK cells into the medium during virus preparation. Spleen cells from BBDR rats incubated for 18 h in the presence of purified genomic KRV DNA induced the production of IL-12p40 whereas the same quantity of genomic DNA from uninfected NRK cells did not (Fig. 4, p < 0.05). On the basis of these results, we hypothesize that the ligand within KRV that induces IL-12p40 secretion in spleen cells is viral DNA.

**KRV-induced IL-12p40 production by spleen cells is PKR and NF-κB dependent**

Given our observation that intact KRV, genomic KRV DNA, and CpG DNA all induce BBDR spleen cells in vitro to produce IL-12p40, we next tested the hypothesis that this process was mediated by TLR ligation. To test this hypothesis, we used inhibitors of TLR-signaling pathways. PKR is a component of both MyD88- and IL-1R domain-containing adaptor protein-dependent TLR-signaling pathways (25, 39). Stimulation of macrophages with CpG DNA activates PKR, implicating this molecule in antiviral defense, stress responses, and TLR signaling (25, 39). Activation of PKR leads to NF-κB and MAPK up-regulation and type I IFN production (40). We reasoned that if KRV-induced activation of spleen cells involves signaling through PKR and NF-κB, it would be possible to suppress this activation using appropriate inhibitors.

We first incubated BBDR spleen cells in the presence of LPS, CpG DNA, poly(I:C), or KRV plus 2AP, an inhibitor of PKR function. As shown in Fig. 5A, incubation with high-dose 2AP led to a statistically significant inhibition of IL-12p40 production in KRV-stimulated cultures (p < 0.001 vs control).

**FIGURE 3.** In vitro peritoneal macrophage production of IL-12p40 induced by KRV virus. Peritoneal macrophages were obtained from BBDR rats injected i.p. 4 days earlier with 3 ml of a 3% thioglycolate solution. Cells were cultured for 18 h in either culture medium alone or culture medium containing LPS (1 μg/ml), CpG DNA (1 μg/ml), poly(I:C) (50 μg/ml), or KRV (2 × 10<sup>5</sup> PFU/ml). The concentrations of IL-12p40, IL-6, and TNF-α in supernatant were then determined by ELISA. Each data point represents mean ± SD of three independent measurements and is shown on a logarithmic scale. *p < 0.025 vs KRV; **p < 0.01 vs KRV; ***p < 0.001 vs KRV.

### Table I. IL-12p40 production by bone marrow-derived DCs<sup>a</sup>

<table>
<thead>
<tr>
<th>DC Type</th>
<th>IL-12p40 (pg/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>46 ± 9 (17)</td>
</tr>
<tr>
<td>KRV (n)</td>
<td>107 ± 34* (6)</td>
</tr>
<tr>
<td>GM-CSF plus IL-4-induced DC</td>
<td>2,578 ± 420 (20)</td>
</tr>
<tr>
<td>Flt-3L-induced bone marrow-derived DC</td>
<td>13,030 ± 3,012** (10)</td>
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<sup>a</sup> IL-12p40 production by BBDR rat DCs. DCs were obtained from BBDR bone marrow cultured in the presence of either GM-CSF plus IL-4 or Flt-3 ligand as described in Materials and Methods. DCs were then cultured for 18 h in medium alone or in medium containing KRV (2 × 10<sup>5</sup> PFU/ml). The concentration of IL-12p40 in culture supernatants was measured by ELISA as described in Materials and Methods. Data are presented as the mean ± SD; the number of individual measurements is shown in parentheses. Statistical comparisons used unpaired t tests. *p < 0.25 and **p < 0.0001 vs control.
to a dose-dependent reduction in the level of IL-12p40 production in response to all four stimuli. At an intermediate dose, 2AP inhibited IL-12p40 production in response to KRV and CpG DNA but not poly(I:C) or LPS. As shown in Fig. 5B, incubation with PDTC, an inhibitor of NF-κB, was also highly effective in suppressing IL-12p40 secretion in response to either KRV or CpG DNA. In a single experiment, we also observed that 20 μM PDTC added to cultures of BBDR spleen cells activated with poly(I:C) led to complete inhibition of IL-12p40 production (D. Zipris, data not shown). These results support the hypothesis that KRV-induced activation of spleen cells is mediated via molecules known to be involved in TLR-signaling pathways.

KRV-induced secretion of IL-12p40 by BBDR spleen cells is inhibited by iCpG and by chloroquine

Our observations that genomic DNA from KRV can activate BBDR spleen cells to produce IL-12p40 and that the pattern of cytokine secretion by T cells and DCs in response to KRV is the same as the pattern observed in response to CpG DNA prompted us to test the hypothesis that these responses are mediated via the TLR9-signaling pathway. To do so, we first used iCpG, an oligonucleotide containing an inhibitory CpG motif that specifically inhibits TLR9 signaling (28). Addition of 1 or 10 μg/ml iCpG to BBDR spleen cells incubated in the presence of either KRV or CpG DNA resulted in substantial reductions in the secretion of IL-12p40 (Fig. 6A). In contrast, iCpG inhibited IL-12 production in response to poly(I:C) only at the highest dose used, and the magnitude of the effect was much less (Fig. 6A).

TLR9 has to be recruited to the lysosomal compartment following CpG-induced activation for signaling activity (41). We reasoned that if KRV signals through TLR9, its recognition would also require endosomal acidification, as previously reported for CpG DNA (41). To investigate this hypothesis, spleen cells from BBDR rats were cultured in the presence of KRV, CpG DNA, or poly(I:C) in the absence or presence of chloroquine, a known inhibitor of endosomal acidification. Addition of chloroquine to BBDR spleen cells incubated in the presence of either KRV or CpG DNA resulted in substantial reductions in the secretion of IL-12p40 in a dose-dependent manner (Fig. 6B). It can be inferred that this reduction in IL-12p40 secretion was not due to simple drug toxicity because IL-12p40 secretion from spleen cells stimulated with poly(I:C), a ligand for TLR3, was not affected by treatment with this inhibitor at any dose tested (Fig. 6B). In their aggregate, these data suggest that KRV activates rat spleen cells via TLR9-signaling pathways.

Genomic DNA isolated from KRV induces the production of IL-12p40 in Flt-3L-induced DCs derived from wild-type but not TLR9-deficient mice

We hypothesized that if the ligand within KRV that activates B lymphocytes and Flt-3L-induced DCs from BBDR rats is viral DNA, we would be able to use DNA extracted from KRV to up-regulate Flt-3L-generated bone marrow-derived pDCs from wild-type but not TLR9-knockout mice to secrete IL-12p40. Cells were cultured for 18 h in the presence of genomic DNA purified from tissue culture supernatants containing KRV, DNA extracted from uninfected NRK cells, or purified TLR ligands (n = 2). We observed that purified genomic KRV DNA, CpG DNA, or R848, but not NRK DNA induced high levels of IL-12p40 secretion in pDCs derived from wild-type mice (Fig. 7). In contrast, Flt-3L-induced pDCs from TLR9-deficient mice failed completely to respond to KRV DNA or CpG DNA but did respond to the TLR7/8 ligand, R848. As predicted, because KRV does not infect mice and mouse DCs do not express TLR9, we did not observe a response to KRV DNA or CpG DNA. However, purified KRV DNA induced IL-12p40 secretion in cultured Flt-3L-induced pDCs derived from TLR9-deficient mice (Fig. 7). Similarly, purified NRK DNA failed to induce IL-12p40 secretion in Flt-3L-induced pDCs derived from TLR9-deficient mice (Fig. 7). These results support the hypothesis that the ligand within KRV that induces IL-12p40 release from spleen cells or Flt-3L-induced DCs from BBDR rats is viral DNA and that this up-regulation is mediated via TLR9-signaling pathways.
Chloroquine reduces the penetrance of KRV-induced diabetes in BBDR rats

Given these observations and the fact that chloroquine has clinical benefit in the treatment of systemic lupus erythematosus (SLE) (42) and rheumatoid arthritis (RA) (43), we tested the hypothesis that it would attenuate virus-induced autoimmune diabetes in the rat. BBDR rats 22–25 days of age were injected with poly(I:C) and KRV to induce diabetes according to our standard protocol (9, 10). They were then randomized to receive a daily injection of either saline or chloroquine (100 μg/g body weight) for 21 days. The results are shown in Fig. 8. As expected (9, 10), injection of poly(I:C) and KRV led to diabetes in seven of eight BBDR rats. In contrast, identically treated littermates given chloroquine developed diabetes at a much lower frequency (p < 0.025).

Chloroquine decreases serum levels of IL-12p40 in KRV-infected BBDR rats

Finally, we tested the hypothesis that chloroquine would modulate the in vivo innate immune response of BBDR rats infected with KRV. To do so, we quantified the serum level of IL-12p40 in animals injected with poly(I:C) plus KRV with or without coadministration of chloroquine. Consistent with previous reports (9), we observed that the concentration of IL-12p40 4 days after infection was statistically significantly higher in the serum of BBDR rats treated with poly(I:C) plus KRV than in untreated controls that it would attenuate virus-induced autoimmune diabetes in the rat. BBDR rats 22–25 days of age were injected with poly(I:C) and KRV to induce diabetes according to our standard protocol (9, 10). They were then randomized to receive a daily injection of either saline or chloroquine (100 μg/g body weight) for 21 days. The results are shown in Fig. 8. As expected (9, 10), injection of poly(I:C) and KRV led to diabetes in seven of eight BBDR rats. In contrast, identically treated littermates given chloroquine developed diabetes at a much lower frequency (p < 0.025).

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Finally, we tested the hypothesis that chloroquine would modulate the in vivo innate immune response of BBDR rats infected with KRV. To do so, we quantified the serum level of IL-12p40 in animals injected with poly(I:C) plus KRV with or without coadministration of chloroquine. Consistent with previous reports (9), we observed that the concentration of IL-12p40 4 days after infection was statistically significantly higher in the serum of BBDR rats treated with poly(I:C) plus KRV than in untreated controls
We observed high levels of IL-12 p40 in sera of KRV-infected animals and tissue culture supernatants derived from KRV-infected splenic B lymphocytes and Flt-3L-induced DCs, whereas levels of IL-12 p70 were undetectable (<2.5 pg/ml). Because IL-12 p40 is part of both IL-12 and IL-23, we were unable to differentiate between these two cytokines and cannot exclude the possibility that both IL-12 and IL-23 are secreted by KRV-activated B cells and DCs.

Our data suggest that KRV activates splenic B lymphocytes and Flt-3L-induced bone marrow-derived DCs through TLR9-signaling pathways to produce IL-6 and IL-12p40 (this report) and to activate STAT-1 (D. Zipris, unpublished observations). It is not yet known whether B lymphocytes are involved directly in adaptive immune system activation leading to KRV-induced diabetes in the BBDR rat. B lymphocytes could alternatively be involved indirectly through the production of proinflammatory cytokines. B lymphocytes are known to be critical for diabetes in the NOD mouse model of T1D (48, 49) and a clinical trial of anti-CD20 Ab therapy for new onset T1D has been initiated (www.diabetesresearch.net.org/anti1.html). The role of B cells in KRV-induced diabetes in BBDR rats merits further investigation.

DCs are important in diabetes pathogenesis in NOD mice (50–57), and our data in the BBDR rat document that Flt-3L-induced but not GM-CSF-induced bone marrow-derived DCs are stimulated by KRV to produce high levels of IL-12p40. The difference in the ability of Flt-3L-induced DCs vs GM-CSF plus IL-4-induced DCs to respond to KRV is probably not a result of difference in KRV infectivity, because KRV mRNA was readily detected in both cell populations (D. Zipris, unpublished observations). Flt-3L-induced bone marrow-derived DCs are thought to be in vitro surrogates of pDCs (31) whereas GM-CSF plus IL-4-induced bone marrow-derived DCs are thought to be in vitro surrogates of myeloid DCs (30). Human and rat pDCs have been reported to express TLR9 (31, 58, 59) and this observation would be consistent with our hypothesis that KRV induces IL-12p40 production by signaling through TLR9 and that Flt-3L-induced bone marrow-derived DCs respond to KRV. Although our results are consistent with the notion that KRV activates pDCs, there are caveats that constrain this interpretation. Flt-3L-induced DC cultures from BBDR rats contain phenotypically heterogeneous cell subsets that match both mDC and pDC populations, as evidenced by cell surface marker expression and the ability to respond to both myeloid DC and pDCs associated TLR ligands (D. Zipris, unpublished observations). We therefore are unable to exclude the possibility that DC subsets other than pDCs are up-regulated by KRV. In addition, in vitro-generated bone marrow-derived DCs induced by Flt-3L may be phenotypically and functionally different from splenic pDCs in vivo. Efforts are underway to isolate primary pDCs and to determine whether they can be stimulated directly by KRV.

Why bone-marrow-derived GM-CSF-induced DCs from BBDR rats do not respond well to KRV in vitro despite the fact that they express TLR9 (D. Zipris, unpublished observations) is unclear. It is not due to the inability of KRV to infect this cell subset (D. Zipris, unpublished observations) but could be due to endosomal trafficking differences between Flt-3L-induced and GM-CSF-induced bone marrow-derived DCs (60, 61).

It has also been proposed that B cell activation plays a critical role in the pathogenesis of SLE and RA (62, 63) and that TLR-signaling pathways also have a major role in these diseases (64). The observation that chloroquine can attenuate diabetes in the BBDR rat is reminiscent of previous reports that this agent is able to attenuate clinical manifestations in SLE (42) and RA (62, 63). TLR9-signaling pathways have also been implicated in the loss of...
self-tolerance in SLE (62, 63). In those studies, the authors proposed that activation of autoreactive rheumatoid factor B cells in SLE is mediated by immune complexes of IgG bound to self DNA. Effective stimulation of these B cells required activation of both the BCR and the TLR9-signaling pathway (62, 63). The beneficial effect of chloroquine observed in some lupus patients could involve the disruption of TLR9-mediated activation of autoreactive B lymphocytes (62, 63).

It remains to be determined whether KRV induces activation of cells of the immune system other than B lymphocytes and DCs. It has been reported that KRV activates macrophages in the BBDR rat to produce NO (65), and we found low levels of IL-12p40 produced by thioglycollate-elicited macrophages activated by KRV in vitro. In addition to B lymphocytes, DCs, and macrophages, KRV could potentially activate NK cells or NKT cells via TLR-signaling pathways to produce proinflammatory cytokines or to activate adaptive immunity, thereby facilitating the expression of diabetes in genetically susceptible hosts. Studies to address these possibilities are currently under way.

TLR and innate immune activation were recently found to be crucial for virus-induced diabetes in a transgenic mouse model (66). However, in another study in transgenic mice, TLR and DC activation were found to be insufficient to cause diabetes in the absence of specific CD4 T cell help (67). Understanding the role of activation were found to be insufficient to cause diabetes in the genetically susceptible animal models remains an important goal for understanding how the environment may modulate the unpredictable penetrance of autoimmune diabetes in genetically susceptible humans.

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Disclosures
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