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Arthritogenic Properties of Double-Stranded (Viral) RNA[1]

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Viral infections often lead to arthralgias and overt arthritic states. The inflammatogenic compound of the viruses giving rise to such an outcome has to date not been identified. Because expression of dsRNA is a common feature of all viruses, we decided to analyze whether this property leads to the induction of arthritis. Histological signs of arthritis were evident already on day 3 following intra-articular administration of dsRNA. Arthritis was characterized by infiltration of macrophages into synovial tissue. It was not dependent on acquired immune responses because SCID mice also raised joint inflammation. NF-κB was activated upon in vitro exposure to dsRNA, indicating its role in the induction/progression of arthritis. Importantly, we found that dsRNA arthritis was triggered through IL-1R signaling because mice being deficient for this molecule were unable to develop joint inflammation. Although dsRNA is typically recognized by Toll-like receptor 3, Toll-like receptor 3 knockout mice developed arthritis, indicating that some other receptors are instrumental in the inducing of inflammation. Our results from in vitro experiments indicate that proinflammatory cytokines and chemokines stimulating monocyte influx were readily triggered in response to stimulation with dsRNA. These findings demonstrate that viral dsRNA is clearly arthritogenic. Importantly, macrophages and their products play an important role in the development of arthritis triggered by dsRNA. ***The Journal of Immunology,*** **2004, 172: 5656–5663.**

Various viruses have been implicated in the pathogenesis of reactive arthritides (1). In addition, participation of various viruses in the etiology of rheumatoid arthritis has been suggested (1). However, the nature of the inflammatogenic stimuli in the induction of inflammation by viruses has not been identified.

The existence of viral infection is signaled by the production of dsRNA, which is formed during replication and transcription of all viruses. In contrast, dsRNA is normally not found in uninfected host cells (2). Viral infection often results in the destruction of infected cells, which is mediated either by virus itself or by immune responses. Intracellular mechanisms that inhibit virus replication and enable viral clearance and cell survival seem to exist (3, 4). Most viral infections are associated with strong Th1 immune responses (5). This ususally results in the production of different cytokines (TNF-α, IFNs, and IL-6) and chemokines (macrophage-inflammatory protein-1α (MIP-1α)[3] and monocyte chemoattractant protein-1 (MCP-1)) activating innate immune responses (6). In clinical setting, it leads to arthralgias and overt arthritic states. The inflammatogenic compound of the viruses giving rise to such an outcome has not been identified.

Infection by virus giving rise to endogenous exposure to dsRNA results in recognition of this molecule by Toll-like receptor 3 (TLR3) (6, 7). Ligation of TLR3 leads to the activation of various transcription factors including NF-κB (8–11). Such an activation results in production of proinflammatory cytokines from macrophages through a signaling pathway dependent on an adaptor protein called MyD88 (6).

In the present study, we investigated the possible role of viral dsRNA in arthritis by injecting genuine viral dsRNA and synthetic dsRNA (polyinosonic-polycytidylic acid (poly(IC))), mimicking viral dsRNA into murine knee joints. Our results indicate that viral dsRNA is arthritogenic, because it induces joint inflammation in a healthy host.

Materials and Methods

Mice

Naval Medical Research Institute (NMRI) and BALB/c mice were purchased from B&K Universal AB (Stockholm, Sweden). C^3H Hej and C_3H Hen mice as well as *SCID* mice and their congenic strain CB17 were purchased from M&B (Bomholtvej, Denmark). IL-1R-deficient mice ($B6129S7\text{-}Il1r1^{tm1\,Imx}$) as well as their wild-type controls (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR3-deficient ($TLR3^{-/-}$) mice as well as their wild-type controls ($TLR3^{+/+}$) (6) were originally generated at Yale University School of Medicine. All mice were housed in the animal facility of Department of Rheumatology and Inflammation Research, University of Göteborg. Female mice 6–8 wk of age were used in all the experiments.

RNA preparations

Synthetic and viral dsRNA and ssRNA. Synthetic (viral) dsRNA consisted of double-stranded copolymer, poly(IC), and polyinosonic-uridylic acid. Synthetic ssRNA polyinosinic acid (poly(I)) was purchased from Sigma-Aldrich (Stockholm, Sweden). Each strand contained dI, dC, and dU in precise alternating sequences. dsRNA and ssRNA molecules were dissolved in 1 ml of sterile water and further in PBS to obtain a stock concentration of 1 mg/ml, which was kept in a freezer at −20°C until use. The LPS concentration was 33 pg/10 μg poly(IC). This amount of LPS has been previously documented not to cause joint inflammation (12).

To ensure a double-stranded configuration, dsRNA was kept in a water

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[3] Abbreviations used in this paper: MIP-1α, macrophage-inflammatory protein-1α; KO, knockout; MCP, monocyte chemoattractant protein; poly(I), polyinosinic acid; poly(IC), polyinosonic-polycytidylic acid; TLR, Toll-like receptor.

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bath at +50°C for 30 min and cooled down to room temperature thereafter. Finally, it was diluted with PBS to the required concentration.

Viral dsRNA was purified from *Rotavirus* and was used as the source of genuine dsRNA. Growing of virus and purification procedure is described briefly below.

Virus culture. The MA104 cell line of fetal rhesus monkey kidney cells was used for virus cultivation. Monolayers were grown to confluence in flat-bottom plastic flasks (125 cm^2) in the presence of Eagle's MEM supplemented with 5–10% FCS. Virus suspension was pretreated with trypsin (10 μg/ml inoculum) for 30 min at 37°C, to activate the virus inoculum before cultivation. MA104 monolayers were washed twice with serum-free Eagle's MEM before adsorption of trypsin-activated *Rotavirus* for 1 h at 37°C. After the adsorption step, the medium was removed and replaced by serum-free Eagle's MEM containing 1 μl/ml trypsin. Cell cultures were held at 37°C and inspected daily for development of cytopathogenic effects.

Virus purification. When cytopathogenic effects were observed, the cell cultures were harvested for viral purification by gentle scraping and dispersal of the cells into the medium. Equal volumes (20 ml) of dispersed infected cells and Freon (trichlor-fluorethan; Sigma-Aldrich) were added to plastic Falcon centrifuge tubes, and shaken vigorously for 10 min. The supernatant was pipetted over to another centrifuge tube and treated once more with an equal volume of trichlor-fluorethan with vigorous shaking and centrifuging, as above. Supernatants were pooled and divided into 2 vol. One volume was inoculated as 1-ml aliquots into 9-ml aliquots of nuclisens lysis buffer (bioMerieux, Durham, NC) before storage at −70°C. The second volume of supernatant was subjected to ultracentrifugation at 45,000 rpm, for 21/4 h, to pellet virus (4 tubes of 13 ml). Each pellet was subsequently resuspended in 9 ml of nuclisens lysis buffer for storage at −70°C.

RNA extraction. The semiautomated nuclisens extractor (bioMerieux) was used to prepare viral RNA from the nuclisens lysated according to Booms silica gel method. RNA concentration and purity were assessed by OD spectrophotometry.

Injection protocol

dsRNA and ssRNA were injected intra-articularly at different concentrations in a volume of 20 μl into knee joints of mice of different strains. The contralateral knee joints were always used as a negative control and were injected with PBS alone.

Histopathologic examination

Histopathologic examination of joints was performed after routine fixation, decalcification, and paraffin embedding. Sections were cut and stained with H&E. All the slides were coded and evaluated blindly. Specimens were evaluated with regard to synovial hyperthrophy, pannus formation, and cartilage and subchondral bone destruction (13). The extent of synovitis was judged on an arbitrary scale from 0 to 3. No signs of inflammation represented 0 grade; grade 1 was characterized by mild inflammation with hyperplasia of synovial lining layer. Grades 2 and 3 represented different degrees of inflammation characterized by influx of inflammatory cells scattered throughout the synovial tissue.

Immunochemical examination

The knee joints from five NMRI mice were removed and demineralized by enzymatic procedures detailed previously (14). The demineralized specimens were mounted on cryostat chucks, frozen in isopentane that had been prechilled in liquid nitrogen, and kept at −70°C until cryosectioned. Serial cryosections, 5-μm thick, were stained with a rat mAb to mouse CD11b (Mac-1, clone M1/70), CD3 (clone 17A2; BD PharMingen, San Diego, CA), and followed by incubation with biotinylated secondary Abs, avidin-biotin-peroxidase complexes, and 3-amino-9-ethyl-carbazole containing H_2O_2. All sections were counterstained with Meyer's hematoxylin.

Depletion of immunocompetent cells

Monocyte depletion. Etoposide (Vepesid; Bristol-Myers Squibb, Bromma, Sweden) is a cytotoxic drug known to selectively deplete the monocyte population in mice (15). BALB/c mice were injected with etoposide (12.5 mg/kg, in a volume of 125 μl, s.c. into the groin) on 3 consecutive days before and after intra-articular injection of dsRNA. Control mice received the same volume of PBS.

Neutrophil depletion. mAb RB6-8C5 is a rat IgG2b Ab that selectively binds to and depletes mature mouse neutrophils (16). BALB/c mice were injected i.p. with 1 mg of mAb RB6-8C5, or the IgG rat anti-OVA mAb as a control 2 h before intra-articular injection with dsRNA.

In certain experiments, BALB/c mice were injected with both etoposide and mAb RB6-8C5 to obtain simultaneous depletion of monocytes and neutrophils. All mice from single and double depletion experiments were killed on day 3 after intra-articular injection with poly(IC), and their knee joints were histopathologically analyzed.

Nuclear extract preparation

Cultures of spleen cells (10^7) were stimulated with different concentrations of poly(IC), or LPS, as described above. After 2 h, the stimulation was stopped with ice-cold PBS, and cells were washed, resuspended in 2 ml of hypotonic buffer (pH 7.9, containing 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermin, 1 M dithiotreitol, and proteinase inhibitors (Complete MiniTab; Boehringer Mannheim, Indianapolis, IN)), and homogenized. Following centrifugation at 14,000 × *g* at 4°C for 10 min, the supernatant was removed. The pellet was resuspended in the ice-cold extraction buffer (pH 7.9, 20 mM HEPES, 0.42 M NaCl, 1 M EDTA, 1 mM EGTA, 25% glycerol, 1 M DTT, and proteinase inhibitors).

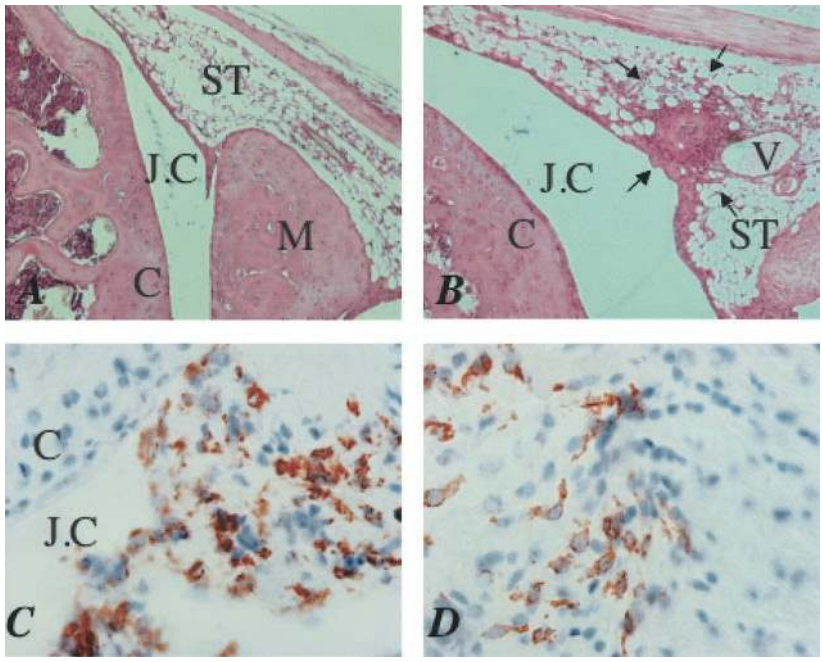

FIGURE 1. Photomicrographs showing the histopathologic and immunhistochemical features of viral dsRNA-induced arthritis. *A*, Normal histologic appearance of a mouse knee joint following injection with control PBS. *B*, Histology of an arthritic knee joint of a mouse 3 days after intra-articular injection with 10 μg of poly(IC). Immunohistochemical analysis of an arthritic knee joint, showing synovial expansion of Mac-1-expressing cells (*C*) and of CD3-expressing cells (*D*) 3 days after intra-articular injection with 10 μg of poly. JC = joint cavity; V = blood vessel; C = cartilage; ST = synovial tissue. Arrow indicates inflammatory cells in the synovium.

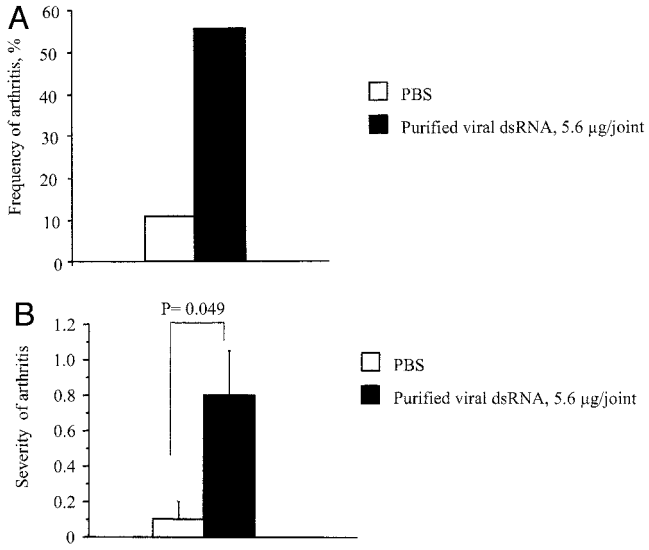

FIGURE 2. Frequency (*A*) and severity (*B*) of arthritis after a single intra-articular injection of purified viral dsRNA (5.6 μg/joint). Mice were killed 3 days after the injection ($n = 9$ per group).

Extraction proceeded at 4°C under continuous rotation for 60 min. The supernatants containing nuclear extracts were collected after centrifugation at 14,000 × *g* for 1 h at 4°C. Protein concentration in the extracts was determined using Bradford reagent (Sigma-Aldrich). Nuclear extracts were aliquoted and stored at −70°C until use.

EMSA

EMSA was performed, as described elsewhere (17), with minor modifications. The sequences for oligonucleotides used for the assay were as follows: NF-κB, sense, 5′-GGCTCAAACAGGGGGCTTTCCCTCCTCAATAT-3′, and antisense, 5′-GGATATTGAGGAGGGAAAGCCCCCTGTTTGAG-3′; AP-1 sense, 5′-GGCTTCCTCCACATGAGATCATGGTTTTCT-3′, and antisense, 5′-GGAGAAAACCATGATCTCATGTGGAGGAAG-3′. Oligonucleotides were annealed at 56°C. The double-stranded product was purified by elution from the electrophoretic gel. Double-stranded oligonucleotides were labeled with [α-^{32}P]deoxynucleotide (Amersham Pharmacia Biotech, Uppsala, Sweden) using Klenow polymerase (5 U/ml; Roche Diagnostic Systems, Somerville, NJ). Binding reactions were performed at room temperature for 20 min. The reaction mixture contained nuclear extract (5–20 μg), 5 μg of poly(dI-dC), 1 mM dithiotreitol, and 1 μl of ^{32}P-labeled double-stranded oligonucleotides (0.1 μg/μl) dissolved in the binding buffer (pH 7.9, 20 mM Tris-HCl, 30 mM NaCl, 5 μM EGTA, 50% glycerol), and supplemented with 0.2 μg/ml BSA.

For competition studies, a 100-molar excess of unlabeled double-stranded oligonucleotides was added to the reaction mixture and incubated for 20 min before the introduction of the ^{32}P-labeled probe. For supershift assays, antiserum to NF-κB p50 (clone C-19; Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with nuclear extracts for another 20 min at room temperature.

Samples containing equal amount of protein were loaded directly onto 2.5% polyacrylamide gel prepared in Tris-borate-EDTA buffer (0.25×), and electrophoresis was performed at 200 V at room temperature. The gel was vacuum dried and exposed to x-ray film for 48 h at −70°C.

Cytokine and chemokine assay

To assess release of cytokines and chemokines in response to dsRNA, murine spleens were obtained aseptically and passed through a nylon mesh. Erythrocytes were depleted by hypotonic lysis. The resulting single-cell suspension was resuspended in Iscove's complete medium (10% FCS, 50 μM 2-ME, 4 mM L-glutamine, and 20 mg/ml gentamicin). Subsequently, 1×10^6 cells/ml were incubated with different concentrations of dsRNA and ssRNA. The cultures were maintained in 24-well plates (Nunc, Roskilde, Denmark) at 37°C in 5% CO_2 and 95% humidity. The supernatants were collected after 3 days for analysis of IL-6, TNF-α, MIP-1α, and MCP-1.

Levels of cytokines and chemokines in supernatants were determined using TNF-α, MIP-1α, and MCP-1 enzyme-linked immunosorbent assay kits from R&D Systems (London, U.K.). The assays were performed as recommended by the manufacturer. The values below the detection limit were considered as zero.

IL-6 levels were measured by a bioassay with cell clone B13.29, subclone B9, which is dependent on IL-6 for growth, as previously described (18). B9 cells were harvested from tissue culture flasks, seeded into microtiter plates (Nunc) at a concentration of 2.5×10^4 cell/ml in Iscove's complete medium. Supernatants of spleen cells stimulated with dsRNA were added. [^{3}H]Thymidine was added after 72 h of culturing, and the cells were harvested on glass-fiber filter (AB Ninolab, Upplands Väsby, Sweden) after 4–5 h and counted in a beta counter. The samples were tested in 2-fold dilutions and compared with standard curve obtained using mouse rIL-6 (Genzyme, Kent, U.K.). B9 cells were previously shown not to react with several recombinant cytokines, including IL-1α, IL-1β, IL-12, IL-3, IL-5, GM-CSF, TNF-α, and IFN-γ. There was only weak reactivity with IL-4 (18).

Statistical analysis

Statistical comparisons were made by using the χ^2 with Yates' corrections. All values are reported as the mean ± SEM. Values of $p < 0.05$ were considered significant.

Results

Induction of arthritis by viral RNA

Intra-articular injection of synthetic dsRNA (poly(IC)), but not PBS, directly into murine knee joints led to development of arthritis (Fig. 1, *A* and *B*). It was important to confirm that the highly purified viral dsRNA is also able to give rise to joint inflammation. Five micrograms of viral dsRNA was injected intra-articularly.

Table I. *Frequency and severity of arthritis in different mouse strains and following different depletion procedures*[a]

Group of Mice	Injected Agent	No. of Mice, *n*	Frequency of Arthritis	Severity Grade, Mean ± SD
NMRI	Poly(IC)	14	9/14[b]	0.9 ± 0.9
NMRI	Poly(I)	14	4/14	0.5 ± 0.8
NMRI	PBS	14	1/14	0.1 ± 0.4
SCID (CB17)	Poly(IC)	7	4/7	0.6 ± 0.5
Non-SCID (CB17)	Poly(IC)	9	5/9	0.9 ± 0.8
IL-IR$^{-/-}$ (C57BL/6)	Poly(IC)	9	1/9	0.1 ± 0.3
IL-IR$^{+/+}$ (C57BL/6)	Poly(IC)	10	5/10	0.5 ± 0.5
C_3H/HeJ	Poly(IC)	9	2/9	0.2 ± 0.4
C_3H/HeN	Poly(IC)	9	2/9	0.2 ± 0.4
Etoposide (BALB/c)	Poly(IC)	9	2/9	0.3 ± 0.7
RB6–8C5 (BALB/c)	Poly(IC)	9	6/9	1.2 ± 0.4
Control IgG + PBS	Poly(IC)	9	5/9	1.0 ± 0.8
Etoposide (BALB/c) + RB6–8C5 (BALB/c)	Poly(IC)	10	3/10	0.4 ± 0.7

[a] Mice were killed 3 days after a single intra-articular injection with 20 μl of dsRNA (poly(IC); 10 μg/knee), ssRNA (poly(I), 10 μg/knee), and PBS (20 μl/knee). BALB/c mice were injected with etoposide (12.5 mg/kg, in a volume of 125 μl, s.c. into the groin) or PBS as a control on 3 consecutive days before and after intra-articula injection of dsRNA. BALB/c mice were injected i.p. with 1 mg of mAb RB6–8C5, or the IgG rat anti-OVA mAb as a control 2 hs prior to intra-articular injection with dsRNA.

[b] Value of $p < 0.05$, as compared with mice injected with PBS.

The mice were killed 3 days after the injection. Just as in the case of synthetic dsRNA, viral dsRNA gave rise to arthritis (Fig. 2).

Histologically, the arthritis was characterized by synovial hyperthrophy and infiltrating mononuclear cells in the synovial lining cell layer, deep in the sublining space, as well as around surrounding synovial vessels. Immunohistochemical examination resulted in good morphologic preservation of synovial and articular tissue. In sections of arthritic joints, a large proportion of synovial cells was stained with Mac-1, which recognizes granulocytes and macrophages, and with CD3-specific Ab. The Mac-1-stained cells, having a morphology of macrophages, were found both within the thickened synovial lining layer and within the deeper synovial tissue. The predominance of Mac-1 cells was evident at day 3 after intra-articular injection with poly(IC), but also $CD3^+$ T lymphocytes were found in these arthritic joints (Fig. 1, *C* and *D*).

To assess the optimal amount of poly(IC) for triggering arthritis, different doses of poly(IC) (0.1, 1, 10, 20 μg/knee) were used in one experiment. We found that 10–20 μg of poly(IC) was the optimal dose because it triggered arthritis in the great majority of animals.

To exclude contribution of LPS contamination in the induction of arthritis, synthetic dsRNA was injected intra-articularly into knee joints of LPS-nonresponder C_3H/HeJ mice. No difference in frequency and severity of arthritis was observed upon injection of dsRNA to LPS-nonresponding mice (strain C_3H/HeJ) and congenic LPS-responding mice (strain C_3H/HeN) (Table I). This indicates that the induction of arthritis was due to dsRNA rather than LPS contamination in the injected vehicle.

Five different mouse strains were assessed for their susceptibility to poly(IC)-induced arthritis, including NMRI, C_3H, BALB/c, CB17, and C57BL/6. We found that arthritis was inducible by dsRNA in all of these strains (Table I).

To study the effects of repeated exposure of dsRNA on joint pathology, we injected 10 μg of poly(IC) intra-articularly on days 0 and 7. On day 10, the mice were killed and their joints were analyzed. The severity of arthritis was not significantly different with repetitive injections than with a single injection (results not shown).

We wanted also to assess the time point for the development of maximal frequency and severity of arthritis induced by a single injection of poly(IC). We found that histological signs of arthritis were most pronounced on day 3 after the injection of poly(IC), and its frequency and intensity diminished on days 7, 14, and 28 (Fig. 3).

Synthetic ssRNA did not give rise to the same frequency or severity of arthritis when provided intra-articularly (Table I). In analogy, intra-articular injection of polyinosonic-uridylic acid did not give rise to arthritis. Indeed, only one of five mice developed histological signs of joint inflammation.

Systemic in vivo inflammatory response following intra-articular exposure to dsRNA

Because viral dsRNA can activate macrophages to release IL-6, we measured its level in sera from mice injected intra-articularly with viral dsRNA (poly(IC)) at day 0, and then they were killed at days 3, 7, 14, and 28. Serum IL-6 levels peaked 7 days after injection of dsRNA and decreased with time to baseline levels at day 28 (Fig. 4).

Macrophages as key mediators for induction of arthritis triggered by dsRNA

To understand the role of different immune cells in the induction of dsRNA-mediated arthritis, we treated mice with etoposide. Etoposide is a cytotoxic drug known to selectively deplete the monocyte cell population in mouse. Pretreatment of mice with etoposide led to a profoundly decreased number of circulating monocytes.

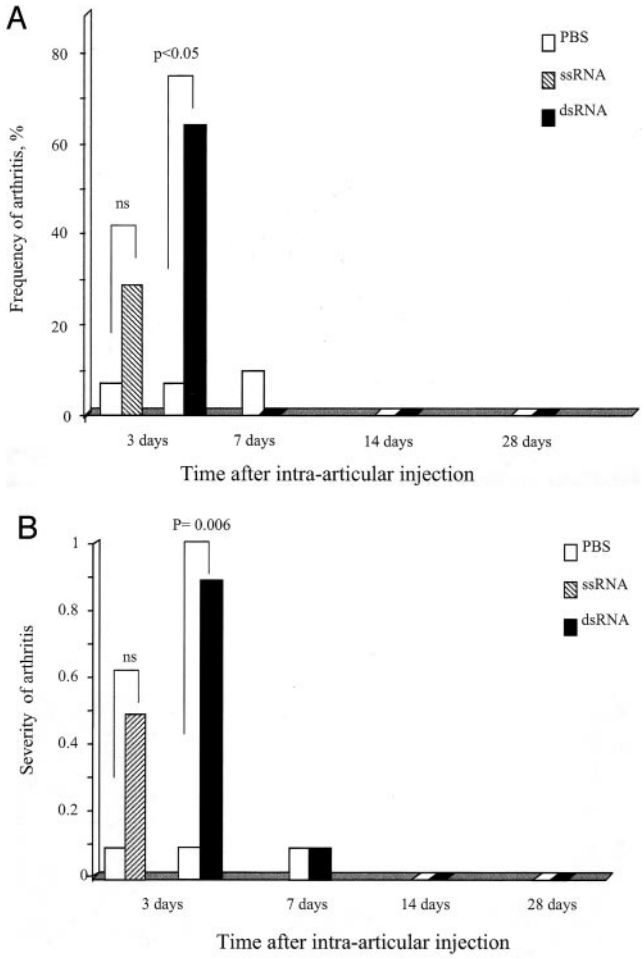

FIGURE 3. Frequency (*A*) and severity (*B*) of arthritis after a single intra-articular injection of dsRNA or ssRNA. Mice were killed 3, 7, 14, and 28 days after a single intra-articular injection with 10 μg of dsRNA or ssRNA (n = 14 per group).

Interestingly, depletion of monocytes almost totally abrogated the dsRNA-triggered arthritis (Fig. 5). In contrast, control mice developed joint inflammation at the expected frequency. These results strongly indicate that monocyte/macrophage population is responsible for the induction of dsRNA-triggered arthritis.

For analysis of the role of neutrophils, which are typically the earliest cells, first to migrate into tissues in response to inflammatory stimuli, we used neutrophil-depleting RB6-8C5 Ab. We found

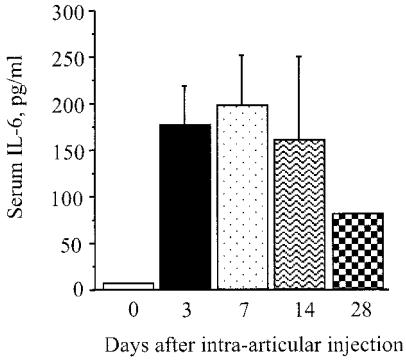

FIGURE 4. IL-6 levels in sera from NMRI mice injected intra-articularly with viral dsRNA (poly(IC); 10 μg) at day 0, and then killed at days 3, 7, 14, and 28 (n = 9).

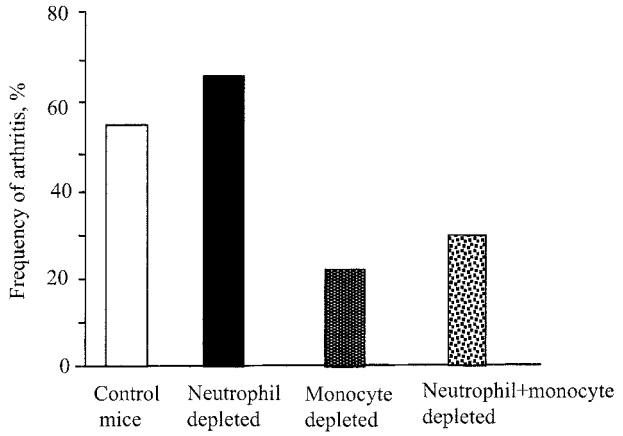

FIGURE 5. Frequency of arthritis after a single intra-articular injection of 10 μg of poly(IC). BALB/c mice were depleted of neutrophils using mAb against RB6-8C5 ($n = 9$). BALB/c mice were depleted of monocytes using etoposide treatment ($n = 9$). BALB/c mice were depleted simultaneously of both neutrophils and monocytes ($n = 10$).

that neutrophil-depleted mice did not differ from control mice in the development of arthritis triggered by dsRNA (Fig. 5). This indicates that neutrophils are not mandatory in the development of this condition. Simultaneous depletion of neutrophils and monocytes did not further decrease the frequency of dsRNA-induced arthritis more than etoposide treatment alone.

Next, we assessed the role of T and B lymphocytes using SCID mice lacking these cell populations, but having an intact population of monocytes/macrophages. Histologic results from SCID mice and their congenic littermates (CB17) demonstrated that the severity and incidence of arthritis were similar (Fig. 6). This finding proves that T and B cells are not pivotal for the development of dsRNA-triggered arthritis. Taken together, these studies provided strong evidence of the role of macrophages in initiating arthritis triggered by dsRNA.

Important regulatory role of NF-κB in dsRNA-triggered arthritis

What controls and regulates the macrophage activity in dsRNA-triggered arthritis? To answer this question, we investigated the role of NF-κB, because this transcription factor controls and regulates gene expression of proinflammatory cytokines at the transcriptional level (19, 20). Another important transcription factor is AP-1, which is a key regulator of matrix metalloproteinases, known to play an important role in rheumatoid joint destruction

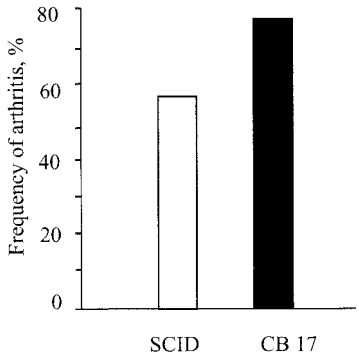

FIGURE 6. Incidence of arthritis in mice being deficient for T and B cells ($n = 9$) in comparison with their congenic strain (CB17) ($n = 9$). Three days after a single intra-articular injection of 10 μg of poly(IC), all the mice were killed.

(21). The impact of dsRNA (direct or indirect) on the activation of transcription factors in the spleen cell cultures was assayed in vitro. The binding of NF-κB and AP-1 to oligonucleotides containing NF-κB and AP-1 sense and antisense in the presence or absence of dsRNA (poly(IC)) was assessed by EMSA (Fig. 7). Increasing concentration of dsRNA (50, 5, 0.5 μg/ml) gave rise to a dose-dependent DNA binding to NF-κB (Fig. 7*A*). Specificity of DNA binding to NF-κB oligonucleotides was proved by a competitive inhibition of the reaction by introduction of unlabeled (cold) NF-κB oligonucleotides to the reaction mixture. In addition, incubation of nuclear extracts with Abs to NF-κB p50 subunit resulted in the formation of an additional band (supershift) in the gel (Fig. 7*B*). Similar pattern was observed when nuclear extracts from spleen cells stimulated with dsRNA were assessed for AP-1 protein (Fig. 7*C*). Intensity of AP-1 binding increased in parallel with increasing concentrations of stimulated dsRNA. Introduction of an excess of unmarked AP-1 oligonucleotides prevented the reaction between nuclear extracts and ^{32}P-marked AP-1 oligonucleotides.

IL-1β is one of the major proinflammatory cytokines produced by monocytes/macrophages and being controlled via NF-κB activation. We decided to assess dependence of dsRNA-mediated signaling through IL-1R on the arthritic process. We found that mice being deficient for IL-1R were totally unable to develop joint inflammation upon injection of dsRNA (Fig. 8). This result implies that signaling through IL-1 is operational in dsRNA-induced arthritis.

Does TLR3 exert any role in dsRNA-triggered arthritis?

It has been previously reported that the mammalian TLRs recognize a variety of microbial components and activate NF-κB and

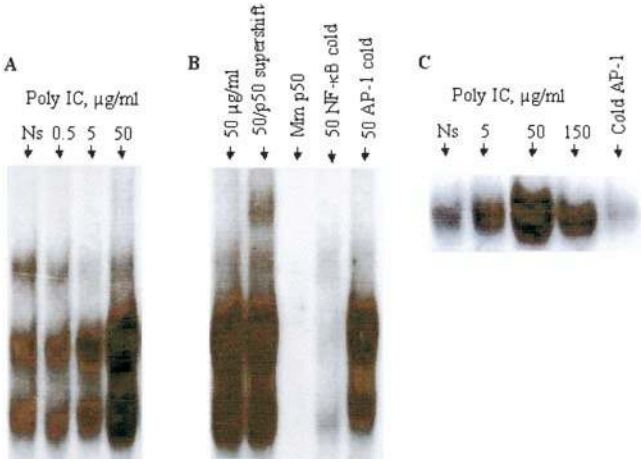

FIGURE 7. The activation of NF-κB and AP-1 in response to dsRNA was tested in cultures of spleen cells stimulated with increasing concentrations of synthetic dsRNA (poly(IC)). Nuclear extracts were prepared after 2 h of stimulation. Ns = Nonstimulated; mm = mastermix. *A*, EMSA was performed using specific probe to NF-κB binding site, and after 20 min at room temperature the complexes were resolved by electrophoresis through a 2.5% polyacrylamide gel. *B*, For competition studies, a 100-molar excess of unlabeled double-stranded oligonucleotides was added to the reaction mixture and incubated for 20 min at room temperature before the introduction of the ^{32}P-labeled probe. To identify the NF-κB subunits, we performed Ab-mediated supershift assays. Specific Abs to p50 subunits of NF-κB were added to nuclear extracts and incubated for another 20 min at room temperature. *C*, EMSA was performed using specific probe to AP-1 binding site, and after 20 min at room temperature the complexes were resolved by electrophoresis through a 2.5% polyacrylamide gel. For competition studies, a 100-molar excess of unlabeled double-stranded oligonucleotides was added to the reaction mixture and incubated for 20 min at room temperature before the introduction of the ^{32}P-labeled probe.

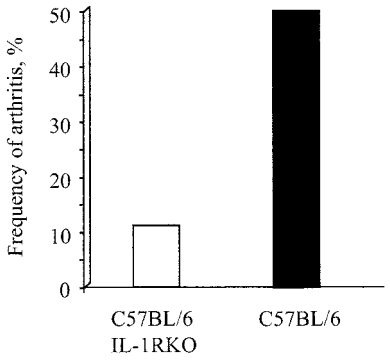

FIGURE 8. Incidence of dsRNA-triggered arthritis in IL-1RKO mice (IL-1R$^{-/-}$) ($n = 9$) in comparison with their wild-type littermates (C57BL/6) ($n = 10$). Three days after intra-articular injection with 10 μg of poly(IC), all the mice were killed.

other signaling pathways (22–24). Indeed, dsRNA interacts with TLR3 (6, 25) and triggers production of proinflammatory cytokines and chemokines such as TNF-α, MIP-1α, MCP-1, and IL-6.

We decided to analyze the role of TLR3 in dsRNA-induced arthritis both in vivo and in vitro. We injected TLR3 knockout (KO) mice and their congenic controls intra-articularly with dsRNA, and 3 days later their knee joints were histopathologically analyzed. The results obtained indicate that TLR3 status did not significantly affect the development of arthritis. Indeed, three of four mice in TLR3$^{+/+}$ group and four of five in TLR$^{-/-}$ group displayed signs of mild-moderate degree of arthritis. Next, we assessed in vitro the induction of proinflammatory cytokines and chemokines in murine spleen cell cultures by exposing the cells from TLR3$^{+/+}$ and TLR3$^{-/-}$ to dsRNA. It was found that dsRNA gave rise to somewhat stronger cytokine and chemokine responses in control mice as compared with TLR3KO mice (Fig. 9*A*).

Cytokine and chemokine induction by dsRNA

To understand how dsRNA-triggered arthritis is initiated, we analyzed the impact of dsRNA on the induction of cytokine/chemokine production following exposure to dsRNA. The rationale for this approach is that local release of cytokines/chemokines has been implicated as a key initiator of the inflammation and joint destruction observed in inflammatory arthritides (26).

Because cytokines/chemokines play an important role in the pathogenesis of aseptic arthritis (26, 27) and septic arthritis (28), we decided to assess the potential of dsRNA to induce production of proinflammatory cytokines and chemokines in murine spleen cell cultures by stimulating the cells from SCID, CB17, C$_3$H HeJ, C$_3$H HeN, C57BL/6, IL-1RKO, and healthy NMRI with dsRNA (0, 50, 150 μg/ml). The supernatants were collected after 3 days of incubation for detection of TNF-α, MIP-1α, MCP-1, and IL-6. We found that production of cytokines and chemokines was clearly dose dependent (Fig. 9*B*), and in certain circumstances dependent on expression of IL-1R (Fig. 9*C*), such as in case of MIP-1α and IL-6 production.

As mentioned before, SCID mice lacking T and B lymphocytes raised joint inflammation upon administration of dsRNA. Therefore, we also decided to analyze in vitro impact of dsRNA on SCID splenocytes even in vitro. We found that levels of both cytokines (IL-6, TNF-α) and chemokines (MCP-1 and MIP-1α) were similar in SCID mice as compared with congenic littermates (CB17) (results not shown). This finding again stressed that dsRNA-induced arthritis is initiated by activated macrophages and that T and B lymphocytes are not involved in the development of this process.

Furthermore, we assessed the role of LPS contamination in vitro by using spleen cell cultures from LPS-nonresponder strain C$_3$H HeJ mice and their congenic LPS-responder C$_3$H HeN mice. The results from this experiment demonstrated no differences between C$_3$H HeJ mice and their congenic littermates regarding the production of IL-6, TNF-α, MIP-1α, and MCP-1 upon the exposure to dsRNA. This finding proves that the inflammation is dependent on dsRNA rather than contaminating LPS (results not shown).

Discussion

This is the first study presenting the central role of viral dsRNA in induction of arthritis. Our results indicate that synthetic dsRNA, mimicking viral dsRNA as well as purified viral dsRNA itself, are able to induce arthritis. dsRNA may, therefore, play an important pathogenic role in virally triggered inflammatory joint diseases. This conclusion is supported by our finding that the proinflammatory effect exerted by viral dsRNA is not caused by endotoxin contamination. It is based on: 1) a low LPS concentration in the dsRNA preparation; and 2) similar frequency and severity of arthritis between the LPS-nonresponder and their congenic LPS-responder mice.

What mechanism triggers induction of dsRNA-mediated arthritis? Because joint inflammation occurs already on day 3 following intra-articular injection with poly(IC), it is strongly suggestive for activation of the innate rather than aquired immunity. Indeed, dsRNA-triggered arthritis occurs in SCID mice lacking T and B cells, almost at the same frequency and magnitude as in their congenic counterparts, supporting mechanisms operating in innate immunity. We believe that monocytes/macrophages are the main cell population that mediates arthritis caused by dsRNA. This is based on abundance of Mac-1$^+$ mononuclear cells in the inflamed synovial tissue. Further support for the role of macrophages is clearly decreased frequency of arthritis upon in vivo depletion of monocyte/macrophage population in mice intra-articularly exposed to dsRNA. In contrast, neutrophils, despite their rapid influx into tissues in response to inflammatory stimuli, are not mandatory in the development of dsRNA-triggered arthritis. Lysis of this cell population in vivo does not affect natural course of the disease.

Some of the major intracellular mediators of inflammatory response in macrophages are NF-κB and AP-1. The activation of NF-κB and AP-1 in dsRNA-exposed macrophages was examined using EMSA. Our findings indicate that dsRNA activates dose dependently NF-κB and AP-1, especially with respect to its p50 subunit. Such an activation is a prerequisite for subsequent induction of proinflammatory cytokine and metalloproteinase production. Indeed, in vitro analysis showed that dsRNA induces both cytokine and chemokine release by leukocytes. Also, a single intra-articular injection of dsRNA leads to systemic production of inflammatory mediators. Finally and importantly, expression of joint inflammation upon exposure to dsRNA is completely abrogated in mice deficient for IL-1R expression.

TLRs are a family of molecules that recognize danger signals associated with microbial pathogens, and induce antimicrobial immune responses (29, 30). It has been shown that TLR3 is an important recognition molecule specific for dsRNA (6). Our data demonstrate that mice lacking TLR3 were still able to develop arthritis, indicating that some other recognition systems for dsRNA may also be operative. One of such systems might be dsRNA-dependent protein kinase (PKR) interaction with its ligand (i.e., dsRNA) giving rise to NF-κB activation in a direct way, as described by Yang et al. (31). In agreement with the previous study (6), dsRNA was more efficient in vitro to give rise to cytokine and chemokine responses in controls as compared with TLR3KO mice. Alltogether, our results suggest that dsRNA is arthritogenic and

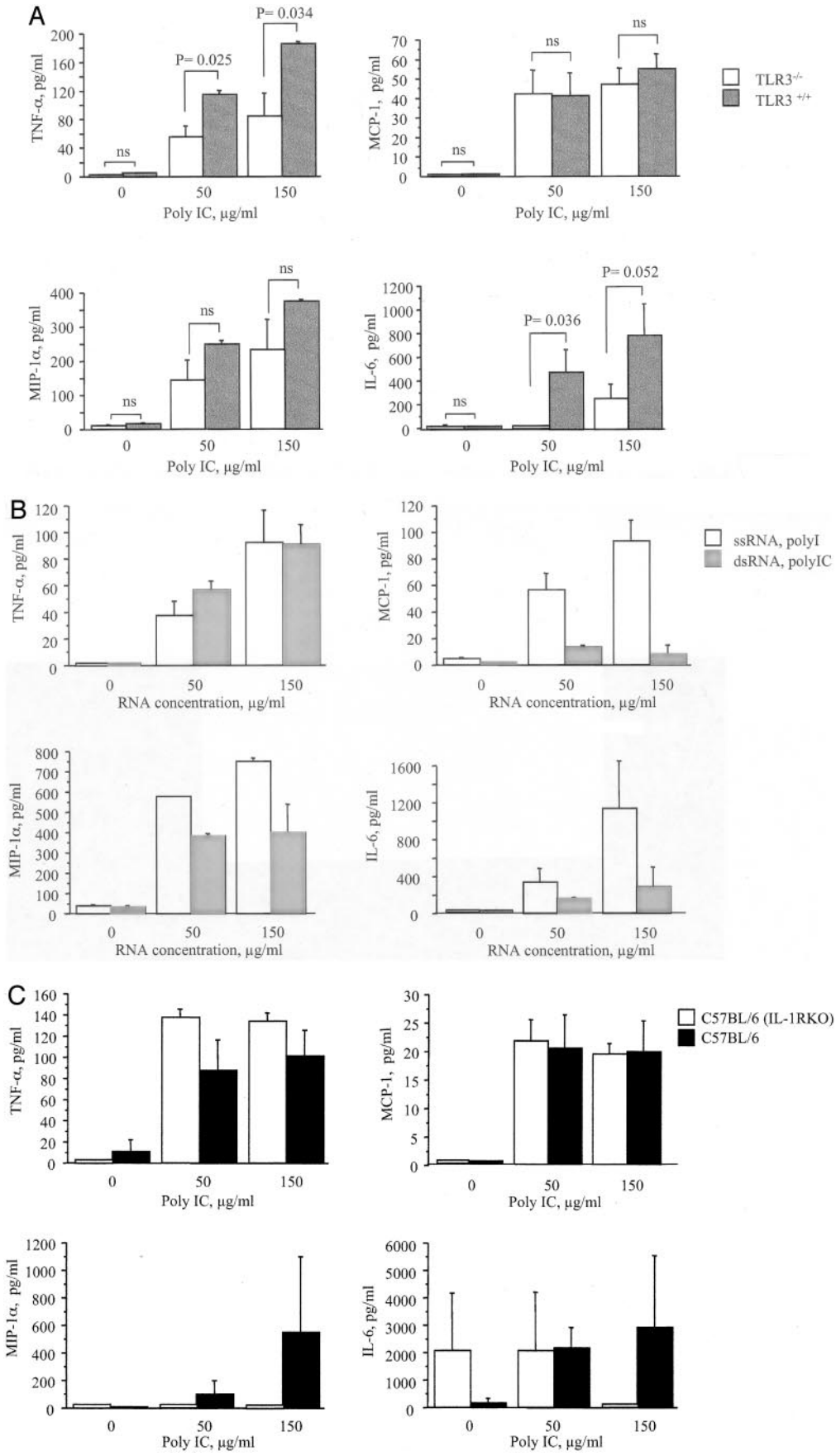

FIGURE 9. *A*, Levels of IL-6, TNF-α, MCP-1, and MIP-1α in vitro by spleen cell cultures from mice being deficient for TLR3 ($n = 4$) or their congenic littermates ($n = 3$) after 2 days of stimulation with poly(IC) (0, 50, 150 μg/ml). *B*, Levels of IL-6, TNF-α, MCP-1, and MIP-1α produced in vitro by spleen cell (obtained from NMRI mice, $n = 2$) cultures after 3 days of stimulation with poly(IC) or poly(I) (0, 50, 150 μg/ml). *C*, Levels of IL-6, TNF-α, MCP-1, and MIP-1α produced in vitro by spleen cell cultures from mice being deficient for IL-1R ($n = 3$) or their congenic littermates ($n = 3$) after 3 days of stimulation with poly(IC) (0, 50, 150 μg/ml).

that its arthritogenic properties are mediated by NF-κB activation and signaling involving IL-1R interaction. This finding is the first molecular evidence of inflammatogenic property of viral constituents once deposited in the joint cavity.

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References

1. Hyrich, K. L., and R. D. Inman. 2001. Infectious agents in chronic rheumatic diseases. *Curr. Opin. Rheumatol. 13:300*.
2. Satoh, M., V. M. Shaheen, P. N. Kao, T. Okano, M. Shaw, H. Yoshida, H. B. Richards, and W. H. Reeves. 1999. Autoantibodies define a family of proteins with conserved double stranded RNA-binding domains as well as DNA binding activity. *J. Biol. Chem. 274:34598*.
3. Guidotti, L. G., R. Roshford, J. Chung, M. Shapiro, R. Purcell, and F. V. Chisari. 1999. Viral clearance without destruction of infected cells during acute HBV infection. *Science 284:825*.
4. Levine, B., J. M. Hardwick, B. D. Trapp, T. O. Crawford, R. C. Bollinger, and D. E. Griffin. 1991. Antibody-mediated clearance of α virus infection from neurons. *Science 254:856*.
5. Kelly, E., M. Kehoe, A. Brown, and F. Imani. 2001. Double-stranded RNA regulates IL-4 expression. *J. Immunol. 167:2496*.
6. Alexopoulou, L., A. Czopik-Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like-receptor 3. *Nature 413:696*.
7. Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol. 1:135*.
8. Iwamura, T., M. Yoneyama, N. Koizumi, Y. Okabe, H. Namiki, C. E. Samuel, and T. Fujita. 2001. PACT, a double stranded RNA binding protein, acts as a positive regulator for type 1 interferon gene induced by Newcastle disease virus. *Biochem. Biophys. Res. Commun. 282:515*.
9. Fujita, T., M. Miyamoto, Y. Kimura, J. Hammer, and T. Taniguchi. 1989. Involvement of a *cis*-element that binds an H2TF-1/NF-κB like factor(s) in the virus-induced interferon-β gene expression. *Nucleic Acids Res. 17:3335*.
10. Lenardo, M. J., C. M. Fan, T. Maniatis, and D. Baltimore. 1989. The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell 57:287*.
11. Visvanathan, K. V., and S. Goodbourn. 1989. Double-stranded RNA activates binding of NF-κB to an inducible element in the human β-interferon promoter. *EMBO J. 8:1129*.
12. Deng, G. M., and A. Tarkowski. 2000. The features of arthritis induced by CpG motifs in bacterial DNA. *Arthritis Rheum. 43:356*.
13. Bremell, T., A. Abdelnour, and A. Tarkowski. 1992. Histopathological and serological progression of experimental *Staphylococcus aureus* arhtritis. *Infect. Immun. 60:2967*.
14. Jonsson, R., A. Tarkowski, and L. A. Klareskog. 1986. Demineralization procedure for immunohistopathological use. *J. Immunol. Methods 88:109*.
15. Calame, W., A. E. Douwes-Idema, M. T. van den Barselaar, R. van Furth, and H. Mattie. 1994. Influence of cytostatic agents on the pulmonary defense of mice infected with *Klebsiella pneumoniae* and on the efficacy of treatment with ceftriaxone. *J. Infect. Dis. 29:53*.
16. Vedrengh, M., and A. Tarkowski. 1997. Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*. *Infect. Immun. 65:2517*.
17. Bergquist, J., B. Ohlsson, and A. Tarkowski. 2000. Nuclear factor-κB is involved in the catecholaminergic suppression of immunocompetent cells. *Ann. NY Acad. Sci. 917:281*.
18. Helle, M., L. Boeije, and L. A. Aarden. 1988. Functional discrimination between interleukin-6 and interleukin-1. *Eur. J. Immunol. 18:1535*.
19. Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol. 12:141*.
20. Shakhov, A. N., M. A. Collart, P. Vassalli, S. A. Nedospasov, and C. V. Jongeneel. 1990. κB-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. *J. Exp. Med. 171:35*.
21. Han, Z., D. L. Boyle, L. Chang, B. Bennett, M. Karin, L. Yang, A. M. Manning, and G. S. Firestein. 2001. c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J. Clin. Invest. 108:73*.
22. Fearon, D. T. 1997. Seeking wisdom in innate immunity. *Nature 388:323*.
23. Medzhitov, R., P. Preston-Hurburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature 388:394*.
24. Imler, J. L., and J. A. Hoffmann. 2001. Toll receptors in innate immunity. *Trends Cell Biol. 11:304*.
25. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nat. Immun. 4:161*.
26. Miossec, P. 1992. Cytokine abnormalities in inflammatory arthritis. *Baillieres Clin. Rhumatol. 6:373*.
27. Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol. 14:397*.
28. Hultgren, O., H. P. Eugster, J. D. Sedwick, H. Korner, and A. Tarkowski. 1998. TNF/lymphotoxin-α double-mutant mice resist septic arthritis but display increased mortality in response to *Staphylococcus aureus*. *J. Immunol. 161:5937*.
29. Akira, S. 2001. Toll-like receptors and innate immunity. *Adv. Immunol. 78:1*.
30. Medzhitov, R. M., and C. A. Janeway. 2000. Innate immune recognition: mechanisms and pathways. *Immunol. Rev. 173:89*.
31. Yang, Y. L., L. F. Reis, J. Pavlovic, A. Aguzzi, R. Schafer, A. Kumar, B. R. Williams, M. Aguet, and C. Weissmann. 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J. 14:6095*.