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Immunoregulatory Role of Nitric Oxide in Kilham Rat Virus-Induced Autoimmune Diabetes in DR-BB Rats[1]

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Macrophages play a critical role in the pathogenesis of Kilham rat virus (KRV)-induced autoimmune diabetes in diabetes-resistant BioBreeding (DR-BB) rats. This investigation was initiated to determine the role of macrophage-derived soluble mediators, particularly NO, in the pathogenesis of KRV-induced diabetes in DR-BB rats. We found that the expression of inducible NO synthase (iNOS), an enzyme responsible for NO production, was significantly increased during the early phase of KRV infection. Inhibition of iNOS by aminoguanidine (AG) treatment resulted in the prevention of diabetes in KRV-infected animals. The expression of IL-1β, TNF-α, and IL-12 was significantly decreased in the spleen of AG-treated, KRV-infected DR-BB rats compared with PBS-treated, KRV-infected control rats. Subsequent experiments revealed that AG treatment exerted its preventive effect in KRV-infected rats by maintaining the finely tuned immune balance normally disrupted by KRV, evidenced by a significant decrease in the expression of IFN-γ, but not IL-4, and a decrease in Th1-type chemokine receptors CCR5, CXCR3, and CXCR4. We also found that iNOS inhibition by AG decreased the KRV-induced expression of MHC class II molecules and IL-2R α-chain, resulting in the suppression of T cell activation, evidenced by the decreased cytolytic activity of $CD8^+$ T cells. We conclude that NO plays a critical immunoregulatory role by up-regulating macrophage-derived proinflammatory cytokines, up-regulating the Th1 immune response, and activating T cells, leading to type 1 diabetes after KRV infection, whereas suppression of NO production by AG treatment prevents KRV-induced autoimmune diabetes in DR-BB rats. ***The Journal of Immunology,*** **2004, 173: 1327–1335.**

Type 1 diabetes results from the progressive loss of insulin-producing pancreatic β cells. Diabetes-resistant BioBreeding (DR-BB) rats, unlike their diabetes-prone forebearers, do not normally develop type 1 autoimmune diabetes (1, 2). However, when DR-BB rats are infected with Kilham rat virus (KRV),[5] approximately one-third become diabetic with a disease manifestation similar to human type 1 diabetes (3). The incidence of diabetes increases to >80% when KRV infections are conducted in the presence of the adjuvant poly(inosinic:cytidylic) acid (poly(I:C)) (4–6).

KRV, a member of the *Parvovirus* family, is tropic for actively dividing cells and specifically targets lymphoid organs such as the spleen, thymus, and lymph nodes (7, 8). KRV does not infect pancreatic β cells; therefore, KRV-induced diabetes in DR-BB rats is not the result of direct cytolysis of β cells due to viral infection (8). It was recently reported that the failure to maintain regulatory $CD4^+CD25^+$ T cell function after KRV infection may cause the development of diabetes in DR-BB rats (9). In addition, we found that KRV infection results in the breakdown of the host's immune balance of T cells through the selective up-regulation of β cell-specific cytotoxic $CD8^+$ and Th1-type $CD4^+CD45RC^+$ T cell populations (6), which destroy β cells, leading to type 1 diabetes in DR-BB rats.

Macrophages have been shown to play an important role in KRV-induced autoimmune diabetes in DR-BB rats (5). The production of macrophage-derived proinflammatory cytokines such as TNF-α, IL-1β, and IL-12 was closely correlated with an elevated Th1 immune response in KRV-infected DR-BB rats (5). It has been demonstrated that NO is required for IL-12 signaling and the expression of proinflammatory cytokines in macrophages, suggesting that NO produced in innate immunity is linked to the T cell response in adaptive immunity (10, 11). Whether or not NO produced by activated macrophages plays a role in the activation of effector T cells that cause autoimmune diabetes in DR-BB rats remains unknown.

This investigation was initiated to determine the role of NO in the regulation of immune responses that control the development of KRV-induced autoimmune diabetes in DR-BB rats. We now report that KRV-activated macrophages produce NO, and treatment of KRV-infected rats with aminoguanidine (AG), an inhibitor of inducible NO synthase (iNOS), results in the following: 1) the down-regulation of the Th1 immune response, evidenced by a decrease in the $CD4^+CD45RC^+$ T cell population, a Th1-type cytokine (IFN-γ), and Th1-type chemokine receptors (CCR5 and CXCR3); 2) a significant decrease in the expression of MHC class II molecules and IL-2R α-chain (IL-2Rα); 3) the suppression of cytotoxic T cell activity; and 4) the prevention of autoimmune

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[5] Abbreviations used in this paper: KRV, Kilham rat virus; poly(I:C), poly(inosinic:cytidylic) acid; AG, aminoguanidine; iNOS, inducible NO synthase; IL-2Rα, IL-2R α-chain; MOI, multiplicity of infection; $TCID_{50}$, tissue culture-infective dose; HPRT, hypoxanthine phosphoribosyltransferase.

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diabetes. These results suggest that NO produced by KRV-activated macrophages plays a critical role in the regulation of immune responses such as the up-regulation of the Th1 immune response and the activation of β cell-CTLs, resulting in the development of autoimmune diabetes in KRV-infected DR-BB rats.

Materials and Methods

Animals

DR-BB rats were produced from breeding stocks purchased from the University of Massachusetts (DR-BB/Wor; Worcester, MA). The animals were maintained under specific pathogen-free conditions at the University of Calgary (Calgary, Alberta, Canada) and used at 21–25 days of age. The use and care of the animals in this study were approved by the Animal Care Committee, Faculty of Medicine, University of Calgary.

Cells and virus

Normal rat kidney (NRK) cells, Chang (human liver) cells, H-1 virus, and KRV were obtained from American Type Culture Collection (Manassas, VA). NRK and Chang cells were cultured in complete medium, DMEM/RPMI 1640 supplemented with 10% heat-inactivated FBS, 5 mM sodium pyruvate, 5 mM L-glutamine, and 5 μg/ml gentamicin, at 37°C under 5% CO_2. KRV and H-1 virus were cultivated in NRK and Chang cells, respectively, and harvested from the supernatant of infected cultures at 3 days after viral infection (multiplicity of infection (MOI) = 1), and kept at −70°C until use. Virus was titered in 96-well plates (Corning Glass Works, Corning, NY), seeded with NRK or Chang cells, and the tissue culture-infective dose ($TCID_{50}$) was determined before use (5, 6).

Preparation of peritoneal macrophages

Peritoneal macrophages from DR-BB rats were harvested by lavage of the peritoneal cavity with 20 ml of RPMI 1640 at 4 days after i.p. administration of 2 ml of 3% thioglycolate. Cells were washed twice and suspended in RPMI 1640 medium (5×10^5 cells/ml; 2 ml/well) in six-well plates (Corning Glass Works), and nonadherent cells were removed 2 h later. Adherent cells were infected 24 h later with KRV or H-1 virus at a MOI of 5. Anti-IFN-γ Ab (BD Pharmingen, San Diego, CA) was added at a final concentration of 1.25 μg/ml, and AG (Sigma-Aldrich, St. Louis, MO) was added at a final concentration of 1 mM.

Nitrite assay

The production of NO was measured as described previously (12). At various time points after in vitro infection of macrophages with KRV or H-1 virus, 50 μl of culture supernatant from KRV- or H-1 virus-infected macrophages was mixed with 100 μl of Greiss reagent and incubated for 10 min at 37°C. The OD_{550} was determined using an Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Nitrite concentration was determined from a standard curve generated using sodium nitrite (Sigma-Aldrich). The absorbance of the medium alone was subtracted from the value obtained for each sample.

Infection of DR-BB rats with KRV or H-1 virus and AG treatment

KRV or H-1 virus (3×10^5 $TCID_{50}$/rat, i.p.) was administered to 21- to 25-day-old DR-BB rats, and poly(I:C) (5 μg/g of body weight, i.p.; Sigma-Aldrich) was administered every 24 h for 3 days after infection. AG (100 mg/kg of body weight, i.p.; Sigma-Aldrich) or PBS as a vehicle control was given to DR-BB rats at 12 and 24 h before infection with KRV and every 12 h thereafter for 24 days or until sacrifice. The development of diabetes was monitored by urine glucose measurement (Diastix, Miles, ON, Canada) and confirmed by blood glucose measurement (Lifescan, Burnaby, BC, Canada) for 4 wk after KRV infection or until sacrifice. Animals were considered diabetic if they showed glycosuria (>2+) and hyperglycemia (>16.7 mM) as previously described (6).

RT-PCR analysis of cytokines, chemokines, and chemokine receptors

Total RNA was extracted from either KRV- or H-1 virus-infected peritoneal macrophages from DR-BB rats or from isolated splenocytes of AG- or PBS-treated, KRV/poly(I:C)-injected DR-BB rats using the acid guanidine thiocyanate phenol-chloroform method (13). Three micrograms of total RNA was converted to cDNA using SuperScript RT II (Life Technologies, Burlington, ON, Canada), and conventional or quantitative real-time PCR was performed using specific primers described previously for several cytokines (5), chemokines (14), chemokine receptors (15), and iNOS (16). Hypoxanthine phosphoribosyltransferase (HPRT) mRNA was measured as an internal standard (5). After amplification, the products were subjected to electrophoresis on 1% agarose containing ethidium bromide and then analyzed under UV light against DNA molecular markers using a GeneGenius bioimaging system (Syngene, Frederick, MD).

For quantitative real-time PCR, the expression of mRNA was analyzed using the LightCycler system (Roche Diagnostics, Mannheim, Germany). PCR were run in glass capillaries in a volume of 20 μl containing 2 μl of FastStart DNA SybrGreen I mix, 2 mM $MgCl_2$, 0.5 μM each primer, and 2 μl of cDNA. After each PCR, a melting curve analysis was performed to confirm the specificity and purity of the amplified PCR product. PCR products were also subjected to electrophoresis on 1% agarose containing ethidium bromide and analyzed by UV light against DNA molecular markers to confirm correct amplicon size and purity. To determine that the efficiency of each reaction was greater than or equal to $E = 1.85$, standard curves were generated by serial dilution of the control templates and assigning relative concentrations. To generate standard curves, the crossing cycle numbers of the logarithmic-linear PCR phase (crossing points) of the standards were plotted against the logarithm of their concentrations (fit points method). The correlation coefficient of the standard curves generated in each measurement was always ~0.95 or better, and the coefficient of variance in triplicate samples was <10%. Each gene was analyzed at least three times, and cDNA levels during the linear phase of amplification were normalized against constitutively expressed housekeeping gene *HPRT* controls. Each real-time PCR was performed with an initial denaturation step of 10 min at 95°C, followed by amplification for 45 cycles using previously described conditions and primers (5, 14–16).

Flow cytometric analysis

Flow cytometric analysis of splenocytes isolated from DR-BB rats at 3 and 7 days after KRV infection was performed as previously described (17). Briefly, cells were incubated for 30 min at 4°C with FITC-conjugated OX-6 mAb (RT1.B; Cedarlane, Hornby, ON, Canada) and PE-conjugated OX-42 mAb (CD11b/c; BD Pharmingen), or biotin-conjugated OX-19 mAb (CD5; Cedarlane) and FITC-conjugated OX-39 mAb (CD25; Cedarlane). The biotin was labeled with PE-streptavidin. The cells were then washed two times with FACS buffer (1% heated-inactivated FBS and 0.1% sodium azide in PBS (pH 7.2)) before fixation with 1% formalin, and were analyzed by FACScan (BD Biosciences, Sunnyvale, CA). The percentage of positive MHC class II and IL-2Rα cells was shown on the gate of $OX\text{-}42^+$ cells and T cells, respectively.

To analyze the $CD4^+CD45RC^+$ population, splenocytes isolated from DR-BB rats at 7 days after KRV infection were stained with biotin-conjugated OX-22 mAb (CD45RC; Cedarlane) and FITC-conjugated OX-35 mAb (CD4; BD Pharmingen) for 30 min at 4°C. The biotin was labeled with PE-streptavidin.

Measurement of viral titer

NRK or Chang cells (2×10^3 cells in 100 μl of complete DMEM medium) were seeded in 96-well microplates. An equal volume (100 μl) of a serially diluted KRV or H-1 virus sample in complete medium was added to each well and incubated for 5 days at 37°C under 5% CO_2. Cells were then fixed with 1% formalin and stained with 1% crystal violet solution to determine $TCID_{50}$. Four wells were used for each dilution.

In vitro $CD8^+$ CTL assay

Splenocytes were isolated from AG-treated, KRV/poly(I:C)-injected, or PBS-treated, KRV/poly(I:C)-injected DR-BB rats at 7 days after KRV infection, and $CD8^+$ T cells were isolated using rat $CD8^+$ T cell negative selection isolation immunocolumns using the manufacturer's protocol (Cedarlane). The purity of the recovered $CD8^+$ T cells was analyzed by flow cytometry and was found to be >90%. Subconfluent NRK cells (MHC matched with DR-BB rat) were infected with KRV (MOI = 5) for 24 h and labeled with ^{51}Cr (200 μCi/2×10^6 cells/ml) for 90 min. Purified $CD8^+$ T cells (2×10^5 cells/well) from AG- or PBS-treated, KRV poly(I:C)-injected DR-BB rats were incubated for 6 h in 200 μl of complete RPMI medium containing 10% FCS, 4 mM glutamine, and 50 μg/ml penicillin/streptomycin with ^{51}Cr-labeled KRV-infected NRK cells (1×10^4 cells/well). The culture supernatant was collected, and the released radioactivity was measured using a TopCount NXT microplate scintillation and luminescence counter (Packard, Meriden, CA). Spontaneous cell lysis was determined from the target cells without addition of effector cells, and total cell lysis was determined by lysing the cells with 5% Triton X-100.

Specific ^{51}Cr release was calculated as follows: percent lysis = 100 × (test cpm − spontaneous cpm)/(total cpm − spontaneous cpm).

Histologic examination

Pancreata were fixed with formalin, paraffin-embedded, serially sectioned at 5 μm, and stained with H&E. The insulitis lesions were classified as early, intermediate, late, or end stage according to morphological criteria described previously (18).

Statistical analysis

Statistical analyses were performed using Fisher's exact test or Student's *t* test. The level of significance was set at $p < 0.05$.

Results

iNOS activity is increased in response to KRV infection in DR-BB splenocytes and isolated peritoneal macrophages

To determine whether KRV can activate iNOS expression leading to NO production, we examined the expression of iNOS mRNA in the splenocytes of KRV/poly(I:C)-injected DR-BB rats. We found that the expression of iNOS was significantly increased at 3 days postinfection. When we administered AG, a selective inhibitor of iNOS, into DR-BB rats during KRV infection, the expression of iNOS mRNA was significantly decreased in the splenocytes compared with splenocytes from PBS-treated, KRV-infected control animals (Fig. 1*A*). To determine whether KRV infection directly

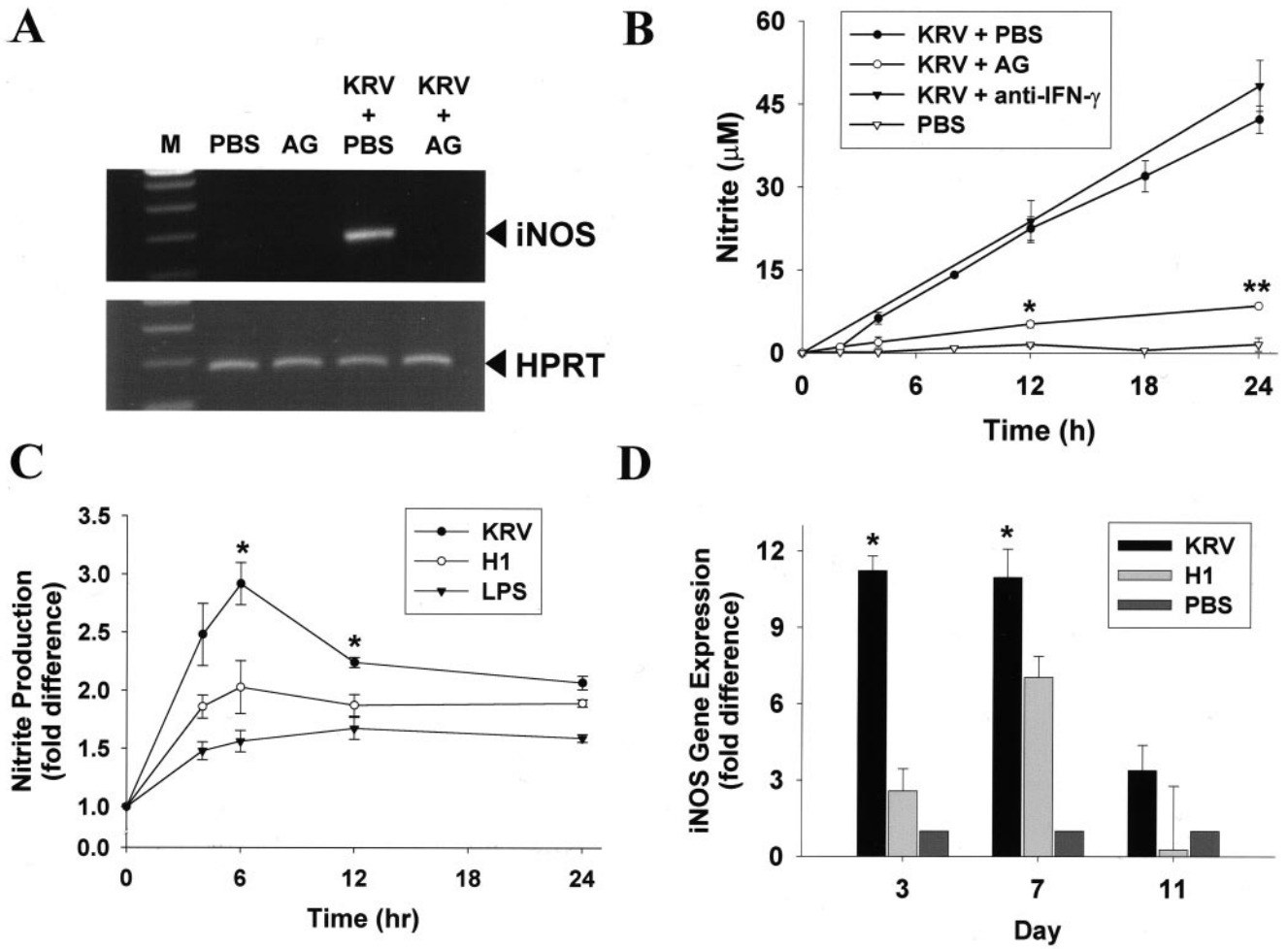

FIGURE 1. KRV induces iNOS expression in DR-BB rats and NO production in peritoneal macrophages. *A*, Splenocytes were isolated from DR-BB rats ($n = 3$/group) treated with PBS, AG, PBS and KRV/poly(I:C) (KRV + PBS), or AG and KRV/poly(I:C) (KRV + AG) at 3 days after infection. Total RNA was extracted from splenocytes, and *iNOS* and *HPRT* gene expression were analyzed by RT-PCR. *Lane M*, 100-bp DNA ladder. *B*, Purified peritoneal macrophages (5×10^5 cells/ml/well) isolated from DR-BB rats were infected with KRV (MOI = 5) either with PBS (KRV + PBS), AG (KRV + AG), or anti-IFN-γ Ab (KRV + anti-IFN-γ). As a control, purified macrophages were treated with PBS alone (PBS). At various times after treatment, supernatants from each group were analyzed for NO production by the Greiss assay. Background NO levels were subtracted from each sample. Background NO levels were determined in the supernatant from the culture of purified macrophages treated with uninfected NRK cell debris. Error bars represent the SEM ($n = 3$). ∗, $p < 0.01$; ∗∗, $p < 0.05$ compared with KRV + PBS group. *C*, Purified peritoneal macrophages (5×10^5 cells/well) isolated from DR-BB rats were infected with KRV or H-1 virus (MOI = 5). As a control, purified macrophages were treated with either LPS or PBS alone. At various times after treatment, supernatants from each group were analyzed for NO production by the Greiss assay. Fold differences were determined by comparing the KRV-, H-1 virus-, or LPS-treated group with the PBS-treated group (1.0). Error bars represent the SEM ($n = 3$); ∗, $p < 0.05$ compared with H-1 group. *D*, Splenocytes were isolated from DR-BB rats ($n = 4$/group) treated with PBS or equal infectious doses of KRV/poly(I:C) or H-1 virus/poly(I:C) at 3 days after treatment. Total RNA was extracted from splenocytes, and *iNOS* gene expression was analyzed by real-time RT-PCR. Values were normalized against HPRT and expressed as the fold difference of the KRV- or H-1 virus-infected group compared with the PBS-treated group. Error bars represent SEM ($n = 4$); ∗, $p < 0.001$ compared with the H-1-infected group.

induces NO production in macrophages of DR-BB rats, we isolated peritoneal macrophages from DR-BB rats, infected them with KRV, and examined NO production at different times after infection. We found that KRV infection clearly induced NO production within 2 h, and NO production increased linearly for 24 h before leveling off. In contrast, AG treatment resulted in the inhibition of NO production in KRV-infected macrophages (Fig. 1*B*).

To determine whether KRV induces iNOS expression through KRV-induced IFN-γ production, we infected macrophages in the presence of anti-IFN-γ Ab and examined NO production. We found that KRV could induce NO production as efficiently as in the absence of anti-IFN-γ Ab (Fig. 1*B*), indicating that KRV can directly induce the expression of iNOS independent of IFN-γ.

To determine whether a nondiabetogenic virus has a similar NO-inducing effect, we next examined the production of NO in macrophages after infection with H-1 virus, which is a rat parvovirus in the same family as KRV but does not induce diabetes. We found that H-1 virus was unable to generate NO production as high as KRV in DR-BB rat peritoneal macrophages in vitro ($p < 0.05$; Fig. 1*C*). Macrophages are highly nonspecific, and most externally delivered pathogens are likely to induce some NO production. Thus, we examined whether H-1 virus was able to induce iNOS expression in vivo. We infected DR-BB rats with the same infectious dose of either KRV or H-1 virus in the presence of poly(I:C) and sacrificed them 3 days later. We found that H-1 virus was unable to elevate iNOS levels to that seen in KRV-infected animals, even though H-1 virus was able to induce some iNOS expression ($p < 0.001$; Fig. 1*D*). These in vitro and in vivo studies indicate that a nondiabetogenic virus has a significantly lower effect on the production of NO in DR-BB rats.

iNOS activity is required for up-regulation of proinflammatory cytokine mRNA after KRV/poly(I:C) treatment

To determine whether iNOS has any effect on the expression of proinflammatory cytokines in DR-BB rats, we injected DR-BB rats with KRV/poly(I:C), with or without AG treatment, and examined the expression of proinflammatory cytokine mRNA in the splenocytes 3 days later. Infection of DR-BB rats with KRV induced a high expression of proinflammatory cytokines such as IL-12, IL-1β, and TNF-α. When KRV-infected DR-BB rats were treated with AG, the expression of IL-12, IL-1β, and TNF-α mRNA was significantly decreased compared with KRV/poly(I:C)-injected controls that were not treated with AG (Fig. 2*A*). The expression of these proinflammatory cytokine mRNAs was closely correlated with *iNOS* gene expression.

To determine whether iNOS inhibition specifically reduced proinflammatory cytokine gene expression, as opposed to a general reduction in gene expression, we examined the expression of several chemokines in the splenocytes of AG-treated, KRV/poly(I:C)-injected DR-BB rats. We found that the expression of MIP-1α, MIP-1β, and RANTES were increased during KRV infection, but AG treatment did not alter the expression of these chemokines by day 7 (Fig. 2*B*), suggesting that iNOS inhibition selectively reduces proinflammatory cytokine expression, but not chemokine expression.

To directly determine whether KRV can induce cytokine and chemokine gene expression in macrophages, we infected isolated peritoneal macrophages with KRV in vitro and examined the expression of IL-1β and RANTES, as representatives. We found that KRV was able to induce the expression of both IL-1β and RANTES, with maximum expression occurring 12 h after infection and declining thereafter, whereas PBS treatment had no effect on gene expression (Fig. 2*C*).

Inhibition of iNOS activity results in a decreased Th1 immune response

To determine whether inhibition of iNOS activity influences Th1 and Th2 immune responses, we analyzed Th1- and Th2-type cytokine gene expression in splenocytes from KRV/poly(I:C)-injected animals, with or without AG treatment, at 3 and 7 days after infection. Splenocytes from KRV/poly(I:C)-injected animals showed an elevation of *IFN-γ* (Th1-type) gene expression as compared with uninfected controls, whereas AG treatment resulted in the inhibition of *IFN-γ* gene expression to a level comparable with splenocytes from uninfected rats (Fig. 3*A*). In contrast, inhibition of iNOS activity did not affect expression of the *IL-4* (Th2-type) gene in KRV/poly(I:C)-injected animals (Fig. 3*A*).

We further examined the expression of Th1-type chemokine receptor genes (*CCR5*, *CXCR3*, and *CXCR4*) and a Th2-type chemokine receptor gene (*CCR4*) in KRV/poly(I:C)-injected DR-BB rats, with or without AG treatment, at 7 days after infection. We

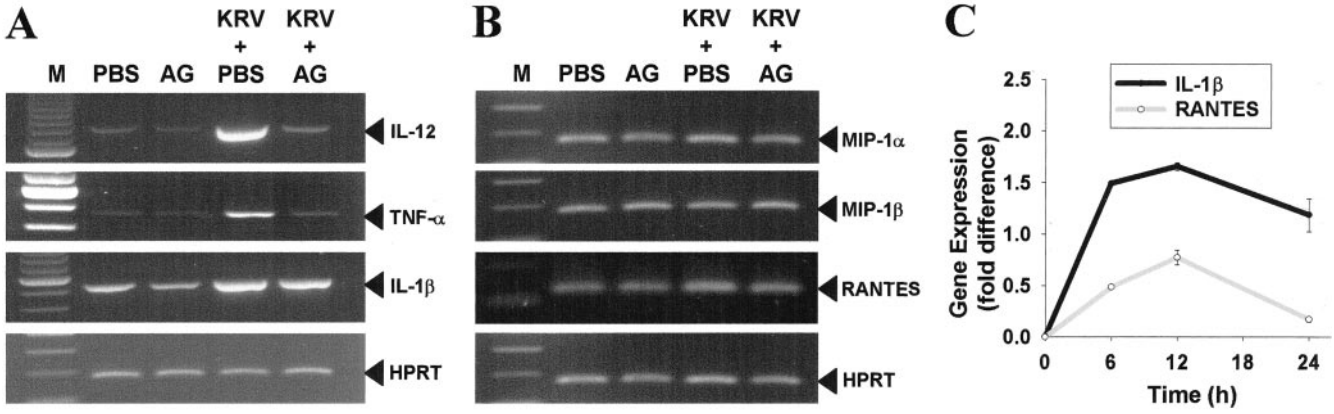

FIGURE 2. iNOS inhibition reduces KRV-induced proinflammatory cytokine but not chemokine gene expression in DR-BB rats. Splenocytes were isolated from DR-BB rats ($n = 3$/group) treated with PBS, AG, PBS and KRV/poly(I:C) (KRV + PBS), or AG and KRV/poly(I:C) (KRV + AG) either 3 or 7 days postinfection. Total RNA was extracted from splenocytes, and either proinflammatory cytokine (*A*) or chemokine (*B*) gene expression was analyzed by RT-PCR. *Lane M*, 100-bp DNA ladder. *C*, Purified peritoneal macrophages (5×10^5/ml/well) isolated from DR-BB rats were infected with KRV (MOI = 5) or treated with PBS and incubated at 37°C. At various times, cells were removed and total RNA was isolated. *IL-1β* and *RANTES* gene expression were analyzed by real-time RT-PCR. Gene expression was determined as the fold difference over the internal standard control, HPRT. Error bars represent SEM ($n = 4$).

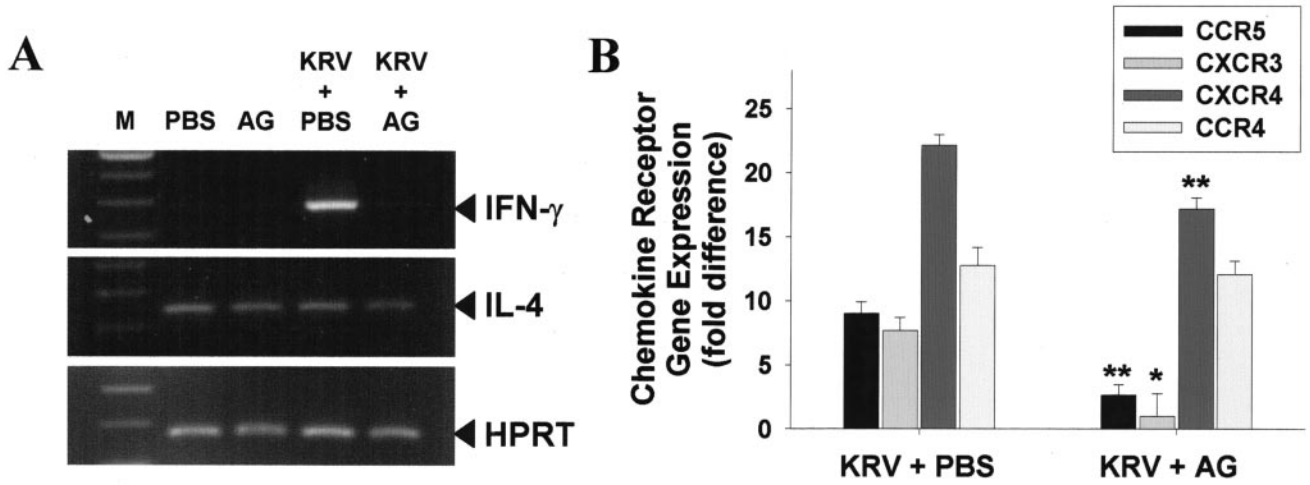

FIGURE 3. Inhibition of iNOS reduces Th1-type cytokine and chemokine receptor gene expression in KRV-infected DR-BB rats. *A*, Splenocytes were isolated from DR-BB rats ($n = 3$/group) treated with PBS, AG, PBS and KRV/poly(I:C) (KRV + PBS), or AG and KRV/poly(I:C) (KRV + AG) 3 days postinfection. Total RNA was extracted from splenocytes, and *IFN-γ*, *IL-4*, and *HPRT* gene expression were analyzed by RT-PCR. *Lane M*, 100-bp DNA ladder. *B*, Splenocytes were isolated from DR-BB rats ($n = 4$/group) treated with PBS and KRV/poly(I:C) (KRV + PBS) or AG and KRV/poly(I:C) (KRV + AG) 7 days postinfection. Total RNA was extracted from splenocytes, and cytokine and chemokine receptor gene expression was analyzed by real-time RT-PCR. Values were normalized against HPRT and expressed as the fold difference between genes. *, $p < 0.005$; **, $p < 0.0005$, compared with the KRV + PBS group. Error bars represent SEM ($n = 4$).

found that AG-treated, KRV/poly(I:C)-injected rats showed a significant decrease in the expression of Th1-type chemokine receptor genes *CCR5*, *CXCR3*, and *CXCR4*, compared with PBS-treated, KRV/poly(I:C)-injected DR-BB rats (Fig. 3*B*). In contrast, AG treatment did not change the expression of *CCR4*, a Th2-type chemokine receptor (Fig. 3*B*). Collectively, these results suggest that inhibition of iNOS activity results in a decrease primarily in the Th1 immune response in KRV/poly(I:C)-injected DR-BB rats.

To determine whether the diminished Th1 immune response was a result of inhibiting an increase in the Th1-type cell population, we examined the proportion of Th1-like $CD4^+CD45RC^+$ T cells in splenocytes of the infected DR-BB rats treated with AG by

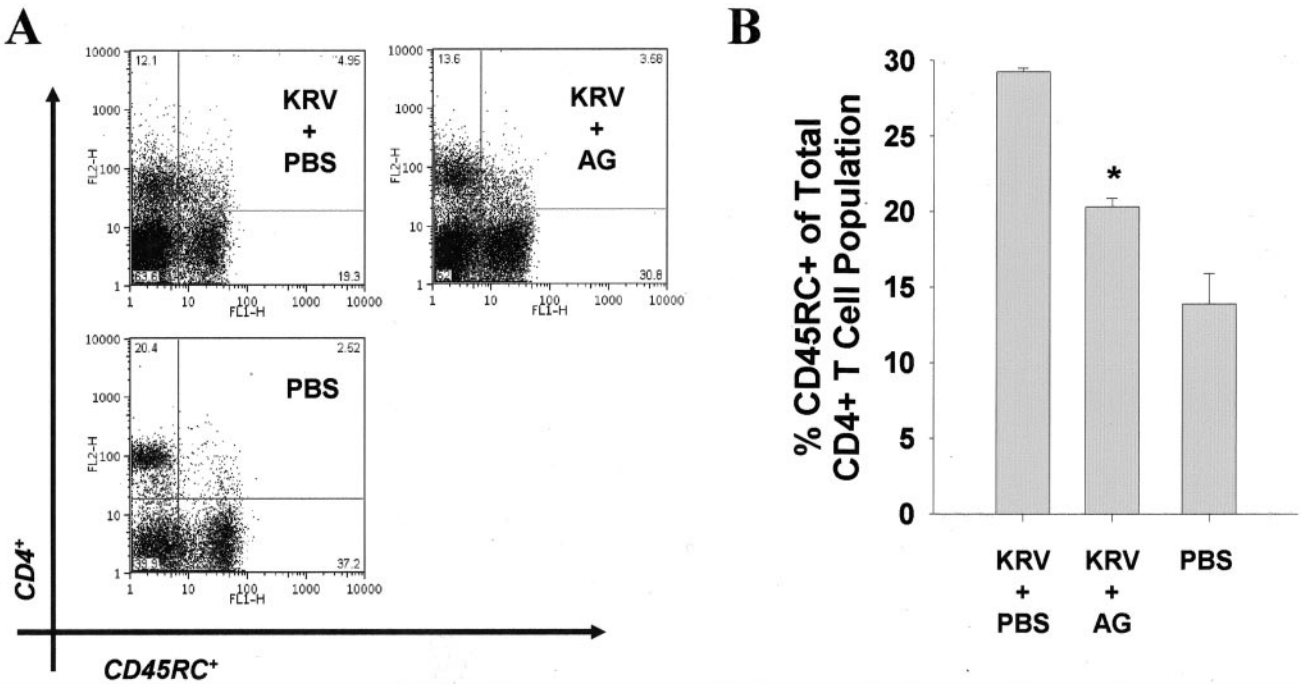

FIGURE 4. iNOS activity is required for the amplification of the Th1-type $CD4^+CD45RC^+$ T cell population in KRV-infected DR-BB rats. *A*, Splenocytes were isolated from DR-BB rats ($n = 2$–4/group; representative data from one animal shown) treated with PBS and KRV/poly(I:C) (KRV + PBS), AG and KRV/poly(I:C) (KRV + AG), or PBS alone at 3 days postinfection. A total of 1×10^6 cells was stained with anti-CD45RC Ab and anti-CD4 Ab. A minimum of 16,500 cells per sample were analyzed by FACS. The percentage of labeled cells of the total cells gated is shown. *B*, The percentage of $CD45RC^+$ T cells in the $CD4^+$ T cell population was calculated using the following equation: total percentage of $CD45RC^+$ T cells = percentage of $CD4^+CD45RC^+$ cells/(percentage of $CD4^+CD45RC^+$ cells + percentage of $CD4^+CD45RC^-$ T cells) × 100. *, $p < 0.01$, compared with the KRV + PBS group. Error bars represent the SEM ($n = 2$–4).

FACS analysis. KRV infection of DR-BB rats resulted in an increase of the Th1-type $CD4^+CD45RC^+$ T cell population compared with uninfected controls (Fig. 4). AG treatment of KRV/poly(I:C)-injected DR-BB rats prevented the amplification of the Th1-type $CD4^+CD45RC^+$ T cell population. These results indicate that inhibition of iNOS prevents the selective activation of Th1-type $CD4^+$ T cells that occurs as a result of KRV/poly(I:C) infection in DR-BB rats.

Inhibition of iNOS activity decreases the interaction between macrophages and T cells and attenuates T cell activity

To determine the effect of iNOS inhibition on the interaction between macrophages and T cells, we examined the expression of MHC class II molecules in macrophages, which are involved in Ag presentation to T cells, and the expression of IL-2Rα in T cells, an indicator of T cell activation (19), in KRV/poly(I:C)-injected DR-BB rats with or without AG treatment. We found that KRV/poly(I:C) injection significantly increased the expression of MHC class II molecules on $OX\text{-}42^+$ cells (macrophages) (Fig. 5*A*) and the expression of IL-2Rα on $OX19^+$ cells (T cells) (Fig. 5*B*) compared with uninfected controls. In contrast, AG treatment of KRV/poly(I:C)-injected rats significantly inhibited the expression of both MHC class II molecules and IL-2Rα compared with KRV/poly(I:C)-injected rats (Fig. 5). Taken together, these results suggest that inhibition of iNOS activity results in a decreased interaction between macrophages and T cells. This may lead to the prevention of T cell activation.

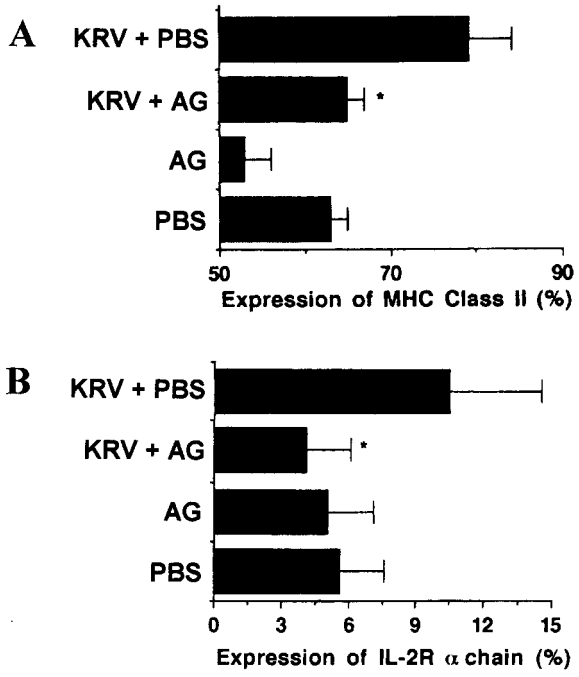

FIGURE 5. Inhibition of iNOS reduces the interactive capabilities of macrophages and T cells in KRV-infected DR-BB rats. Splenocytes were isolated from DR-BB rats (n = 3/group) treated with PBS, AG, KRV/poly(I:C) and PBS (KRV + PBS), or KRV/poly(I:C) and AG (KRV + AG) at 3 and 7 days postinfection. *A*, For macrophage MHC class II expression analysis at 3 days postinfection, the splenocytes were labeled with PE-conjugated OX-42 mAb and FITC-conjugated OX-6 mAb, and analyzed with by FACScan flow cytometer. *B*, For T cell IL-2Rα analysis at 7 days postinfection, the splenocytes were labeled with FITC-conjugated OX-39 mAb and PE-conjugated OX-19 mAb, and analyzed by FACScan flow cytometer. The percentage of positive MHC class II and IL-2Rα cells is shown on the gate of $OX\text{-}42^+$ cells and T cells, respectively. *, $p < 0.05$ compared with the KRV + PBS group.

To determine whether inhibition of iNOS activity affects the population of $CD8^+$ T cells, which are known to be cytotoxic effectors for KRV-induced diabetes, we examined the population of $CD8^+$ T cells in KRV/poly(I:C)-injected DR-BB rats with or without AG treatment at 7 days after KRV infection. We found that AG treatment resulted in a significant decrease in the $CD8^+$ T cell population compared with PBS-treated, KRV/poly(I:C)-injected control DR-BB rats (Fig. 6*A*). To examine whether inhibition of iNOS activity inhibits the cytolytic activity of $CD8^+$ T cells, we performed a ^{51}Cr release assay. We isolated $CD8^+$ T cells from KRV/poly(I:C)-injected DR-BB rats with or without AG treatment at 7 days after KRV infection, incubated them with ^{51}Cr-labeled, KRV-infected NRK cells as target cells, and measured the specific lysis. We found that target cells incubated with $CD8^+$ T cells from KRV/poly(I:C)-injected rats treated with AG showed 5% lysis, whereas those incubated with $CD8^+$ T cells from KRV/poly(I:C)-injected rats treated with PBS showed 42% lysis (Fig. 6*B*). These results indicate that iNOS inhibition decreases not only the $CD8^+$ T cell population, but also the activity of $CD8^+$ T cells.

Inhibition of iNOS activity prevents KRV-induced autoimmune diabetes in DR-BB rats

To determine whether inhibition of iNOS activity prevents KRV-induced autoimmune diabetes in DR-BB rats, we treated the rats with AG at 12 and 24 h before infection with KRV and every 12 h thereafter for 24 days. All PBS-treated, KRV/poly(I:C)-injected rats (six of six) developed diabetes by 16 days postinfection, whereas only two of nine (22%) of AG-treated, KRV/poly(I:C)-treated DR-BB rats became diabetic by 24 days postinfection (Fig. 7*A*).

To determine whether the inhibition of iNOS activity prevented the development of insulitis, the islets were examined. More than 40% of the examined islets remained intact, and ~30% of the islets showed early- or intermediate-stage insulitis in AG-treated, KRV/poly(I:C)-injected DR-BB rats. In contrast, ~50% of the examined islets from PBS-treated, KRV/poly(I:C)-injected DR-BB rats showed end-stage insulitis, and >30% of the islets showed late-stage insulitis (Figs. 7, *B* and *C*). This suggests that the inhibition of iNOS results in the prevention of diabetes and reduces the severity of lymphocyte-mediated insulitis in KRV-infected DR-BB rats.

Discussion

KRV can induce autoimmune type 1 diabetes in DR-BB rats without directly infecting pancreatic β cells (3, 8). Macrophages and T cells have been shown to be involved in the destruction of β cells in KRV-infected DR-BB rats. Inactivation of macrophages by liposomal dichloromethyl diphosphonate, which selectively induces apoptosis in macrophages, results in the near-complete prevention of insulitis and diabetes in KRV-infected DR-BB rats (5). Cytokines produced from activated macrophages were shown to create the immune environment for the activation of effector T cells that destroy β cells, resulting in the development of autoimmune diabetes in NOD mice (20) and DR-BB rats infected with KRV (5). In addition to cytokines such as IL-12, IL-1, TNF-α, and IFN-γ, activated macrophages also produce other soluble mediators such as NO.

NO is known to be an effector molecule released by islet-infiltrating, activated macrophages or monocytes that can induce the destruction of β cells (21). The production of NO within islet lesions was found to be correlated with an increase in the number of Fas molecules that are recognized by islet-infiltrating T cells expressing Fas ligand, which then allows the transfer of Fas signals, eventually resulting in β cell apoptosis (22, 23). In addition

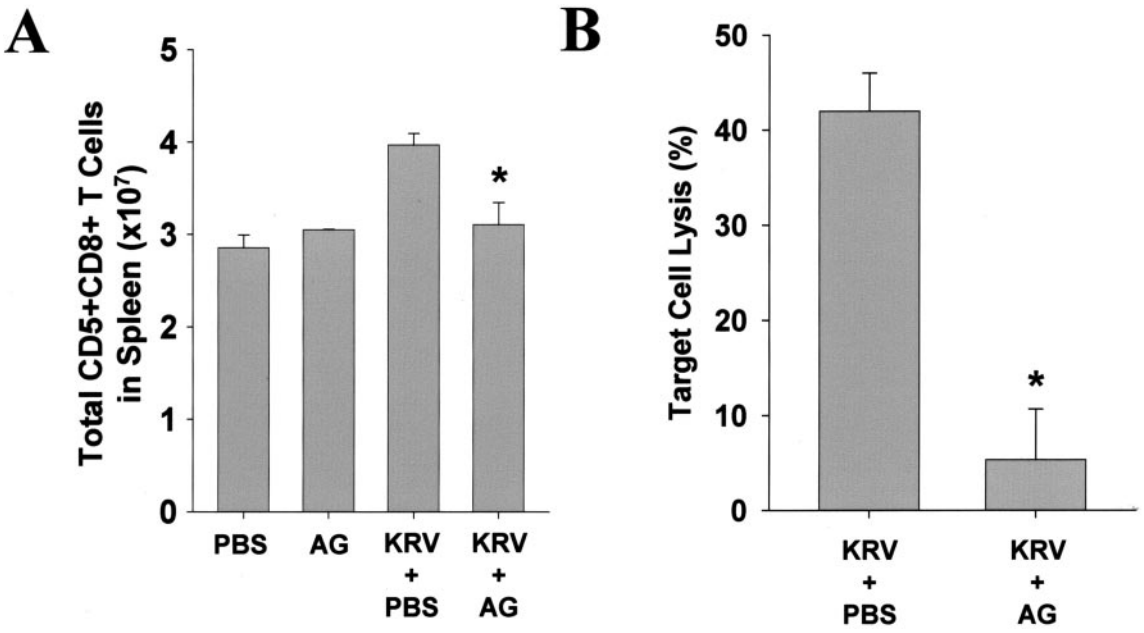

FIGURE 6. Inhibition of iNOS attenuates the cytotoxic activity of $CD8^+$ T cells in KRV-infected DR-BB rats. Splenocytes were isolated from DR-BB rats ($n = 3$/group) treated with PBS alone, AG alone, KRV/poly(I:C) and PBS (KRV + PBS), or KRV/poly(I:C) and AG (KRV + AG) at 7 days after infection. *A*, A total of 1×10^6 cells was stained with anti-CD8 Ab and anti-CD5 Ab and analyzed by FACS. The number of $CD5^+CD8^+$ T cells was then determined. Error bars represent SEM. *, $p < 0.05$ compared with the KRV + PBS group. *B*, $CD8^+$ T cells (2×10^5 cells/200 μl/well) were purified and incubated with ^{51}Cr-labeled, KRV-infected NRK cells (1×10^4 cells/well) for 6 h, and the specific cell lysis was determined. Error bars represent SEM. *, $p < 0.05$, compared with the KRV + PBS group.

to the role of NO as an effector molecule in the destruction of β cells, NO has been shown to play another role as a modulator of immune responses (10, 11).

One enzyme responsible for the production of NO is iNOS, which is expressed in islet-infiltrating macrophages as well as by islet cells (24). The expression of iNOS mRNA in the islets was found to be closely correlated with mRNA expression of proinflammatory cytokines such as IL-1β and IFN-γ (24). Rats treated with iNOS inhibitor and iNOS-knockout mice showed alleviated liver injury during hemorrhagic shock, due to down-regulation of IL-6 and G-CSF mRNA levels in vivo, indicating that NO is essential for the up-regulation of the inflammatory response (11). In addition, iNOS is required for IL-12 signaling in innate immunity (10), and the production of IL-12 promotes the activation of Th1-type $CD4^+$ T cells by the selective expression of IL-12Rs (25). Thus, iNOS activation is thought to play an important role in the generation of the Th1 immune response through IL-12 signaling in adaptive immunity.

In this study, we first asked whether KRV could induce the expression of iNOS and production of NO in macrophages from DR-BB rats. We infected macrophages isolated from DR-BB rats with KRV and examined the production of NO. Complete virions were not packaged and released in KRV-infected macrophages (data not shown), suggesting that KRV cannot replicate in macrophages of DR-BB rats. We found that KRV-infected macrophages clearly produced a significant amount of NO. It is known that iNOS expression can be induced by IFN-γ (26); therefore, it is possible that KRV indirectly induces iNOS expression via IFN-γ produced by KRV-infected macrophages, rather than by direct induction of iNOS. To examine this possibility, we measured NO production in KRV-infected macrophages in the presence of anti-IFN-γ Ab in vitro, and found that removal of IFN-γ did not significantly alter the level of NO production, indicating that KRV can directly induce NO production in macrophages from DR-BB rats. We further found that KRV can directly induce cytokine and chemokine gene expression in isolated macrophages.

We previously found that proinflammatory cytokines from activated macrophages play an important role in KRV-induced diabetes (5), and it was shown that NO is required for the expression of proinflammatory cytokines (11). Second, we asked whether NO is required for the induction of proinflammatory cytokines in KRV-infected DR-BB rats. We infected DR-BB rats with KRV/poly(I:C), treated them with AG, an iNOS inhibitor, and examined the expression of proinflammatory cytokine mRNA in the splenocytes. We found that the expression of IL-12, IL-1β, and TNF-α mRNA was significantly decreased by AG treatment, indicating that NO is required for the induction of these proinflammatory cytokines in KRV-infected DR-BB rats.

In our previous study, we found that KRV infection resulted in the up-regulation of Th1-type $CD4^+$ T cells, which are involved in the destruction of pancreatic β cells (6). In addition, it was recently found that NO can selectively enhance the differentiation of Th1 cells, but not Th2 cells (27). Third, we asked whether NO plays a role in the regulation of the KRV-induced immune response. We injected DR-BB rats with KRV/poly(I:C), treated them with AG, and examined the expression of IFN-γ (a Th1-type cytokine) and IL-4 (a Th2-type cytokine) mRNA. We found that AG treatment significantly decreased Th1-type cytokine gene expression, but not Th2 cytokine gene expression. It is known that Th1- and Th2-type cells have different chemokine receptor profiles (28–31); therefore, we further analyzed the expression of CCR5, CXCR3, and CXCR4 (for Th1) and CCR4 (for Th2) in splenocytes from KRV/poly(I:C)-infected DR-BB rats treated with or without AG. We found that KRV/poly(I:C) injection resulted in the significant induction of Th1 chemokine receptor gene expression (*CCR5*, *CXCR3*, and *CXCR4*), but did not induce Th2 chemokine receptor gene expression (*CCR4*). However, inhibition of iNOS by AG

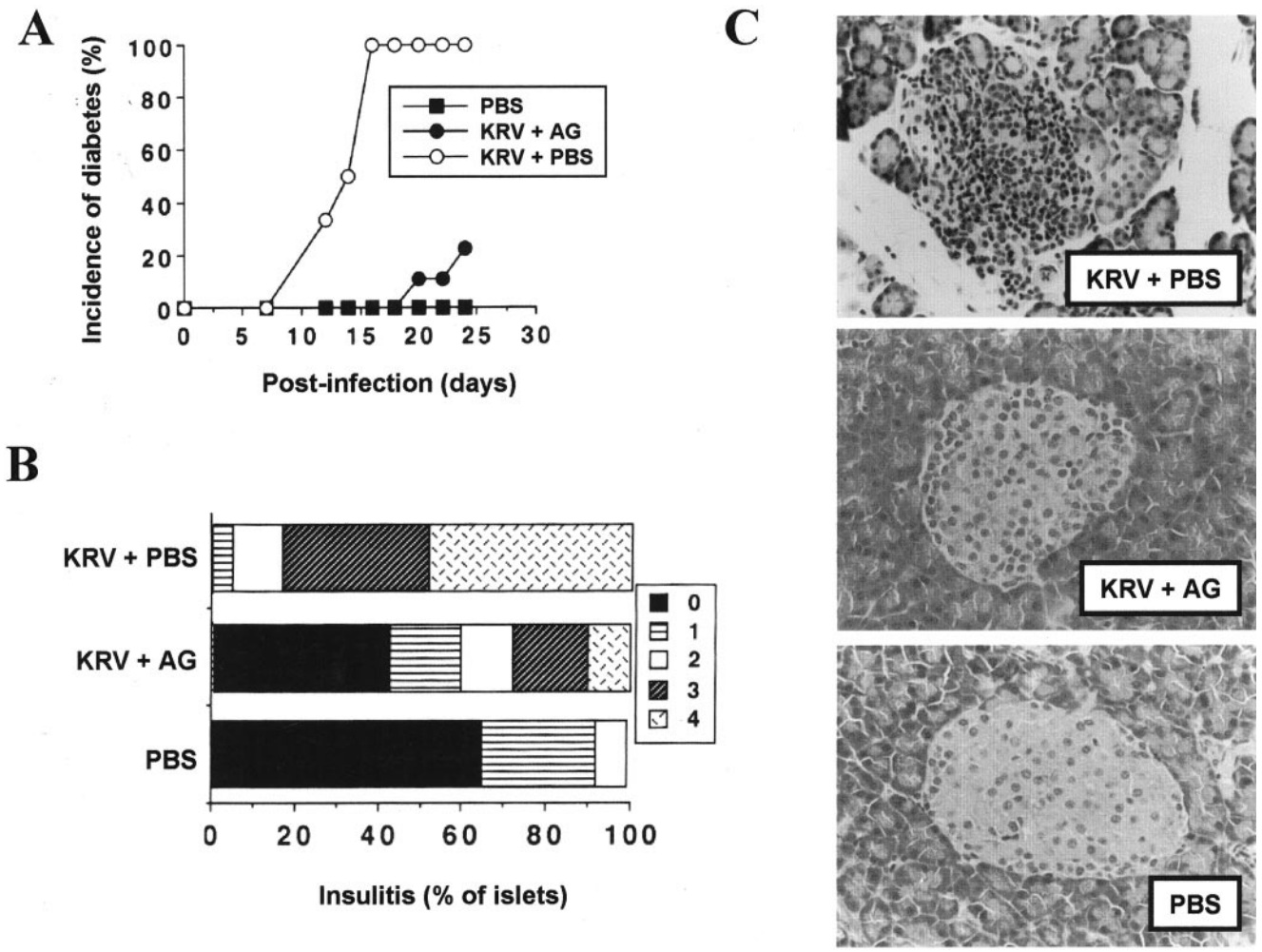

FIGURE 7. iNOS activity is required for the induction of diabetes in KRV-infected DR-BB rats. *A*, KRV (3×10^5 $TCID_{50}$) was administered to 21- to 25-day-old DR-BB. Poly(I:C) (5 μg/g of body weight) was administered every 24 h for 3 days postinfection. AG or PBS, as a control, was administered 12 and 24 h before KRV infection and every 12 h thereafter. The development of diabetes was monitored for 4 wk by urine and blood glucose analysis. *B*, Animals were sacrificed 4 wk postinfection, and 25–30 islets per nondiabetic rat that was treated with KRV/poly(I:C) and PBS (KRV + PBS), KRV/poly(I:C) and AG (KRV + AG), or PBS (n = 4/group), were examined. The insulitis score was determined according to the following categories: 0, normal islets; 1, early insulitis; 2, intermediate insulitis; 3, late insulitis; and 4, end-stage insulitis. *C*, Photomicrographs of representative pancreatic islets from rats treated with KRV/poly(I:C) and PBS (KRV + PBS), KRV/poly(I:C) and AG (KRV + AG), or PBS.

treatment resulted in the suppression of Th1-type chemokine receptor gene expression. We previously found that the $CD4^+CD45RC^+$ Th1-type cell population was significantly upregulated in KRV-infected DR-BB rats (6). Thus, to determine whether inhibition of iNOS results in a decrease in the Th1-type T cell population, we examined the proportion of Th1-like $CD4^+CD45RC^+$ T cells in the spleen of KRV/poly(I:C)-injected, AG-treated DR-BB rats by FACS analysis. We found that AG treatment resulted in the selective decrease of Th1-like $CD4^+CD45RC^+$ T cells. Taken together, these results indicate that NO plays a critical role in the regulation of the Th1 immune response.

Fourth, we asked whether NO affects the activation of T cells in KRV-infected DR-BB rats. The expression of MHC class II molecules was shown to be dependent on the expression of proinflammatory cytokines such as IFN-γ and TNF-α (32). Thus, we hypothesized that the decreased expression of proinflammatory cytokines by inhibition of iNOS expression may result in a decrease in the expression of MHC class II molecules. We examined the expression of MHC class II molecules by FACS analysis in KRV/poly(I:C)-injected DR-BB rats and found that KRV infection resulted in the increase of the MHC class II-expressing macrophage population, and treatment with AG inhibited this increase. We then examined the expression of IL-2Rα on T cells, a marker of T cell activation. We found that the population of IL-2Rα-expressing T cells in AG-treated, KRV/poly(I:C)-injected DR-BB rats was reduced to the level of the uninfected control group. These results suggest that inhibition of iNOS in KRV/poly(I:C)-infected DR-BB rats results in the prevention of MHC class II production, which subsequently prevents T cell activation. To further investigate whether iNOS inhibition in KRV-infected DR-BB rats truly prevents T cell activation, we examined the $CD8^+$ T cell population and its cytolytic activity in AG-treated, KRV/poly(I:C)-injected DR-BB rats. We found that AG treatment significantly decreased the number of $CD8^+$ T cells and their activity, confirming that iNOS inhibition prevents T cell activation.

Finally, we asked whether AG treatment can prevent the development of insulitis and diabetes in KRV/poly(I:C)-injected DR-BB rats. Our results revealed that inhibition of NO production by AG treatment resulted in a significant decrease in the incidence of diabetes and development of insulitis compared with PBS-treated, KRV/poly(I:C)-injected DR-BB rats. There are several

possible mechanisms for the destruction of pancreatic β cells by NO. Proinflammatory cytokines, possibly involving NO, can induce Fas expression in β cells and generate apoptotic signals, leading to β cell death (22). NO can inhibit DNA synthesis and damage β cell mitochondria by inactivating specific iron-sulfur-containing enzymes (33, 34). In addition, NO may be involved in the regulation of the Th1 immune response, resulting in the amplification of β cell CTLs that contribute to the destruction of β cells.

In conclusion, KRV can induce the production of NO in macrophages of DR-BB rats, and KRV-induced NO plays a critical role in the up-regulation of the Th1 immune response and activation of effector T cells leading to autoimmune type 1 diabetes in KRV-infected DR-BB rats. In contrast, suppression of NO production by treatment with AG, an iNOS inhibitor, results in the down-regulation of the Th1 immune response and cytotoxic T cell activity, leading to the prevention of autoimmune diabetes in KRV-infected DR-BB rats.

Acknowledgments

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