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Polyinosinic-Polycytidylic Acid Treatment of Friend Retrovirus-Infected Mice Improves Functional Properties of Virus-Specific T Cells and Prevents Virus-Induced Disease

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During viral infections, the initial response of the immune system is the induction of type I IFN, which mediates an antiviral, antiproliferative, and immunomodulatory activity. Infected cells produce type I IFN, which acts in an autocrine or paracrine manner to signal the presence of a viral infection and induces cell-intrinsic apoptosis (1, 2). The induction of type I IFN leads to the expression of several hundred IFN-stimulated genes that form the antiviral state in virus-harboring cells (3). Some of these genes code for antiviral proteins, including protein kinase R (PKR), 2'-5'-oligoadenylate synthetases, and Mx proteins. PKR inhibits the initiation of translation (4), whereas 2'-5'-oligoadenylate synthetases activates the degradation of viral RNA (5). Apart from the antiviral activity, type I IFN also has immunomodulatory effects. It stimulates NK and CD8+ T cells, and induces the maturation of APCs. Type I IFN induces the production of IL-15 by dendritic cells (DCs), which are critical in proliferation and maintenance of NK cells (6). It is known from lymphocytic choriomeningitis virus infection that type I IFN also plays a pivotal role in the activation of virus-specific T cells, because CD8+ T cells require type I IFN for an optimal clonal expansion (7). It has also been shown to facilitate cross-presentation of viral Ag to CD8+ T cells by DCs (8).

Most viruses have evolved strategies to overcome the host immune response. One common mechanism is the suppression of the type I IFN response by the virus, showing how important this response is for antiviral immunity (9). Vaccina virus encodes multiple genes that antagonize with the action of type I IFN (10). Also, other viruses like influenza A virus (11, 12), rotavirus (13), rabies virus (14), Ebola virus (15), HSV type 1 (16), Epstein–Barr virus (17), hepatitis C virus (18–22), and adenovirus (23–25) have evolved means to downregulate the IFN response at different phases of infection. During retroviral infections, such as HIV, SIV, or Friend retrovirus (FV), the type I IFN response is quite weak. It was shown that during primary HIV infection, type I IFNs are barely detectable because of a decrease of plasmacytoid DCs (26), the most important IFN-α producers. This was also observed in chronic HIV infections (27). In SIV infections, a transient peak of IFN-α was detectable in the blood and lymph nodes of rhesus macaques, but the viral burden was not significantly suppressed by the IFN-α levels (28). In previous studies of our group we could show that during FV infection of mice, IFN-α protein was not detectable in the serum (29, 30). It was also found in in vitro studies that specific DC subsets can activate HIV-specific T cells by the induction of type I IFN or IL-12p70 (31), and a stimulation with the TLR3/MDA5 ligand polyinosinic-polycytidylic acid [poly (I:C)] can inhibit HIV replication in vitro, which is mediated by type I IFNs (32). In vivo studies of type I IFN in HIV-infected...
patients showed controversial results from disease protection to progression. A beneficial effect of type I IFN in the asymptomatic phase of HIV-1 infection was observed by stabilizing CD4+ T cells, but this was not confirmed in later phases of the infection (33, 34). Other studies suggest that during HIV-1 infection, an unresponsiveness to type I IFN occurs, as the expression of the receptor of IFN-α/β was decreased during infection (35). The underlying mechanisms wherefore the type I IFN response cannot control retroviral infections or how retroviruses suppress type I IFN responses are still unclear.

In this study, we developed a therapeutic approach that caused a strong induction of type I IFN during acute retroviral infections and consequently augmented the initial immune response. For that purpose, poly(I:C), an artificial TLR3/MDA5 ligand, was used because it was already described as one of the most potent inducers of type I IFN (36). Poly(I:C) mimics dsRNA and is recognized by TLR3 in the endosomal compartment of specialized cells or by MDA5 that is expressed in the cytosol of all somatic cells (37). The binding of poly(I:C) to its receptor leads to the induction of type I IFN, IL-12p70, inflammatory cytokines like IL-1β, IL-6, and TNFα, and various chemokines like RANTES or MIP-1β (38).

Until now, several in vivo and in vitro studies with poly(I:C) were performed to treat viral infections. However, little is known about the antiviral effect of poly(I:C) in retroviral infections (39–43). In addition, none of the previous studies in any infection model investigated the underlying mechanisms of the antiviral activity of poly(I:C) in vivo. It is especially not known whether the poly(I:C)-induced type I IFN acts by inducing antiviral enzymes or by its immunomodulatory properties. In this study, we used the FV model to develop a therapeutic approach that induces type I IFN responses during an acute retroviral infection. The FV complex is composed of two retroviruses: the replication-competent helper virus called Friend murine leukemia virus (F-MuLV), which is nonpathogenic in adult mice; and the replication-defective, pathogenic spleen focus-forming virus (44). The induction of adult mice with the FV complex can induce acute splenomegaly because of rapid polyclonal erythroblast proliferation, which is followed by the development of a lethal erythroleukemia at 3 to 4 wk postinfection (wpi) (45).

The aim of this study was the induction of type I IFN during an acute retroviral infection, which resulted in direct antiviral and immunomodulatory effects, and we report on the mode of antiviral action of type I IFN during retroviral infections.

Materials and Methods

Mice and virus

Three- to 6-mo-old female (B10.A × A.BY) F1 mice (H-2b) and C57BL/6 mice were used for the experiments. Experiments were also done with IFNAR−/− (46) (kindly provided by Dr. K. S. Lang, Heinrich Heine University, Düsseldorf, Germany) and MDA5−/− mice (2) (kindly provided by Dr. W. Barchet, University Bonn, Bonn, Germany). Additional experiments to investigate the role of TLR3 in poly(I:C) therapy during acute FV infection were performed with TLR2−/− (47, 48) (kindly provided by Prof. Dr. C. J. Kirschning, University of Duisburg-Essen, Essen, Germany), because only these double-knockout mice were available at the time. However, TLR2 should not play any role in the recognition of poly(I:C) and viral infections. The B-tropic, polyclonally-inducing FV complex used in all experiments was taken from uncloned virus stocks obtained from 15% spleen cell homogenates as described previously (49). The progression of disease was monitored by spleen weights and virus assays as indicated.

In all experiments, (B10.A × A.BY) F1 mice were injected intravenously with 0.5 ml PBS containing 5000 spleen focus-forming units of the FV complex. C57BL/6, IFNAR−/−, MDA5−/−, and TLR2−/−/TLR3−/− mice were injected intravenously with 0.5 ml PBS containing 20,000 spleen focus-forming units of the FV complex. The used stock was not contaminated by lactate dehydrogenase-elevating virus.

Poly(I:C) treatment

Mice were injected i.p. twice with 125 µg poly(I:C) (Invivogen, Toulouse, France) diluted in 500 µl sterile H2O. Treatment was performed on 4 and 8 d postinfection (dpi). Control mice were injected i.p. with 500 µl sterile H2O. Ten dpi, the mice were sacrificed and analyzed for disease progression and viral loads.

IFN-α concentration in the sera

The IFN-α concentration of (B10.A × A.BY) F1 mice treated with 500 µl of 125 µg poly(I:C) at 4 and 8 dpi was determined. Sera were harvested at 24 h after poly(I:C) injections. Cytokine concentrations were measured by an IFN-α ELISA (PBL Biomedical Laboratories, Piscataway, NJ). Six mice per group were analyzed.

Detection of virus-infected cells in the spleen

Infectious centers (ICs) from spleens were detected by 10-fold dilutions of single-cell suspensions onto Mus dunni cells. Cultures were incubated for 3 d, fixed with ethanol, stained with F-MuLV envelope-specific Ab 720, and developed with peroxidase-conjugated goat anti-mouse Ab and amniotylocarbazol to detect foci (50).

Palpation for splenomegaly

FV disease progression was followed weekly by spleen palpation of each individual mouse under general anesthesia, a standard procedure used to follow progression of FV infection as previously described (49). Palpations were done in a blinded manner, and splenomegaly was rated on a scale from 1 to 4, with 1 being normal, 2 being moderately splenomegaly (three to five times enlarged), and 3 and 4 being severely splenomegaly (weighting 10–20 times a normal spleen).

B lymphocyte cultures

Mice were infected with FV, and 10 dpi they were sacrificed, and the spleens were removed and homogenized. The single-cell suspensions were stained with allophycocyanin-labeled anti-CD19 (1D3), and the CD19+ B cells were sorted by FACS Aria (BD Biosciences, San Jose, CA). B cells were cultured in RPMI 1640 supplemented with 10% FCS, 500 nM 2-ME, and 100 U/ml penicillin/streptomycin for 2 d. Cells were incubated with 100 µg/ml poly(I:C) or remained unstimulated for up to 2 d. Every day, 1 × 106 cells were harvested and resuspended in TRIzol Reagent (Invitrogen, Paisley, U.K.) to isolate RNA.

RNA isolation

Total RNA was isolated from total spleen cells utilizing TRIzol Reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany) including a DNase digestion step (DNase-free DNase set; Qiagen). Isolated RNA was dissolved in 40 µl RNase-free water and stored at −80°C.

Real-time-PCR

Real-time PCR (RT-PCR) analysis to quantify FV infection levels of cells was performed as described previously (51). RT-PCR for detection of OAS1a and PKR mRNA was performed using Qiagen One Step RT-PCR kit and Qiagen Quanti Tect Primer assay for OAS1a and PKR. The quantitative mRNA levels were performed by using StepOne Software v2.0 (Applied Biosystems, Carlsbad, CA) and were normalized to β-actin mRNA expression level.

Lymphocyte depletion

Mice were injected i.p. with 0.5 ml supernatant fluid containing CD8-specific mAb 169.4. NK cells were depleted by injection of 50 µg NK1.1-specific Ab (BD Pharmingen, San Diego, CA). For depletion of CD4+ T cells, mice were injected with 0.5 ml supernatant fluid obtained from hybridoma cells producing the CD4-specific mAb YTS 191.1. Mice were injected every other day, for five times, starting on the day of FV infection. The treatment depleted more than 95% of the targeted cells in all investigated organs (at 10 dpi).

Tetramers and tetramer staining

For quantification of virus-specific CD8+ T cells, 1 × 106 nucleated spleen cells were stained with eFluor 450-labeled anti-CD8 (Ly-2), FITC-labeled anti-CD43 (1B11) and PE-labeled MHC class I H2-D8 tetramers specific for the FV Gag epitope (52) for 30 min at room temperature. For detection of virus-specific CD4+ T cells, 1 × 106 nucleated spleen cells were stained with 1 µl allophycocyanin-labeled MHC class II-Ab tetramer-
specific F-MuLV envelope fn20 (53) for 3 h at 37˚C. Subsequently, cells were stained with FITC-labeled anti-CD11b (M1/70) and Alexa Fluor 700-labeled anti-CD4 for an additional 30 min at 37˚C. Cells were washed twice, resuspended in buffer containing 7-aminoactinomycin D, and analyzed by flow cytometry.

### Cell surface and intracellular staining

Cell surface staining was performed with the following Abs: anti-CD43 (1B11; Biolegend, San Diego, CA), anti-CD4 (RM4-5; eBioscience, San Diego, CA), anti-CD69 (H1.2F3; Invitrogen), anti-CD8 (53-6.7; eBioscience), and anti-CD107a (1D4B; BD Biosciences). Dead cells (7-aminoactinomycin D-positive) were excluded from analysis. Intracellular granzyme B (monoclonal anti-human granzyme B Ab allophycocyanin-conjugated, clone GB12; Invitrogen) staining was performed as described previously (54). Intracellular cytokine stainings were performed as described previously (55, 56) with the following Abs: anti–IFN-γ (XMG1.2; eBioscience), anti–TNF-α (MP6-XT22; eBioscience), and anti–IL-2 (JES6-5H4; eBioscience). Data were acquired on an LSR II flow cytometer (BD Biosciences) from 100,000–300,000 lymphocyte-gated events per sample. Analyses were performed using FACSDiva (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

### In vivo cytotoxicity assay

The in vivo CTL assay was performed as previously described (57). Labeled target cells were transferred into naive, FV-infected or poly(I:C)-treated FV-infected mice at 8 dpi. Poly(I:C) treatment was performed as mentioned earlier. Two hours postinjection of labeled target cells, mice were sacrificed, and spleen and blood were analyzed for killing of target cells.

### Statistical analyses

Statistical analyses and graphical presentations were computed with GraphPad Prism version 5. Statistical differences (p value) were analyzed by unpaired Student t test.

### Results

#### Poly(I:C) induced IFN-α in FV-infected animals

Previous studies of our group showed that the IFN-α response during FV infection is very weak. However, we also demonstrated that endogenous IFN-α has a strong suppressive effect on FV repli-

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**FIGURE 1.** IFN-α induction in FV-infected mice by poly(I:C) treatment. Mice were infected with FV, and at 4 and 8 dpi, the mice were treated with poly(I:C). IFN-α levels were determined in the plasma by ELISA. The means of six mice per group + SEM are shown.

**FIGURE 2.** Effect of poly(I:C) treatment on FV-induced disease. Mice were treated twice with poly(I:C) at 4 and 8 dpi with FV. Ten dpi, disease progression and viral loads were analyzed. Virus-induced disease was documented by spleen weights (A), and viral loads at 10 dpi were measured in the spleen (B) by using an IC assay. To analyze disease progression, we treated mice three times with poly(I:C) at 4, 8, and 12 dpi with FV. At 7 wpi, splenomegaly and viral loads were analyzed. Virus-induced disease was documented over time by palpating the spleen size as an indicator for the severity of splenomegaly (C), and viral loads were measured in the spleen at 7 wpi (D). To analyze the onset of erythroleukemia, we stained splenocytes for expression of Ter119 and analyzed by flow cytometry (E). Thirty mice per group were analyzed during acute infection, and the mean values for each group are indicated by a bar. For the long-term study, four to six mice per group were analyzed. At least two independent experiments were performed. Differences between the untreated control group (FV) and the group of treated mice [FV + poly(I:C)] were analyzed by using the unpaired Student t test. Statistically significant differences between the groups are indicated as follows: ***p < 0.005; ****p < 0.0005.
tion in vitro and in vivo (29). Therefore, we wanted to analyze whether poly(I:C), which is known to be an effective inducer of type I IFN (40), can modulate FV infection in vivo. For this purpose, we infected mice with FV, and at 4 and 8 dpi, half the animals received poly(I:C), whereas the other half received sterile H2O. At 1 d posttreatment, we analyzed the serum for IFN-α protein concentration by ELISA. During FV infection, the TLR3/MDA5 ligand was able to induce high amounts of IFN-α. At 24 h post-poly(I:C) injection, up to 50-fold greater protein concentrations were measured in the serum of treated animals compared with the untreated control animals (Fig. 1). At 5 dpi, a mean concentration of ~1800 pg/ml was detectable after treatment and ~2300 pg/ml at day 9. In FV-infected and untreated animals, the IFN-α protein concentration (mean: 50 pg/ml) was as low as in naive mice. Therefore, a strong induction of IFN-α in FV-infected animals could be observed after treatment with poly(I:C).

**Poly(I:C) prevented FV-induced acute splenomegaly and the development of erythroleukemia**

To analyze the therapeutic effect of poly(I:C) in retroviral infection, susceptible (B10.A X A.BY) F1 mice were infected with FV, and at 4 and 8 dpi, the animals were treated with poly(I:C). At 10 dpi, the mice were sacrificed, and disease progression and viral loads were analyzed. The virus-induced disease was determined by measuring the spleen weights. Treatment with poly(I:C) prevented acute splenomegaly in all mice. The treated animals had 7-fold smaller spleens (mean: 0.23 g; Fig. 2A) compared with the FV-infected control animals, which got H2O injections (mean: 1.65 g). Moreover, the spleen weight of the treated animals was not significantly different from that of uninfected mice (mean: 0.13 g). The treated animals also showed a strong reduction in spleen viral loads (Fig. 2B). At 10 dpi, high viral loads were measured in the spleen of control mice (mean: 3.22 X 10^7 IC/spleen). After the poly(I:C) therapy, a >230-fold reduction in viral loads was found (mean: 1.38 X 10^5 IC/spleen). Thus, a strong antiretroviral effect of poly(I:C) treatment could be observed in the acute phase of FV infection.

To determine whether the animals benefit in the long term from the temporary therapy during acute infection, we infected mice with FV, treated them three times at 4, 8, and 12 dpi with poly(I:C), and analyzed disease progression over 7 wk. Severity of splenomegaly was followed by weekly palpations. At 7 dpi, the mice were sacrificed, and spleen weights and viral loads were analyzed. All control animals developed splenomegaly within 2 wpi (spleen size: 3-4; Fig. 2C) and remained splenomegalyic over the 7-wk observation period. In contrast, the treated animals did not show enlarged spleens during the long-term study (spleen size: 1; Fig. 2C). Poly(I:C) also had a positive effect on viral loads at 7 dpi. In control mice, high viral loads were measured (mean: 9.6 X 10^4 IC/spleen), whereas the therapy resulted in a 100-fold reduction in spleen viral loads (mean: 9.6 X 10^2 IC/spleen; Fig. 2D), demonstrating a long-lasting effect of the poly(I:C) treatment.

During FV infection of susceptible mice, Ter119^+ erythroid precursor cells are transformed by the virus, which results in the development of a lethal erythroleukemia (44). Thus, increased numbers of Ter119^+ cells in FV-infected mice are indicative for leukemia development. The percentages of Ter119^+ erythroblasts in the spleen of untreated and poly(I:C)-treated animals were analyzed at 7 wpi. In treated animals, a low percentage of Ter119^+ cells (4%) was found in the spleen, which was comparable with that of naive animals (5%; Fig. 2E). In FV-infected animals, up to 72% of the splenocytes were positive for Ter119 (Fig. 2E), which demonstrated the development of an erythroleukemia. Taken together, the temporary treatment with poly(I:C) during acute infection induced a long-lasting protection against FV-induced splenomegaly and onset of lethal leukemia. However, treated animals could not completely eliminate virus and developed a chronic infection (Fig. 2D).

**FIGURE 3.** Effect of poly(I:C) on FV-induced disease in mice deficient of IFNAR, MDA5, or TLR3. A, Mice deficient in IFN-α/β (IFNAR^−/−), MDA5^−/−, or TLR3^−/− (B) and wt mice (C57BL/6) were treated twice with poly(I:C) at 4 and 8 dpi with FV. Ten dpi, viral loads were analyzed in the spleen by using an IC assay. At least two independent experiments were performed. Differences between the poly(I:C)-treated animals and control animals were analyzed by using the unpaired Student t test. Statistically significant differences between the groups are indicated as follows: *p < 0.05; **p < 0.005.
effect, we infected mice lacking TLR3 (TLR3−/−) or MDA5 (MDA5−/−) with FV and subsequently treated them with poly(I:C). As control animals, we used untreated knockout mice and FV-infected wt mice (C57BL/6). In both poly(I:C)-treated knockout mouse strains, we found a significant decrease in viral loads compared with untreated control mice. In MDA5−/− mice, the treatment resulted in an almost 100-fold reduction of the viral burden compared with untreated FV-infected knockout mice (mean: 3.1 × 10^5 IC/spleen in untreated and 3.2 × 10^3 IC/spleen in poly(I:C)-treated MDA5−/−; Fig. 3B). This was similar to the effect of poly(I:C) in wt C57BL/6 mice. The mice deficient in TLR3 showed an only 18-fold reduction of the viral loads in the spleen after injection of poly(I:C) compared with untreated TLR3−/− mice (mean: 7.5 × 10^5 IC/spleen in untreated and 4.1 × 10^4 IC/spleen in poly(I:C)-treated TLR3−/−; Fig. 3B).

The data suggest that the TLR3 and the MDA5 pathways can compensate for each other if one of the pathways is missing. However, the MDA5 receptor seems to be less efficient, because only the lack of TLR3 reduced the efficacy of the antiviral effect of poly(I:C).

Poly(I:C) inhibited FV replication in B cells

To investigate the mechanism of the antiretroviral activity of poly(I:C), the TLR3/MDA5 ligand was tested for its ability to suppress FV replication in vitro. Therefore, CD19+ B cells were analyzed because they are a reservoir for FV (58) and they express TLR3 and MDA5, which was verified by RT-PCR (data not shown). In susceptible (B10.A × A.BY) F1 mice, up to 20% of all splenic CD19+ B cells were infected with FV during the acute phase of infection (data not shown). At 10 dpi, CD19+ B cells were isolated by FACS from the spleen of these FV-infected mice. We stimulated 5 × 10^5 cells with poly(I:C) or they remained unstimulated for up to 2 d in cell culture. Every day, cells were harvested and RNA was isolated. The samples were then analyzed for FV-RNA by RT-PCR. As is shown in Fig. 4A, cells incubated with poly(I:C) showed a strong reduction in viral RNA compared with the unstimulated cells. To test the role of type I IFN in poly(I:C)-mediated suppression of FV replication, we used B cells from IFNAR−/− mice. These B cells were not affected by poly(I:C); therefore, no inhibition of FV replication was measured. Because IFNAR−/− mice were on the resistant C57BL/6 background, their B cells had less viral RNA compared with B cells, which were from susceptible (B10.A × A.BY) F1 mice. Using infected B cells, we also analyzed whether the antiviral enzymes OAS1a and PKR were induced by poly(I:C) stimulation. An induction of mRNA for both antiviral enzymes was found in B cells from wt mice stimulated with poly(I:C) (Fig. 4B, black bars). An inhibition of FV replication in vitro and the induction of antiviral enzymes

![FIGURE 4. Antiretroviral activity of poly(I:C) in vitro. (B10.A × A.BY) F1 mice and IFNAR−/− mice were infected with FV. Ten dpi, CD19+ B cells were isolated from the spleen of four infected mice per group by FACS. B cells were stimulated with poly(I:C) for 2 d in cell culture or remained unstimulated. Every day, cells were harvested and total RNA was isolated using TRIzol. Levels of FV-RNA (A), Oas1a mRNA, and PKR mRNA (B) were measured by quantitative real-time PCR. At least two independent experiments were performed and the samples were run in duplicate. Means of four mice per group + SEM are shown. Differences between the groups were analyzed by using the unpaired Student t test. Statistically significant differences between the groups are indicated as follows: *p < 0.05; ***p < 0.0005.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

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by poly(I:C) was demonstrated, requiring the induction of type I IFN.

Depletion experiments suggested that CD4⁺ and CD8⁺ T cells may be involved in the antiretroviral effect of poly(I:C)

Because a direct antiviral effect of poly(I:C) that depended on IFN-α was observed, it was of interest whether also immunomodulatory effects of poly(I:C) were important for the reduced splenomegaly and decreased viral loads in FV-infected mice. To this end, the effector cell populations that were relevant for immunity to FV after poly(I:C) therapy were determined. At day 10, no binding or neutralizing Abs could be found in the serum of infected, poly(I:C)-treated animals (data not shown). This indicated that B cells did not play an important role in poly(I:C) therapy. To analyze components of the cellular immune system, we performed experiments in which NK, CD4⁺ T, or CD8⁺ T cells were depleted during treatment with the ligand. For lymphocyte depletion, specific Abs were injected on the day of infection and every other day thereafter until 8 dpi. At the time points of poly(I:C) treatment (4 and 8 dpi) and at the day of the final examination (10 dpi), the efficacy of depletion was analyzed. At all these time points, at least 99% of the NK cells (NK1.1⁺) and CD4⁺ T cells were ablated. The CD8⁺ depletion resulted in at least 96% reduction of the cells throughout the 10 dpi (data not shown). The depletion of NK cells did not influence the outcome of the therapy, as NK cell-depleted, poly(I:C)-treated mice showed low spleen weights (mean: 0.42 g) and viral loads (mean: 2.96 × 10⁷) comparable with those of nondepleted, poly(I:C)-treated mice (mean spleen weight: 0.32 g; mean viral loads: 8.04 × 10⁷; Fig. 5). This suggests that NK cells were not required for successful poly(I:C) therapy in FV-infected mice. In contrast, the depletion of T cells during poly(I:C) treatment of FV-infected mice resulted in a severe splenomegaly (mean spleen weights of 1.8 g for CD4⁺ T cells and 2.42 g for CD8⁺ T cells; Fig. 5A) and high viral loads (mean IC/spleen 1.55 × 10⁶ for CD4⁺ T cells and 6.86 × 10⁷ for CD8⁺ T cells; Fig. 5B). These results indicate that poly(I:C) has no therapeutic effect in mice lacking CD4⁺ or CD8⁺ T cells, suggesting that these cells might play a role in poly(I:C)-mediated immunity to FV infection. However, the experiments do not finally prove that CD4⁺ or CD8⁺ T cells were required for the therapy because T cell depletion always results in enhanced infection in FV-inoculated mice (Fig. 5B) (59). Thus, other assays were used to confirm the important role of T cells during antiretroviral treatment with poly(I:C).

Poly(I:C) therapy induced functional activation of CD4⁺ T cells

To investigate the role of virus-specific CD4⁺ T cells in poly(I:C) therapy of FV-infected mice in more detail, we analyzed stimulation and function of these cells. We wanted to examine whether an expansion of virus-specific CD4⁺ T cells occurred after poly(I:C) treatment. Thus, class II tetramer staining was performed that labels CD4⁺ T cells that are specific for a F-MuLV envelope epitope (53). No significant increase in the absolute number of virus-specific CD4⁺ T cells could be detected after TLR3/MDA5 ligand treatment of FV-infected mice (Fig. 6A). However, the function of the CD4⁺ T cells was clearly improved after treatment. The most important function of CD4⁺ helper T cells is the production of cytokines. Thus, the expression of IFN-γ, TNF-α, and IL-2 was measured after poly(I:C) therapy, and the numbers of multifunctional cytokine-producing cells in the spleen were determined. Because intracellular cytokine staining could not be combined with tetramer staining, cytokine expression was measured in the population of activated CD4⁺ T cells. As a marker for effector T cells, the activation-associated glycoform of CD43 was used (54, 60). Increased numbers of single cytokine producers in treated mice were observed compared with the untreated control group (Fig. 6B). Approximately 6-fold more TNF-α–producing CD4⁺ T cells were observed in the poly(I:C)-treated mice compared with the untreated control mice. For IL-2 a 4-fold and for IFN-γ a 2-fold increase was found in treated mice. Furthermore, a significant increase in double cytokine producers was detected in the poly(I:C)-treated mice (3-fold more than in control mice; Fig. 6C). Therefore, poly(I:C) therapy did not induce an expansion of virus-specific CD4⁺ T cells but increased the functional properties of activated CD4⁺ T cells.

Poly(I:C) therapy induced functional activation of virus-specific CD8⁺ T cells

The depletion experiments (Fig. 5) suggested that CD8⁺ T cells might be important during the poly(I:C) therapy. Therefore, we wanted to analyze which role virus-specific CD8⁺ T cells play during treatment with the TLR3/MDA5 ligand. First, we checked whether the decrease in viral loads during the therapy might be

![FIGURE 5](http://www.jimmunol.org/)
a result of more virus-specific CD8$^+$ T cells. A class I tetramer staining was performed to label the CD8$^+$ T cells, which were specific for the immunodominant FV GagL epitope (52). During the therapy with poly(I:C), no expansion of virus-specific CD8$^+$ T cells was observed, as no significant difference in the absolute numbers of virus-specific CD8$^+$ T cells between the treated mice and the control mice was found (Fig. 7A). To determine functional properties of CD8$^+$ T cells, we measured intracellular expression of the cytolytic molecule granzyme B in virus-specific CTL. A significantly greater mean fluorescence intensity for granzyme B expression was detected in tetramer$^+$ CD8$^+$ T cells during the therapy with the TLR3/MDA5 ligand, we tested the production of cytolytic molecules, such as granzyme B and antiviral cytokines, like IFN-γ and TNF-α in activated CD43$^+$CD8$^+$ T cells. Treated mice had a 2-fold increase in the absolute numbers of TNF-α–producing activated CD43$^+$CD8$^+$ T cells detected in the poly(I:C)-treated animals (5-fold increase; Fig. 7D) compared with the
control animals. The expression of IFN-γ by CD8+ T cells was very low, but a slight increase in the absolute numbers of virus-specific CD8+ T cells (data not shown). The intracellular expression of granzyme B (GzmB) in FV-specific CD8+ T cells was shown. Flow cytometry was also used to detect intracellular granzyme B or the degranulation marker CD107a in activated CD8+ T cells (CD43+). To measure cytokine production, we measured TNF-α expression in total CD8+ T cells (D). A minimum of five mice per group were analyzed, and the mean value for each group is indicated by a bar. At least two independent experiments were performed. Differences between the untreated control group (FV) and the poly(I:C)-treated mice were analyzed by using the unpaired Student t test. Statistically significant differences between the groups are indicated as follows: *p < 0.05; **p < 0.0005. MFI, mean fluorescence intensity.

Discussion

Therapeutic targeting of the innate immune system by TLR ligands is an effective treatment against cancer, allergies, and also viral infections. Poly(I:C), the ligand for TLR3 and MDA5, is known to be a strong inducer of type I IFN (36). After poly(I:C) treatment, the induced type I IFN leads to an antiviral state in cells that is mediated by antiviral enzymes like PKR or OAS1a (4, 61). Apart from its direct antiviral activity type I IFN has also a modulatory effect on immune cells like DCs, NK cells, or T cells (7, 8, 62–64). Thus, poly(I:C) might be a promising drug candidate for the therapy of retroviral infections.

In this study, treatment with poly(I:C) led to reduced viral loads in FV-infected mice and prevented the onset of a lethal leukemia. As expected, the therapeutic effect of poly(I:C) depended on the induction of type I IFN (Fig. 1). The expression of IFN-α after TLR3 activation induced antiviral enzymes that led to an inhibition of FV replication (Fig. 4). Several other studies with non-retroviruses have shown that poly(I:C) has an antiviral effect in vivo (39–43). An induction of IFN-α and antiviral enzymes was observed, but the effect of the TLR ligand on the virus-specific adaptive immune response was not investigated in any of these studies. In human infections with hepatitis B and C virus, IFN-α is used as a standard treatment. During the first phase of this therapy, IFN-α induces a strong antiviral effect that results in a rapid decline of HBV or HCV viral loads. However, the virus is not completely cleared during this phase of the treatment (65). During the ongoing IFN-α treatment, viral loads decrease slowly, which can result in a total clearance of the virus. This suggests that in the first phase of IFN-α treatment, the rapid decrease in viral loads is mediated by the induction of antiviral enzymes; but in the later phase, the immunomodulatory effects of IFN-α seem to be responsible for virus clearance (66). Thus, type I IFNs have a dual role in antiviral therapy, and this has to be taken into account when investigating the antiviral mechanisms of poly(I:C) therapy.

In this paper, we demonstrate that an immunomodulatory effect of poly(I:C) during FV infection contributed to successful antiretroviral therapy (Figs. 5, 6). Interestingly, poly(I:C) treatment augmented the effector functions of CD4+ and CD8+ T cells but did not increase the overall numbers of virus-specific T cells. In a previous study of our group, we could show that the IFN-α subtype 1 was able to enhance the magnitude of the virus-specific
CD8+ T cell response in FV-infected mice (67), which seems contradictory to our current data. However, other IFN-α subtypes, such as 4 and 9, were also effective in reducing FV loads but did not increase absolute numbers of virus-specific CD8+ T cells (67). The most predominant IFN-α subtype that is produced after poly(I:C) treatment in vivo is IFN-α5 (data not shown), but the influence of this particular subtype on CD8+ T cell activity during FV infection has not been investigated so far. Other reports from studies on the influence of type I IFN on T cell responses support our current findings. It was shown that type I IFN can induce the maturation and the survival of DCs that results in an enhanced bridging between innate and adaptive immune systems (62). From lymphocytic choriomeningitis virus infection it is known that type I IFN plays a pivotal role in T cell activation because it is required for an optimal clonal expansion of CD8+ T cells (7). It has also been demonstrated to facilitate cross-presentation of viral Ag to CD8+ T cells by DCs (8). Therefore, not just the antiviral activity of type I IFN but also its immunomodulatory effects on CD8+ T cells are important for an optimal therapeutic effect against pathogens.

Also for CD4+ T cells, improved functionality was observed after poly(I:C) treatment. It was shown that CD4+ T cells should express a variety of different cytokines, such as IFN-γ, TNF-α, and IL-2, to efficiently clear viral, bacterial, or parasitic infections (68). Studies in other infection models demonstrated that triple cytokine-producing CD4+ T cells had the strongest protective effect against infection (69). We measured a significant increase in single and double cytokine producers after poly(I:C) treatment, but no changes in the absolute numbers of triple cytokine-producing CD4+ T cells. In previous studies of our group, it was already shown that IFN-γ, which was produced by CD4+ T cells during FV infection, had a strong antiretroviral activity (70). Thus, the augmentation of the cytokine response of CD4+ T cells after poly(I:C) treatment likely contributed to the therapeutic effect. This is also supported by the data from the CD4+ depletion experiment shown in Fig. 5. Several direct effects of type I IFN on CD4+ T cells were reported previously. During the induction phase of an immune response, the presence of IFN-α favors the differentiation of CD4+ T cells into Th1 cells (71). In human donors, the stimulation of purified CD4+ T cells with IFN-α led to an induction of IFN-γ and IL-10 (72–74). In addition, a critical effect of type I IFN on clonal expansion of CD4+ T cells was observed in a viral infection (75). Thus, our observations correspond to published results.

In previous studies, our group could show that treatment with TLR9 ligands (CpG-oligodeoxynucleotide class B) induced a decrease in viral loads that was mediated by FV-specific CD8+ T cells (76). Furthermore, with adoptive T cell transfer into chronically FV-infected animals, the CpG treatment could prevent a functional loss of the transferred CD8+ T cells and resulted in the decrease of viral loads (77). Thus, similar therapeutic effects in FV infection with different TLR ligands were observed. Because CpG-oligodeoxynucleotide class B induce low amounts of IFN-α, but in this study the induction of type I IFN by poly(I:C) was key for a successful therapy (Fig. 3A), the antiretroviral effects of TLR9 ligands were most likely independent of type I IFN signaling.

We have shown that TLR3 and MDA5 can compensate for each other in response to poly(I:C), as mice deficient in TLR3 or MDA5 showed decreased FV loads after poly(I:C) injections (Fig. 3B). However, the lack of TLR3 resulted in a reduction of the antiviral effect of poly(I:C) compared with mice deficient in MDA5. In other studies, it was demonstrated that both receptors have distinct roles in response to poly(I:C). For the activation of NK cells, MDA5 seems to be more important than TLR3 (78). This was supported by another study that demonstrated that NK cell activation is TLR3 independent (79). The involvement of TLR3 and MDA5 in poly(I:C)-mediated activation of CD8+ T cells is more controversial. Ngoi and collaborators (79) described that the differentiation of effector CD8+ T cells by poly(I:C) does not require TLR3. Another study demonstrated that both MDA5 and TLR3 are important for an effective Ag-specific CD8+ T cell response during immunization with poly(I:C) and OVA (80). Wang and colleagues (81) showed that during immunization with OVA and poly(I/C), the memory CD8+ T cell responses were enhanced by MDA5 signaling, whereas primary CD8+ T cells were boosted in a TLR3-dependent manner. One explanation for the contradictory results could be the different expression and localization of both receptors on cells of the immune system. MDA5 is ubiquitously expressed, whereas TLR3 is predominantly expressed in APCs (reviewed in Ref. 82). Thus, both receptors may well be involved in the augmentation of the CD8+ T cell response during retroviral infection after poly(I:C) therapy.

 Taken together, poly(I:C) showed strong antiretroviral activity in a mouse model and has therefore the potential for clinical treatment of retroviral infections. In addition, the immunomodulatory effects of the poly(I:C)-induced IFN response suggests that new immunotherapeutic strategies using type I IFN against several viral infections should be developed. For immunotheories, it is crucial to...
clarify the mode of action that mediates the antiviral effects of a ligand.

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Disclosures

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References


