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 Fli-3-ligand-induced bone marrow-derived dendritic cells (DCs) but only low levels of IL-12p40 production by thiglycolate-elicted 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 Fli-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.
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, Mycoplasma 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% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μM 2-ME (Invitrogen Life Technologies). KRV was harvested at multiplicity of infection of 1 and used to infect NRK cells; virus was harvested from supernatant of infected cultures at 2 days after infection and kept at –70°C until use. Viruses were titered using a plaque assay as previously described (10).

In vitro stimulation 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.

Polyinosinic-polyricitydic acid (poly(I:C)) was purchased from Sigma-Aldrich. Contaminating endotoxin concentration was <0.5 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′-TCGTTGTTTTGCTGTTTGCCTG-3′ (27) and was synthesized by TriLink BioTechnologies. The sequence of inhibitory CpG (iCpG) used was 5′-TCAGGAGGAGGTTGTTGCCTG-3′ (28) and was synthesized by Invitrogen 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 instruction. 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, pyrrolidine dithiocarbamate (PDTC), and the protein kinase R (PKR) inhibitor, 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/gm body weight and then injected i.p. with 1 × 10^7 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 dose of poly(I:C) by itself is incapable of inducing diabetes in the BBDR rat (9). Animals were screened three 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 poly(I:C) and infected with virus, animals were treated with poly(I:C) on days 0, 1, and 2 and 1 × 10^7 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 injection for 7 weeks. A second group was injected with PBS on the same schedule.

To determine whether chloroquine activated the injection 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 × 10^7 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 injection for 7 weeks.

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-CD45R 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-3-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% thioglycollate (BD Biosciences). Recovered macrophages were incubated at a concentration of 2 × 10^6 cells/ml for 18 h in the presence of 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 rat rGM-CSF plus 10 ng/ml rIL-4 (both from BD Pharmingen) (30). Flt-3L-induced DC, i.e., mature dendritic cells (DCs) 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 by the 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. This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK 54754 to M. A. M. A.), the Juvenile Diabetes Research Foundation, and the Diabetes Research Development Fund.
that no other paired comparisons were statistically significant.

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).

IL-12 p40 in tissue culture supernatants were measured by an optimized method of Kaplan and Meier; the equality of diabetes-free survival times among groups was compared by life-table analysis using the log-rank statistic (32). Comparisons of two means used unpaired Student’s t tests; comparisons of three or more means used one-way ANOVA and Bonferroni adjusted unpaired t tests for a posteriori contrasts (version 4.0; GraphPad Software).

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 RT1u 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 associated with robust production of IL-12p40 (Fig. 1A), also stimulated BBDR spleen cells to secrete IL-6 but not TNF-α (Fig. 1B).

Interestingly, ligation of TLR9 with CpG DNA induced the same pattern of cytokine production: increases in IL-12p40 and IL-6 but not TNF-α. These differential patterns suggest the possibility that KRV could be a ligand of TLR9.

B cells cultured in vitro in the presence of KRV produce IL-12p40

To begin to identify specific subsets that respond to KRV, we generated spleen cell populations enriched for B (CD45R+B) and non-B (CD45R−) cells and cultured them in vitro in the presence of medium alone, 2 × 10⁷ PFU/ml KRV, or various purified TLR ligands. We observed that the secretion of IL-12p40 in response to both KRV and CpG DNA was statistically significantly greater by

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

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 × 10⁹ 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.

in different treatment groups was tested using the log-rank statistic (32). Comparisons of two means used unpaired Student’s t tests; comparisons of three or more means used one-way ANOVA and Bonferroni adjusted unpaired t tests for a posteriori contrasts (version 4.0; GraphPad Software).

FIGURE 2. Production of IL-12p40 by B cells cultured in the presence of KRV or TLR ligands. Purified CD45R+B cells and CD45R− non-B cells were prepared from BBDR spleens as described in Materials and Methods and incubated for 18 h in the presence of culture medium alone or culture medium containing LPS (1 μg/ml), CpG DNA (1 μg/ml), poly(I:C) (50 μg/ml), or 2 × 10⁷/ml PFU KRV. The concentration of IL-12p40 in 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.
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 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 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 each 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.

CD45R B cells than by CD45R 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.

Table I.  IL-12p40 production by bone marrow-derived DCs

<table>
<thead>
<tr>
<th>IL-12p40 (pg/ml)</th>
<th>Control (n)</th>
<th>KRV (n)</th>
</tr>
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<tbody>
<tr>
<td>Flt-3L-induced bone marrow-derived DC</td>
<td>46 ± 9 (17)</td>
<td>107 ± 34* (6)</td>
</tr>
<tr>
<td>GM-CSF plus IL-4-induced bone marrow-derived DC</td>
<td>2,578 ± 420 (20)</td>
<td>13,030 ± 3,012** (10)</td>
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* 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 × 10⁷ 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 < 0.001 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 dendritic cells do not respond to TLR7/8 ligands, Flt-3L-induced pDCs from TLR9-deficient mice also failed to respond. These data strongly support our hypothesis that the ligand within KRV that induces IL-12p40 release in 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 4 days after infection. Consistent with previous reports (9), we observed that the concentration of IL-12p40 in serum 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 ($p < 0.025$).

FIGURE 6. Production of IL-12p40 by spleen cells incubated in the presence of KRV, TLR ligands, and specific inhibitors of TLR9 signaling. BBDR spleen cells were cultured for 18 h in culture medium alone or culture medium containing CpG (1 μg/ml), poly(I:C) (50 μg/ml), or KRV (2 × 10^7 PFU) as described in Materials and Methods. To some of the cultures were added the indicated concentrations of the TLR9-signaling pathway inhibitors iCpG ($A, n = 6$ at each data point) or chloroquine ($B, n = 3–5$ at each data point). The concentration of IL-12p40 in supernatant was determined by ELISA. All data represent the mean ± SD. *, $p < 0.01$ and **, $p < 0.001$ vs control.

FIGURE 7. Production of IL-12p40 by mouse FLT-3L-induced DCs derived from wild-type and TLR9-knockout mice incubated in the presence of genomic KRV DNA or TLR ligands. Bone marrow from wild-type and TLR9-knockout mice bone marrow was cultured in the presence of Flt-3 ligand to generate a population of pDCs as described in Materials and Methods. The DCs were then cultured for 18 h in medium alone or in medium containing 2.5 μg/ml KRV DNA or NRK DNA, or purified TLR ligands, as described in Materials and Methods. The concentration of IL-12p40 in culture supernatants was measured by ELISA. Data presented are the mean of two independent experiments.
In contrast, treatment with chloroquine beginning on the day of infection significantly attenuated the increase in serum IL-12p40 concentration, although it did not reduce it to the level observed in untreated controls (Fig. 9). These data suggest that chloroquine may attenuate KRV-induced diabetes in the BBDR rat by down-modulation of innate immune system activation.

**Discussion**

These data document that KRV infection in the rat activates B cells and Flt-3L-induced bone marrow-derived DCs and induces production of IL-12p40 by spleen cells. They also document that genomic KRV DNA can directly induce IL-12p40 production and that KRV induction of IL-12p40 production can be inhibited by both inhibitors of TLR-signaling pathways (2AP and PDTC) and specific TLR9 inhibitors (iCpG and chloroquine). Finally, the data document a role for TLR9 signaling in diabetes expression using the TLR9 inhibitor, chloroquine (44), to inhibit the in vivo production of IL-12p40 and to decrease the frequency of diabetes in KRV-infected BBDR rats. In their aggregate, the data implicate signaling via TLR9 as a major mechanism by which KRV infection induces diabetes in BBDR rats.

How KRV-induced activation of the TLR9-signaling pathway subsequently leads to autoimmune diabetes in the BBDR rat is not yet clear. It has been hypothesized that interactions of T cells with immature APCs lead to the generation of Treg and tolerance, whereas T cell interactions with activated mature APCs lead to the generation of effector T cells and adaptive immunity (45). Based on the present observations and data we have reported previously (9, 10), we hypothesize that treatment of BBDR rats with low doses of poly(I:C) followed by infection with KRV activates APCs and induces inflammation in the microenvironment of the pancreatic lymph nodes (9). This would then enhance the presentation of islet autoantigen(s) to the immune system by mature APCs, facilitating the generation of autoreactive cytotoxic CD8 T cells that then migrate to the islets leading to insulitis, β cell destruction, and diabetes.

In vitro and in vivo data both support the hypothesis that TLR9-signaling pathways mediate KRV-induced innate immune system activation. However, there are several caveats that constrain this interpretation of our data. First, rat spleen cells that are deficient of TLR9 are not currently available, and we are, therefore, unable to rule out the possibility that TLRs other than TLR9 are involved in the signaling pathway by which KRV induces innate immune activation and autoimmune diabetes. We have, however, tested the ability of genomic KRV DNA to activate mouse bone-marrow derived Flt-3L-induced DCs and have observed robust IL-12p40 production by cells from wild-type but not from TLR9 knockout mice, lending further support to our hypothesis that KRV-induced up-regulation of B lymphocytes and Flt-3L DCs is mediated by viral DNA through the TLR9 pathway. Second, like TLR9, TLR3, and TLR7/8, are also located in endosomal compartments and serve as receptors for viral RNA (22, 46). These TLRs could potentially recognize KRV-derived RNA generated during viral replication, leading to innate immune activation and diabetes. However, we observed that chloroquine did not inhibit poly(I:C)-induced production of IL-12p40. Additional experiments to determine the subcellular localization of TLR3 in rat cell subsets will be required to determine why TLR3-induced up-regulation of IL-12p40 was not inhibited by chloroquine. Third, it is also possible that chloroquine, in addition to its known inhibitory effect on TLR9 signaling (44), could interfere with diabetes expression in the BBDR rat by altering Ag processing and presentation (47).

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.diabetestrialnet.org/oanti1.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 Igs 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 thioglycolate-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 TLR signaling in the expression of autoimmune disease in 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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