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Inflammatory arthritis can be reined in by CpG-induced DC–NK cell cross talk

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Unmethylated CpG-oligodeoxynucleotides (ODNs) are generally thought of as potent adjuvants with considerable therapeutic potential to enhance immune responses against microbes and tumors. Surprisingly, certain so-called stimulatory CpG-ODNs strongly inhibited the effector phase of inflammatory arthritis in the K/BxN serum transfer system, either preventively or therapeutically. Also unexpected was that the inhibitory influence did not depend on the adaptive immune system cells mobilized in an immunostimulatory context. Instead, they relied on cells of the innate immune system, specifically on cross talk between CD8 α^+ dendritic cells and natural killer cells, resulting in suppression of neutrophil recruitment to the joint, orchestrated through interleukin-12 and interferon- γ . These findings highlight potential applications of CpG-ODNs and downstream molecules as antiinflammatory agents.

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Abbreviations used: Ab, antibody; AP, alkaline phosphatase; CAE, chloroacetate esterase substrate; DT, diphtheria toxin; DTR, DT receptor; GPI, glucose-6-phosphate-isomerase; HE, hematoxylin and eosin; HEK, human embryonic kidney; IC, immune complex; IDO, indoleamine 2,3-dioxygenase; IKDC, interferon-producing killer dendritic cell; OD, optimal density; ODN, oligodeoxynucleotide; PAM, pathogen-associated molecule; pDC, plasmacytoid DC; PGN, peptidoglycan; RA, rheumatoid arthritis; TLR, Toll-like receptor.

The inflammatory arthritides are an important health problem—rheumatoid arthritis (RA) affects ~1% of the world's population. Genetic factors are known to have a substantial impact on the incidence and progression of RA, but the low concordance rate of this disease in monozygotic twins (~20%) raises the possibility of environmental factors also exerting a crucial influence (1). Indeed, there have been many reports of correlations between viral or bacterial infections and the onset of RA in humans (2). Furthermore, several animal models of inflammatory arthritis are initiated by one or another microbial constituents (3).

Cells of the innate immune system detect the intrusion of pathogens by recognizing conserved sequence motifs embedded within molecules expressed by the intruders (4). Some examples of such motifs are LPS, peptidoglycans (PGNs), double- and single-stranded RNAs, and unmethylated DNA CpG motifs (CpGs). These “pathogen-associated molecules (PAMs)” are specifically recognized by sets of receptors, the best characterized of which are the toll-like receptor (TLR) family members (4, 5).

PAMs and their receptors provide powerful molecular probes for assessing a potential impact of microbes on the initiation and progression of arthritis. Indeed, administration of PAMs promoted disease development in several mouse models of arthritis (6–8).

Given their potential importance, we decided to explore the roles of TLRs and their ligands in the K/BxN mouse model of inflammatory arthritis, which is a particularly convenient system for performing mechanistic dissection. This model has the additional advantage of not requiring adjuvants, which often contain TLR ligands, for disease induction, thereby avoiding potential problems of synergy. K/BxN mice express a transgene-encoded TCR that is reactive to a self-peptide derived from the ubiquitously expressed enzyme glucose-6-phosphate-isomerase (GPI), presented by the MHC class II molecule Ag^{s7} (3). Beginning at 3–5 wk of age, K/BxN mice spontaneously develop an autoimmune/inflammatory disease with many features in common with those of human RA (3). Conveniently, transfer of serum from arthritic mice into healthy nontransgenic animals induces arthritis in a highly synchronized and reproducible fashion (3). Importantly, this system does not require LPS to

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be effective, unlike collagen antibody-induced arthritis (7), again, simplifying the interpretation of results. Arthritis is a very complex, polygenic, and multifactorial disorder, so a major attraction of this serum-transfer system is that it allows one to focus on the inflammatory effector phase of disease, without the complicating influences of the autoimmune initiation phase.

To delineate points of potential environmental impact specifically in the arthritis effector phase, we examined the influence of selected TLRs and TLR ligands in the K/BxN serum-transfer system. Results from these experiments confronted us with two surprises: a potent antiinflammatory effect exerted by so-called stimulatory TLR9 ligands, and

a mechanism that relies solely on cells of the innate immune system, without the requirement for cells of the adaptive immune system seen with other CpG-mediated immunotherapeutic activities.

RESULTS

Certain CpGs inhibit K/BxN serum-transferred arthritis

As a first approach to exploring the role of TLRs and their ligands in the effector phase of inflammatory arthritis, we screened several TLRs for the ability to influence K/BxN serum-transferred disease. As might have been expected (7, 8), LPS exacerbated arthritis development in B6 mice when administered i.p. 2 d before K/BxN serum transfer (Fig. 1 A),

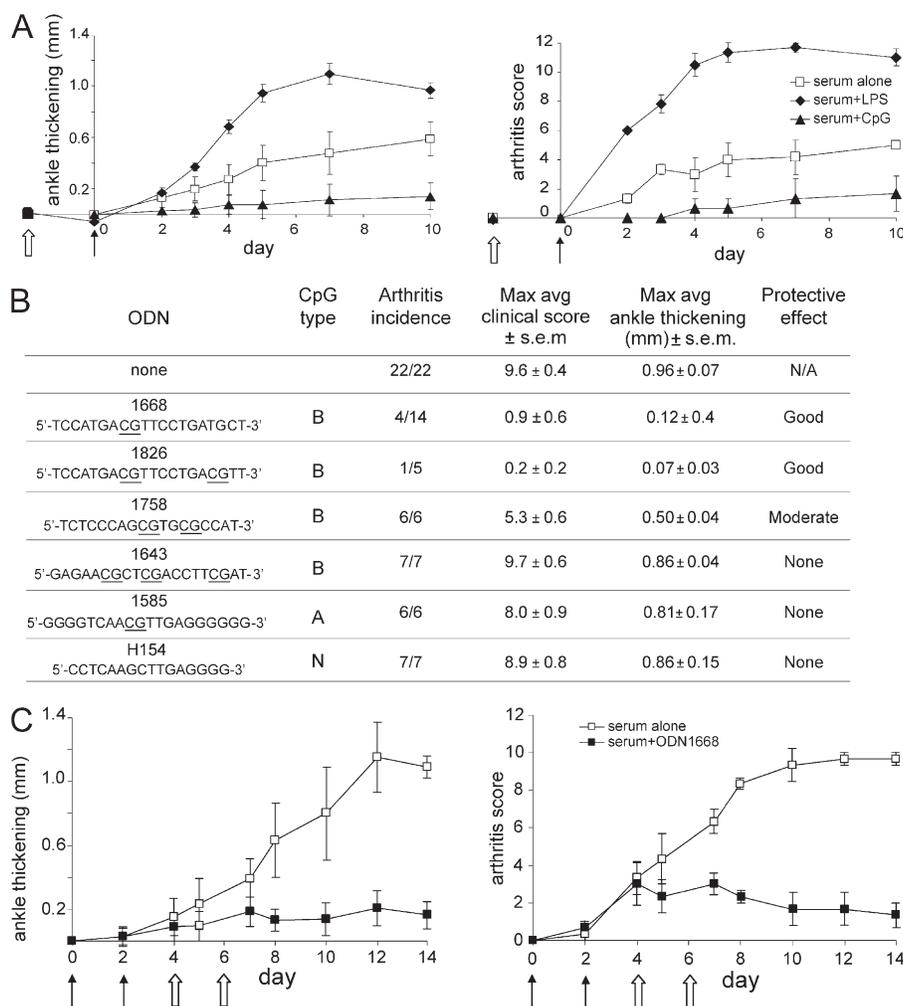


Figure 1. Some CpGs inhibit K/BxN serum-transferred arthritis. (A) Mean ankle thickening (left) or clinical score (right) of mice injected i.p. with 50 μ g LPS, 10 nmol CpG-ODN (ODN1668), or nothing on day -2 and with 100 μ l of K/BxN serum i.p. on day 0. The black and white arrows indicate administration of serum and various TLR ligands, respectively. Representative of two independent experiments ($n = 3$ mice/group). (B) Ankle thickening of mice treated with the optimum serum dose (150 μ l K/BxN serum on day 0 and 2), together with stimulatory ODNs (ODN1668, ODN1826, ODN1758, ODN1643, and ODN1585), a suppressive ODN (H154), or nothing on day 0 and 2. Except for ODN1585, all bases of the ODNs used in this paper were phosphorothioate modified. Effects were evaluated as disease incidence, mean of maximal clinical score, and mean of maximal ankle thickening. CpG dinucleotides are underlined. (C) Mean of ankle thickening (left) or clinical score (right) of mice treated with 150 μ l K/BxN serum on day 0 and 2, either with or without ODN1668 on day 4 and 6 ($n = 3$). The black and white arrows indicate administration of serum and ODN1668, respectively. Representative of two independent experiments.

whether assayed as ankle thickening (left) or as a clinical score (right). Preparations of PGNs, Pam3Cys4, and poly I:C had a similar effect when tested under the same protocol (unpublished data). In contrast, and surprisingly, injection of the CpG oligodeoxynucleotide (ODN) 1668 (Fig. 1 B) severely inhibited development of disease (Fig. 1 A). None of the TLR ligands was able to induce arthritis when injected alone i.p. (unpublished data), in contrast to what has been reported for intraarticular injection of unmethylated CpGs (6), for example.

Interestingly, although some CpG-ODNs were capable of inhibiting K/BxN serum-transferred arthritis, others were

not (Fig. 1 B). ODNs 1826 and 1758 also had a suppressive effect, although in the latter case it was only a moderate one; ODNs 1585, D19 (unpublished data), 1643, and H154 had no detectable influence. The two CpG-ODNs with the strongest influence on disease development are the most similar in sequence, differing by only two nucleotides at the 3' end. Both of these are so-called CpG-B-ODNs, which are characterized by having one or more CpG sequences on a fully phosphorothioate-modified backbone, and by being particularly stimulatory for B cells (9). The ODNs with no effect on arthritis included ones classified as CpG-B (1643);

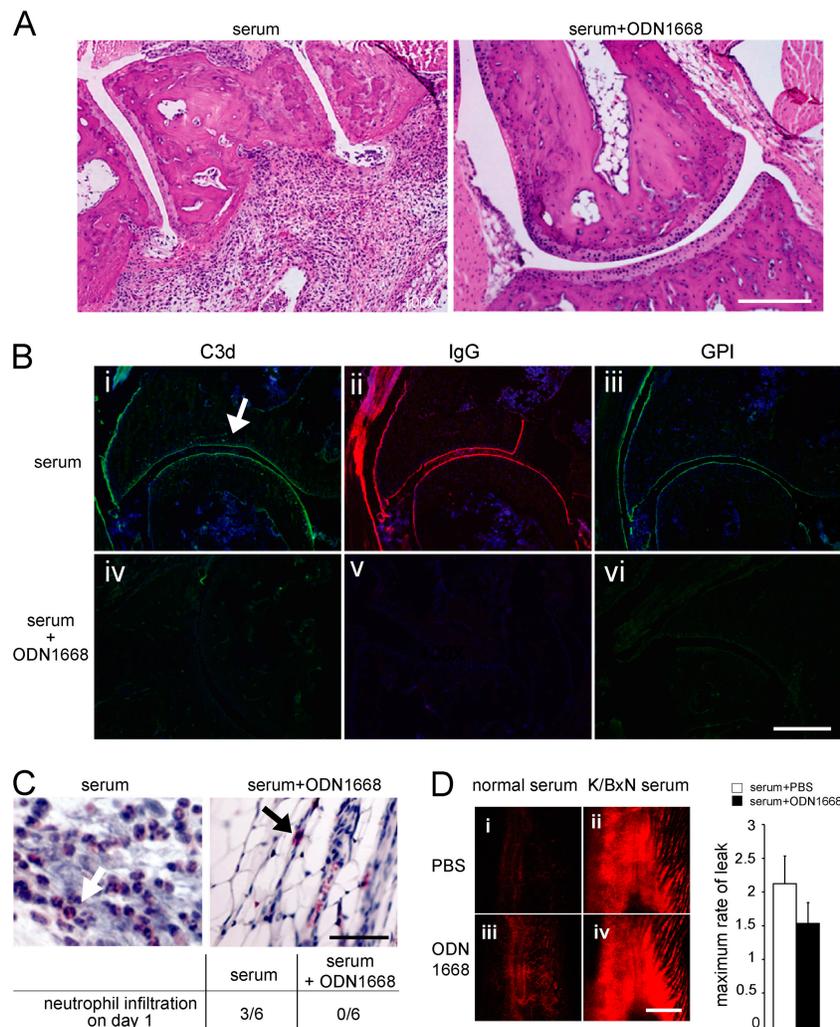


Figure 2. ODN1668 impacts early in arthritogenesis. (A) HE-stained ankle sections from mice 8 d after K/BxN serum transfer with or without ODN1668. Bar, 0.25 mm. $n = 2$. (B) Serial ankle cryostat sections from B6 mice 8 d after K/BxN serum transfer with or without ODN1668. Stained with anti-C3d Ab (i and iv), anti-IgG Ab (ii and v), or anti-GPI Ab (iii and vi). Arrow indicates the interface between the cartilage and articular cavity, where ICs can accumulate. Bar, 0.25 mm. $n = 2$. (C) CAE-stained ankle sections from mice 1 d after K/BxN serum (200 μ l) transfer with or without ODN1668. Many hypersegmented neutrophils with red cytoplasmic staining (white arrow) can be observed in the serum-treated group; in contrast, only resident mast cells (black arrow) and normal loose connective tissue can be seen in the serum+CpG-treated group. Bar, 25 μ m. Results on the incidence of neutrophil infiltration from six mice in each group are tabulated. (D) Mice were treated with PBS (i and ii) or CpG (iii and iv), followed 1 h later with Cy5.5 vascular probe. 10 min after probe injection, 200 μ l of normal (i and iii) or K/BxN (ii and iv) serum was transferred. Confocal micrographs of hindpaws were obtained every 3 s for 5 min before and 10 min after serum injection. (left) Images taken at 10 min after serum injection. The mean maximal leakage rate (right) was calculated by using the first derivative of rate curves generated by plotting the mean fluorescence intensity of individual confocal micrographs obtained every 3 s after serum injection. Bar, 0.5 mm. $n = 5$ mice/group. Results are plotted as the mean \pm the SEM.

CpG-A, with their mixed phosphorothioate/phosphodiester backbone, central CpG-containing palindrome, and terminal polyG motifs (1585); and CpG-N, which can neutralize the effects of immunostimulatory CpGs, including their induction of arthritis in certain contexts (10). The one CpG-N assessed, H154, was also unable to suppress the negative effect of CpG-Bs on arthritis in our system (unpublished data).

ODN1668 was optimally effective at inhibiting K/BxN serum-transferred arthritis when introduced coincident with, or shortly before injection of, the arthritogenic serum. However, it also had efficacy in a therapeutic mode, when administered as long as 4 d after serum transfer (Fig. 1 C). It did not seem to revert symptoms to the predisease state, but rather to stop them in their tracks.

CpG-ODNs operate over a time window between the opening of the vasculature and the influx of inflammatory cells

Mechanisms of pathogenesis in the K/BxN serum-transfer system have been extensively dissected (3, 8, 11–14). Quite similar to a reverse-passive Arthus reaction, arthritis development entails the following, sequentially: GPI–anti-GPI immune complex (IC) formation; activation of inflammatory and probably endothelial cells; induction of macromolecular leakage in joint vessels; complement activation; inflammatory cell influx; an amplification loop resulting in massive cytokine production; leukocyte recruitment and synovial hyperplasia; and, ultimately, joint destruction. To delineate precisely when CpGs impinge on the arthritogenic process, we evaluated their effect on the different disease subphenotypes that had been instrumental in constructing this pathogenetic scenario.

Consistent with the observed dampening of inflammation signaled by a reduction in ankle swelling, hematoxylin and eosin (HE)–stained ankle sections taken from mice 8 d after coadministration of K/BxN serum and ODN1668 revealed an absence of synovial hyperplasia and leukocyte infiltration (Fig. 2 A). IC deposition and complement activation, detected as GPI/IgG/C3d costaining on cryostat sections, was also absent from the ankles of serum/CpG-cotreated animals, although there was weak GPI binding at the cartilage–articular cavity interface (Fig. 2 B), as expected (15). Neutrophil recruitment was assayed 1 d after serum \pm CpG administration by staining ankle sections with HE and chromogenic chloroacetate esterase substrate (CAE), which specifically identifies granulocytes/neutrophils. At this time point, the disease is still at an early stage, so only 3 out of 6 mice injected with serum alone showed infiltration of neutrophils; in contrast, none of the animals coinjected with ODN1668 exhibited neutrophil recruitment (Fig. 2 C).

However, in serum/CpG-cotreated animals the typical vascular permeability to macromolecules that can be visualized by laser-scanning confocal microscopy of an immobilized heel in vivo in real time, subsequent to injection of a Cy5.5-labeled graft copolymer imaging reagent, was present (Fig. 2 D, left; compare iv and ii). Specifically, the maximum rate of leak was the same in K/BxN serum-transferred mice with or without

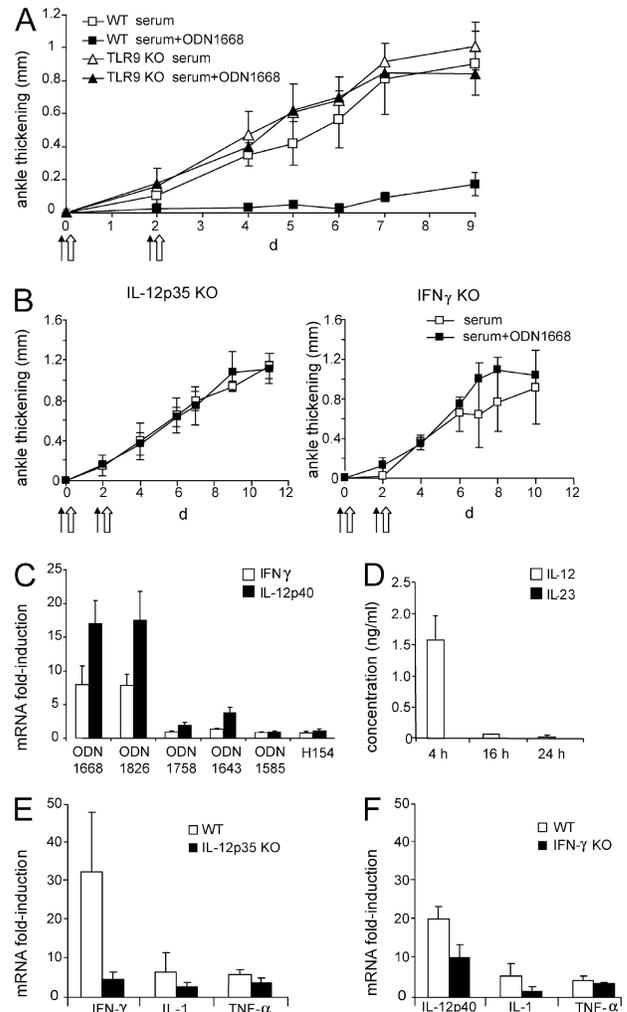


Figure 3. The molecular elements required for ODN1668-mediated protection. (A) Ankle thickening of WT or TLR9-KO mice treated i.p. with 10 nmol of ODN1668 and 150 μ l of K/BxN serum on day 0 and 2 or with serum alone. Results are plotted as the mean \pm the SEM. $n = 3$ –4/group. IL-12 and IFN- γ are critical for the CpG-mediated inhibitory effect. (B) Mean ankle thickening of IL-12p35 $^{-/-}$ and IFN- γ $^{-/-}$ mice treated with K/BxN serum alone or together with ODN1668. Representative of two independent experiments. $n = 3$ mice/group. The black and white arrows indicate administration of serum and ODN1668, respectively. (C) Various ODNs were injected i.p. into B6 mice, and the spleens were collected 4 h later. Fold induction of *IFN-g* and *IL-12p40* transcripts in spleens were quantitated by semiquantitative RT-PCR as described in the Materials and methods. Results were compiled from three independent experiments with four mice per group. (D) Serum levels of IL-12 and -23 were measured at the indicated time points after ODN1668 injection via ELISA. $n = 2$ mice/group for each time point. IFN- γ induction is a driving element. (E and F) B6, IL-12p35 $^{-/-}$, or IFN- γ $^{-/-}$ mice were treated with ODN1668 for 4 h, and fold induction of cytokine transcripts in splenocytes was determined as in D, shown as IL-12p35 $^{-/-}$ versus WT (E) or IFN- γ $^{-/-}$ versus WT (F). Results were compiled from four independent experiments with 6–7 mice/group.

coadministration of ODN1668 (Fig. 2 D, right). Injection of this CpG in the absence of serum did not provoke vessel leakage (Fig. 2 D, compare iii and i). Because the vascular permeability

provoked by serum transfer depends on ICs (11), it would appear that ODN1668 did not compromise GPI/anti-GPI IC formation per se, which is consistent with the observation of indistinguishable anti-GPI antibody (Ab) titers in the blood in the presence or absence of this ODN (unpublished data).

Thus, the window within which CpGs impinge on the unfolding of arthritis in this system is delimited on one side by opening of the vasculature (>2 min after serum injection) and on the other side by inflammatory cell influx subsequent to IC deposition, and the consequent activation of complement (<24 h after injection).

The CpG-mediated inhibitory effect is signaled through TLR9

CpGs are known to exert their immunostimulatory effects through binding to TLR9 (16). Yet, because it has been hypothesized that other receptors might be involved in CpG-induced activation (17), and because binding of ODNs 1668 and 1826 to TLR9 has generally been associated with stimulatory rather than suppressive influences (9), we wished to verify that CpG-mediated inhibition of K/BxN serum-transferred arthritis actually does result from their signaling through TLR9. First, *TLR9*-transfected human embryonic kidney (HEK) 293 cells were stimulated with the various ODNs whose properties are depicted in Fig. 1 B, and induction of a *luciferase* reporter gene driven by NF- κ B-responsive regulatory elements was measured. There was a clear correlation between the strength of the TLR9-transduced signal and its disease-dampening capacity, ODNs 1668 and 1826 being strongest according to both criteria, ODN1758 being intermediate, and ODN1643 and H154 being the weakest (Fig. S1, left, available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>). None of the CpG-ODNs elicited a signal in control *TLR2*-transfected HEK-293 cells (Fig. S1, right). Second, according to data on KO mice, TLR9 was not required for arthritis to develop in response to K/BxN serum transfer; however, it was needed for the ODN1668-induced inhibition of this process (Fig. 3 A).

CpG-mediated inhibition of arthritis requires IL-12 and IFN- γ

To begin dissecting the molecular mechanisms underlying the dampening of K/BxN serum-transferred arthritis by ODN1668, we screened disease inhibition in several mouse strains deficient in the expression of cytokines, particularly some of those previously reported to operate downstream of TLR9 signaling. These experiments were, of course, limited to an assessment of strains that could develop disease in the first place, i.e., not those devoid of IL-1 or TNF- α .

Both IL-12p35 and IFN- γ (Fig. 3 B) were required for ODN1668 to exert its suppressive effect, as transfer of serum plus CpG, versus serum alone, into the corresponding KO strains provoked arthritis with the same kinetics and severity, i.e., with no sign of a CpG-mediated inhibitory effect. In contrast, IL-6 was not needed for effective inhibition (unpublished data).

There was an excellent correlation between the ability of a given ODN to inhibit arthritis and its capacity to induce *IFN- γ* and *IL-12p40* gene expression in spleens of recipient mice 4 h after injection (compare Fig. 1 B and Fig. 3 C). In contrast, splenocyte transcription of several other cytokine genes (e.g., *TNF- α* , *IL-15*, *IL-2*, and *IL-1*) demonstrated no such correlation (unpublished data). The increase in *IL-12p40* expression was associated with a transient augmentation in secretion of the IL-12 cytokine, itself, rather than of IL-23, which also contains the p40 subunit, as indicated by ELISA measurements of serum-cytokine levels (Fig. 3 D).

IL-12p40 expression was important for induction of *IFN- γ* transcription, although some increase in transcript levels was

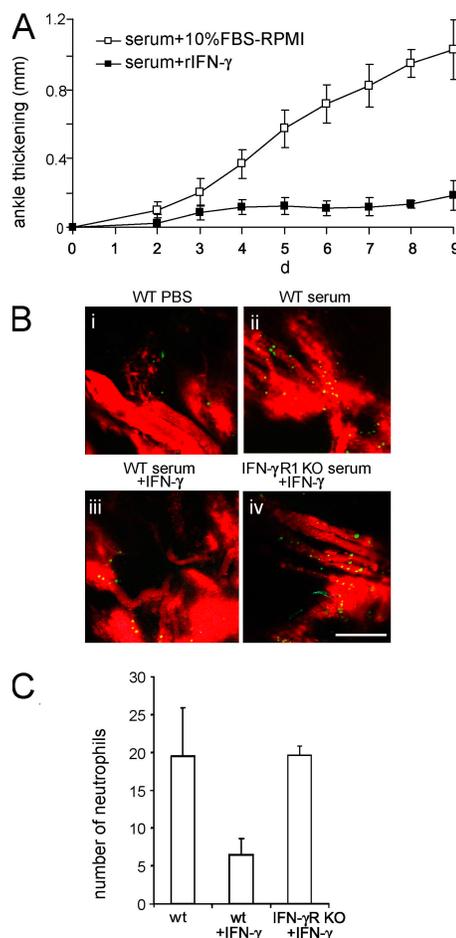


Figure 4. Administration of IFN- γ prevents arthritis development and blocks neutrophil recruitment by binding to their IFN- γ receptors. (A) Mean ankle thickening of mice treated with 150 μ l K/BxN serum on day 0 and 2, together with daily i.p. injection of 50 μ g IFN- γ or vehicle control (10% FBS-RPMI) for 7 d. Results were compiled from two independent experiments. $n = 4$ mice/group. (B) Purified neutrophils from WT B6 (i, ii, and iii) or IFN- γ R KO (iv) mice were treated with IFN- γ (iii and iv) or not (i and ii), and were transferred into arthritic B6 mice. Bar, 0.5 mm. Results shown are images of neutrophils (green) in the inflamed ankles. (C) The bar graphs depict averages of neutrophil number minus background (no serum group [B, i]; mean number is 5.3 from 4 mice). $n = 4-6$ for each group. Error bars represent the SD.

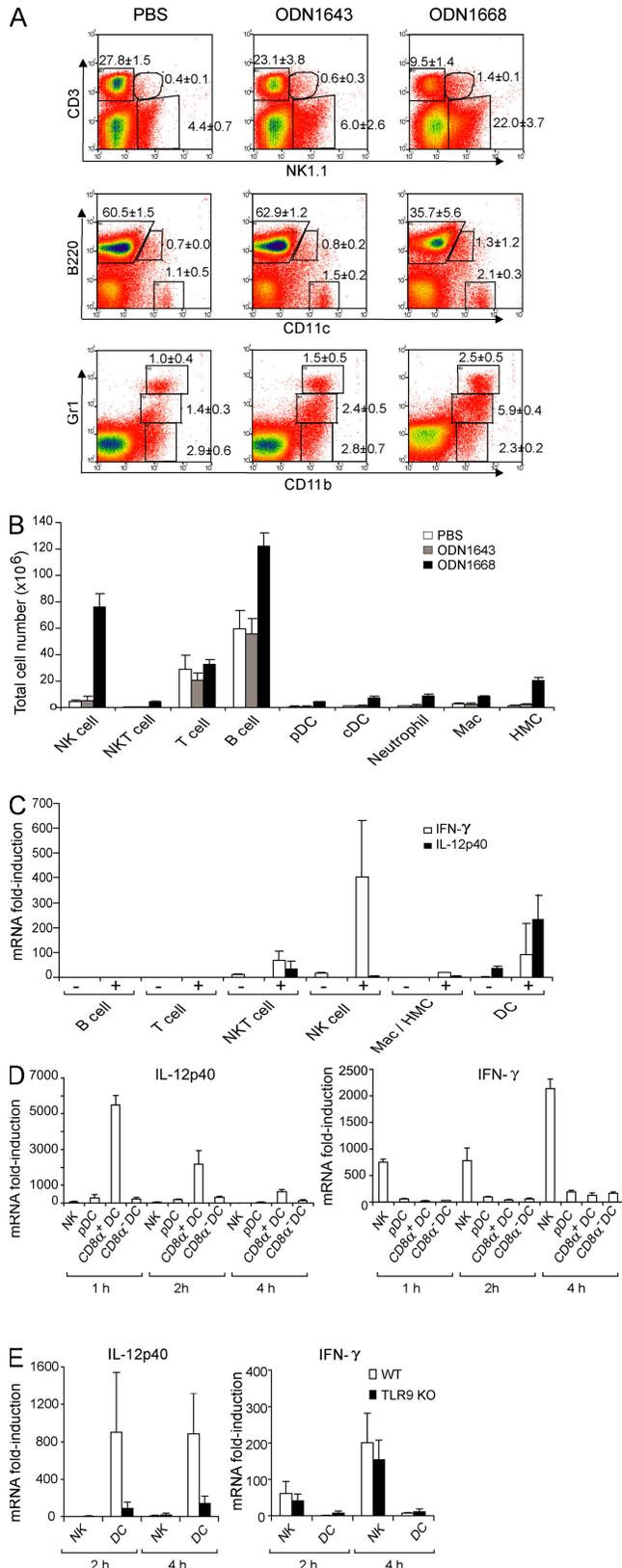


Figure 5. DCs and NK cells are critical players in CpG-mediated inhibition. (A) Mice were treated with PBS, 10 nmol ODN1643, or 10 nmol ODN1668 on day 0 and 2. Spleens were harvested on day 8, and cell types

still evident in its absence (Fig. 3 E); *IFN-γ* expression also promoted the induction of *IL-12* transcription (Fig. 3 F). As discussed below, this mutual dependence might be taken as evidence of a feed-forward loop.

Strikingly, systemic administration of *IFN-γ* was an effective inhibitor, by itself, of serum-transferred arthritis, arguing that systemic mechanisms are probably in play (Fig. 4 A). Because CpG blocked neutrophil accumulation in the joints, and *IFN-γ* was identified as the downstream effector of CpG, we next examined whether *IFN-γ* prevented joint inflammation by acting directly on neutrophil migration. Using a novel laser-scanning, time-lapse, intravital imaging system, we found that *IFN-γ* administration did inhibit recruitment of WT neutrophils into inflamed joints; however, this inhibition was lost when neutrophils from *IFN-γR* KO animals were used (Fig. 4, B and C). Note that the absence of *IFN-γR* on neutrophils did not, in and of itself, influence their migration into the joint under the influence of serum alone (unpublished data). Therefore, *IFN-γ* directly blocks neutrophil migration by binding to their *IFN-γ* receptors. Without neutrophil migration, arthritis cannot set in.

The case for DC–NK cell crosstalk

The combined requirement for *IL-12* and *IFN-γ*, and their interdependent expression, evoke recent findings on the

were identified by their surface markers as follows: T cells ($CD3^+NK1.1^-$), NKT cells ($CD3^+NK1.1^+$), NK cells ($CD3^-NK1.1^+$), B cells ($B220^+CD11c^-$), plasmacytoid DCs (pDCs; $B220^+CD11c^{int}$), conventional DCs (cDC; $B220^-CD11c^{high}$), macrophages (Mac; $Gr1^-CD11b^+$), heterogeneous myeloid cells (HMCs; $Gr1^{dull}CD11b^+$), and neutrophils ($Gr1^{high}CD11b^+$). A dot plot from one representative mouse out of three is shown. The percentage of total live cells (mean \pm the SD) from 3 mice is indicated. Representative of two experiments. (B) Same as in A; absolute cell number is shown (mean \pm the SD). Total splenocyte counts were as follows: in PBS-treated mice, 128, 84, and 84 million; in ODN1643-injected mice, 100, 68, and 96 million; and in ODN1668-injected mice, 328, 384, and 328 million. All cell types, except for T cells, are statistically significant ($P < 0.05$, Student's *t* test) in both the PBS versus ODN1668 and ODN1643 versus ODN1668 comparisons. (C) B6 mice were injected i.p. with ODN1668 or PBS, and 4 h later, spleens were harvested and sorted into various cell types as defined in A; except for DCs, NK cells were excluded before sorting into the Mac and HMC populations; the DC population includes both the plasmacytoid and conventional subsets. Fold induction of *IL-12p40* and *IFN-g*. Transcripts in various cell types were measured by RT-PCR as in Fig. 3 C. Results shown are the average of fold inductions from two independent experiments with a pool of 3 spleens/group. (D) Mice were injected i.p. with ODN1668, and spleens were harvested after 1, 2, or 4 h. NK cells, pDC, $CD8\alpha^+$ DCs, and $CD8\alpha^-$ DCs were sorted (Figs. S2 and S9) and examined for their *IL-12p40* and *IFN-γ* transcription induction. Results shown are the average fold inductions from two independent experiments. (E) Splenocytes from *TLR9*^{-/-} $CD45.2$ and WT $CD45.1$ congenic mice were mixed at a 1:1 ratio and cultured with ODN1668 for 2 or 4 h. *TLR9*^{-/-} and WT DC or NK cells were sorted based on their *CD45* alleles, and were examined for their *IL-12p40* and *IFN-γ* transcript induction. The results shown are the average of fold inductions from two independent experiments. Figs. S2 and S9 are available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>.

importance of the interplay between dendritic and NK cells, and the molecular crosstalk between them, in initiating responses by the innate immune system (18, 19). On one hand, DCs can prime, further the activation of, augment the expansion of, and enhance the activities of NK cells through the production of cytokines like IL-2, -12, -15, IFN- α/β , and TNF- α . On the other hand, mostly through the intermediary of IFN- γ , NK cells can enhance the differentiation, survival, and function of DCs, especially their expression of costimulatory molecules and production of IL-12 and TNF- α . Because most of the studies responsible for these conclusions were performed with cultured cells, the *in vivo* significance remains somewhat controversial. Nevertheless, we hypothesized that such a scenario underlies the CpG-mediated inhibition of K/BxN serum-transferred arthritis, and set out to test this notion.

As a first step, we performed a cytofluorimetric comparison of splenocytes from mice injected with PBS, the control CpG 1643, or the arthritis-inhibitory CpG 1668. As detailed in Fig. 5 (A and B), most of the examined cell populations had increased in number by 8 d after ODN1668 injection, a time point when the expansion of spleen cells was maximal and the inhibitory effect on arthritis was clearly evident. In particular, there was a striking overrepresentation of NK cells (CD3⁻NK1.1⁺; top) augmenting 18-fold in number and rising fractionally from an average of 4.4–22%.

Next, we examined cytokine mRNA induction in the different splenocyte populations (Fig. 5 C). The dominant IL-12-producing cells 4 h after injection of ODN1668 were DCs; NK cells were the major producers of IFN- γ , although NKT and DCs also made low amounts.

In mice, splenic CD8 α^+ and CD8 α^- DCs both express TLR9, and both can be induced to secrete IL-12 after stimulation in culture with CpG (20). However, we found that *in vivo*, CD8 α^+ , but not CD8 α^- , DCs were the major cell type responsible for CpG-induced IL-12 induction (Fig. 5 D and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>). Maximum expression of IL-12 occurred at 1 h after CpG injection; in contrast, induction of IFN- γ expression by NK cells maxed later at 4 h.

We next sought to determine where TLR9 needed to be expressed for DCs to turn up IL-12 expression and NK cells to up-regulate IFN- γ transcription. CD45.2 TLR9^{-/-} splenocytes were mixed 1:1 with splenocytes from WT CD45.1 congenic B6 mice, and the mixture was stimulated with CpG. TLR9^{-/-} DCs were not induced to express IL-12 (Fig. 5 E, left); a similar result was obtained by staining for intracellular IL-12 (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>). There was no difference in IFN- γ induction in TLR9^{-/-} versus WT NK cells (Fig. 5 E, right). Thus, CpG engagement on CD8 α^+ DCs appears to be the gateway for this antiinflammatory effect, inducing IL-12 by the DCs and subsequent activation of NK cells to make IFN- γ .

Lastly, the requirement for DCs and NK cells was assessed. Punctual depletion of DCs was performed by exploiting a

mouse strain wherein DCs can be killed off by diphtheria toxin (DT) treatment of transgenic animals expressing the simian DT receptor (DTR) primarily on DCs, under the dictates of the mouse CD11c promoter (21). These mice were found to lack all CD11c⁺ DC populations, which reduced their capacity to control microbial infections. Because it was reported that the DC compartment began to reemerge 3–5 d after DT treatment, we needed to modify our protocol as per Fig. 6 A (i) to enable us to assay arthritis induction and CpG-mediated inhibition of it during this short time frame. The DC populations were strongly reduced by this protocol, whereas other cell-types, e.g., NK, T, and B cells, were not (Fig. 6 A [ii] and not depicted). Loss of DCs during this time frame had no detectable effect on arthritis progression; however, it did strongly compromise the inhibitory effect of ODN1668 (Fig. 6 A, iii).

IL-15-deficient mice have a strong deficiency in NK cells, but also have deficits in some populations of T lymphocytes, particularly memory T and NKT cells (Fig. S4 A, available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>) (22). However, the CpG-induced, arthritis-inhibitory effect was independent of all α/β T and B cell populations, as was evident with mice carrying a mutation of the *TCR α* locus or in animals lacking B cells caused by the μ MT mutation (Fig. S5). IL-15 KO mice were fully susceptible to K/BxN serum-transferred arthritis, but not to inhibition of disease by ODN1668 (Fig. 6 B, left). Because it was recently reported that mice lacking IL-15 also have defects in some DC populations (although we did not find a numerical deficit; Fig. S4) (23), we tried a second strategy for eliminating NK cells. The polyclonal Ab anti-asialo-GM1 will deplete NK cells, though there are some reports that a subset of CD8⁺ T cells may be removed as well. Again, however, we know that T cells are not required for the CpG-induced suppression of arthritis (Fig. S5, middle). Anti-asialo-GM1 treatment reduced NK cells to \sim 30% of the normal level, although not depleting the T or NKT cell populations (Fig. S4 B), but did not affect serum-induced arthritis. Such treatment did, on the other hand, compromise the ability of ODN1668 to inhibit disease unfolding (Fig. 6 B, right). Together, these two sets of findings on NK cell depletion argue that this cell type has a critical role in CpG-mediated inhibition of serum-transferred arthritis. Again, consistent with the proposed scenario of DC–NK cell cross talk were results on cytokine gene expression in the DTR and IL-15 KO mice; a strong reduction in both *IL-12* and *IFN- γ* expression in the former case, with residual levels probably being attributable to incomplete DC depletion (Fig. 6 C, left), but a drop in only *IFN- γ* transcripts in the latter case (Fig. 6 C, right).

DISCUSSION

The therapeutic potential of CpGs has excited great interest of late (24). Their potent adjuvant activity can be applied to eliciting more effective immune responses to infectious agents or tumors. Their ability to bias responses in a T_H1 direction can be harnessed to redirect pathogenic T_H2 responses, e.g., in the

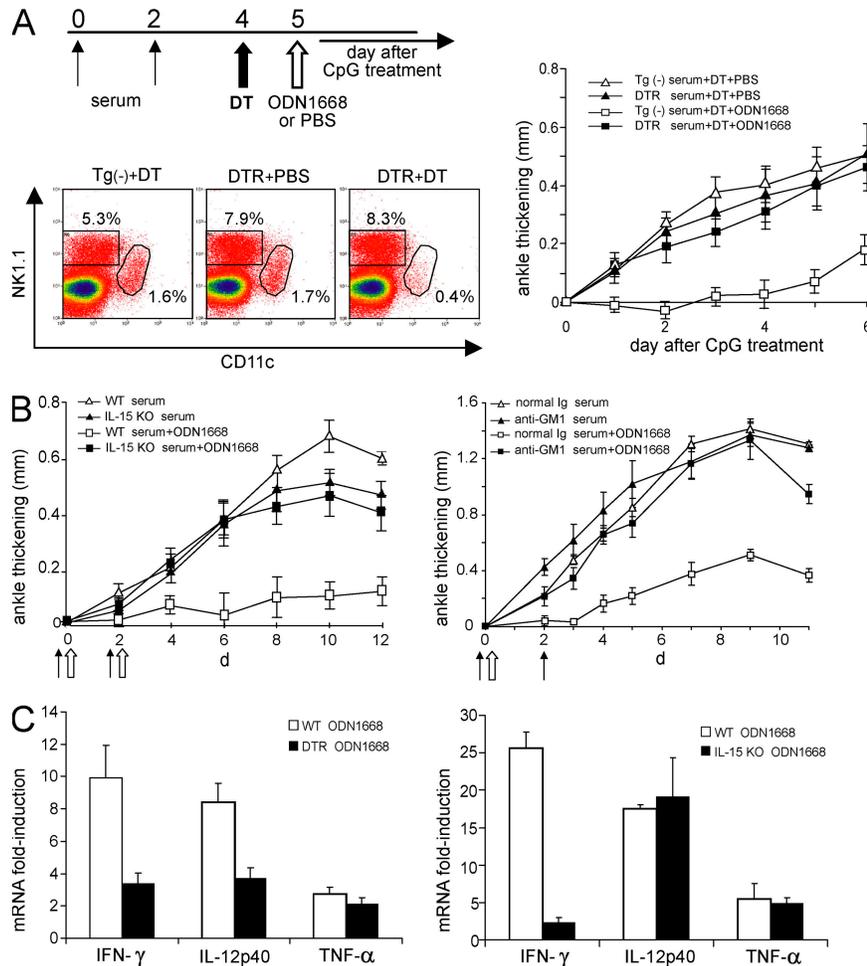


Figure 6. DCs and NK cells are required for CpG-mediated protection. (A) DCs needed. (top left) Protocol for DC depletion and serum+ODN1668 treatment. DTR mice and littermate controls (Tg [-]) were injected with serum on day 0 and 2, followed by DT treatment on day 4; animals were treated with ODN1668 or PBS on day 5. (bottom left) Flow cytometric analysis was performed on the splenic DC populations (CD11c⁺) of DTR mice after 20-h treatment with DT. Tg (-) littermates treated with DT and DTR mice treated with PBS served as negative controls. (right) DC-deficient (DTR) mice treated with serum+PBS or serum+CpG, or Tg (-) control littermates were monitored for arthritis progression. Average ankle thickening subsequent to day 0 (the commencement of CpG or PBS treatment) is plotted. DT-treated DTR mice with serum+PBS, $n = 8$; DT-treated DTR mice with serum+CpG, $n = 8$; DT-treated Tg (-) mice with serum+PBS, $n = 11$; DT-treated Tg (-) mice with serum+CpG, $n = 11$. (B) NK cells needed. Mean ankle thickening of NK cell-deficient (IL-15^{-/-}) or WT mice after administration of K/BxN serum alone or with ODN1668 in addition. Results compiled from three independent experiments with 8–9 mice/group. In a similar set-up, NK cell-deficient mice were generated by injection of anti-asialo-GM1 Ab (anti-GM1). Mean ankle thickening of NK cell-deficient (anti-GM1) or control (normal Ig) mice after treatment with K/BxN serum alone on day 0 and 2 or with ODN1668 on day 0 in addition. $n = 4$ mice/group. (C) Cytokine consequences. IL-15^{-/-} and B6 mice, or DT-treated DTR animals, and their littermate controls were treated with ODN1668 for 4 h. (For both DTR and control mice, ODN1668 was injected 20 h after DT treatment.) Fold induction of cytokine transcripts in splenocytes shown as IL-15^{-/-} versus WT, or DTR versus WT, respectively.

context of allergy or asthma. Given this backdrop, the observation that other PAMs are known to have an enhancing effect on diverse rodent models of inflammatory arthritis (7, 8), and the precedent that CpGs, themselves, have been reported to promote arthritis in more than one model (6, 25), we were surprised to find that ODN1668 could block arthritis development in the K/BxN serum-transfer system. Even more striking, ODN1668 is one of the few agents thus far found to be capable of reversing ongoing arthritis in this system.

Our mechanistic studies afforded a second surprise. The original scenario constructed to account for the immunostimulatory

activities of CpG-B-ODNs began with TLR9-signaled activation of B and DCs and ended with a potent response by the adaptive immune system, entailing Abs, T_H1 cells, and cytotoxic CD8⁺ T cells (Fig. 7 A) (9). More recently, this scenario was enriched to include DC-induced stimulation of NK cells, but it still culminated in and highlighted the promotion of more effective T cell and Ab responses (24, 26). Cases of CpG-ODNs dampening immune responses have been described in the past—autoimmune (27–29), asthmatic (30), or standard T cell (31)—in nature, but, in all of these studies, data, or sometimes just mere assumption, focused attention on

the response of the adaptive immune system (Fig. 7 B). In contrast, the arthritis-inhibitory property of ODN1668 described in this study did not depend on either B or T cells, and thereby reflected an influence solely on the operation of the innate immune system (Fig. 7 C). The key turned out to be cross talk between $CD8\alpha^+$ DCs and NK cells, which relied critically and interdependently on IL-12 produced by the former and IFN- γ made (mostly) by the latter. The eventual outcome of this “conversation” was suppression of the recruitment of neutrophils, a direct effect of IFN- γ on that cell type. At first glance, our data may also appear reminiscent of the recent observations of Katakura et al. on experimental colitis (32). However, these authors described only a protective effect; in fact, when administered therapeutically, ODN1668 exacerbated colitis (33). Moreover, in this case, IFN- γ was pro- rather than antiinflammatory (33).

If one considers the extensive body of existing information on events downstream of CpG-ODN engagement of TLR9 on immune cells, one is faced with a complex, often-conflicting set of data (18, 19). An example is the differential reliance on priming and facilitating cytokines, such as IL-2, IL-15, IFN- α/β , and TNF- α , to optimally activate NK cells. Part of the difficulty in assessing and comparing results is that many of them emanate from *in vitro* experiments on cells derived from various tissues according to diverse expansion protocols. For example, this factor could easily explain why we saw no influence of IL-2 in either gain-of-function or loss-of-function *in vivo* experiments (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>), although it appeared to have a critical role in certain *in vitro*

contexts (34, 35). Another issue is that the various experiments did not always use the same CpG-ODN; sometimes CpG-A versus -B, sometimes a different one of the many available CpG-Bs. This element is a critical one, as we saw from Fig. 1 B that even CpGs of the same class can have very different effects *in vivo*. Third, the picture that emerges is complicated by the difficulty for any study to be comprehensive in light of a constantly increasing cast of players. In this respect, we have briefly explored the roles in CpG-mediated protection of several elements that have elicited interest of late, including the following: indoleamine 2,3-dioxygenase (IDO) (31), interferon-producing killer DCs (IKDCs) (36), and plasmacytoid DCs (pDCs). Under a dose of 1-methyltryptophan, which is an IDO inhibitor previously reported to reverse the immunoresistance of IDO-expressing tumors (37), we did not see any effect on CpG-mediated arthritis inhibition (Fig. S7). This result is consistent with the observation that the immunosuppressive effect of IDO is known to work through T cells (31, 38), whereas CpG-mediated protection from arthritis is T cell-independent (Fig. S5). Likewise, the recently identified IKDC population may not have a substantial role in our system, as the majority of NK1.1-positive cells expanded after CpG treatment were CD11c-negative (Fig. S8). In contrast, we found that pDCs did have a partial effect on CpG-mediated protection from arthritis (Fig. S9). Ostensibly, this effect does not operate through the NK-DC/IL-12-IFN- γ axis we have focused on in this study, as pDCs do not produce either of these two cytokines (Fig. 5 D). The role of pDC is instead through a secondary axis involving Type I IFN (unpublished data). Lastly, existing

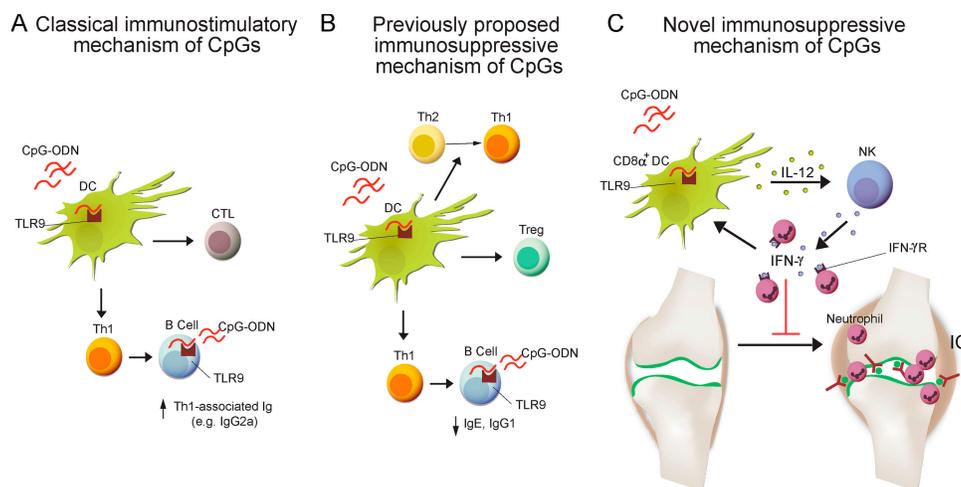


Figure 7. A comparison of proposed immunotherapeutic mechanisms of CpG. (A) The now classical CpG-mediated therapeutic mechanism involved activation of adaptive immunity, such as enhancement of Th1, CTL, and Ab response. This mechanism has been shown to play a key role in disease protection in infection and tumor contexts (24). (B) More recent studies revealed an immunosuppressive effect of CpG. CpG-mediated suppression of an undesired immune response was achieved by regulating acquired immunity in certain allergic reactions or autoimmune diseases. Some examples are switching of a Th2 to a Th1 response (30), activating T regulatory T cells (31), and inducing Ig isotype switching (53). (C) The CpG-mediated therapeutic mechanism proposed here is an antiinflammatory response that relies solely on cells of the innate immune system. ODN1668 signals through TLR9 on $CD8\alpha^+$ DCs to induce IL-12 secretion; IL-12 promotes expression of IFN- γ by NK cells. The process is amplified through a feed-forward loop entailing DC-produced IL-12 and NK cell-produced IFN- γ ; the resulting elevated systemic levels of IFN- γ inhibit arthritis development by preventing neutrophil recruitment into the joint.

information comes from a mix of mouse and human studies and, although the general outline is very similar in the two cases, details of the scenario certainly diverge, reflecting species differences such as the precise cell types that express TLR9 (9).

One of the key elements in the CpG-mediated inhibitory effect was IL-12. This dependence may explain the divergent results with the other TLR ligands we tested, as CpG is a much stronger IL-12 inducer than LPS or polyI:C (unpublished data) (39, 40). IFN- γ is the other important molecule mediating the protective effect. Systemic administration of IFN- γ prevented arthritis, and this effect appeared to be via blockade of neutrophil recruitment to the joint. Neutrophils were the direct targets of the IFN- γ -mediated inhibitory effect, as migration of those devoid of IFN- γ R was not inhibited. One attractive hypothesis for IFN- γ 's downstream mode of action is through down-regulation of CXCR4, as this cytokine has been described to control neutrophil migration in different contexts by posttranslational down-regulation of this chemokine receptor (41, 42).

Why did CpG-ODNs dampen arthritis in the K/BxN serum-transfer system, whereas they promoted disease in other arthritis models (6, 25)? One explanation is that local CpG injection (6) may be proinflammatory, but systemic delivery antiinflammatory. Such behavior would parallel observations on the effect of administering IFN- γ in the collagen-induced arthritis model; injection into the paw promoted disease, although back delivery was inhibitory (43, 44). An explanation in another case (25) is that the influence of CpGs may differ according to when they are introduced, with the lymphocyte-mediated initiation phase being more prone to an enhancing effect. One strength of the K/BxN serum-transfer system is that it focuses uniquely on the effector phase mediated by cells of the innate immune system, which is the phase when most patients would present. Lastly, it may be relevant that the K/BxN system does not entail other potentially synergistic TLR ligands, such as adjuvant (25) or LPS (7).

We hope that our findings will be translatable to the human arthritis context. Differences in TLR9 expression (or other factors) between mice and humans may mean that translation will not be entirely linear, but there should be many commonalities in the molecules, cells, pathways, and networks involved, so that their identification and characterization should provide points of access to the inhibitory process beyond CpG-ODNs themselves. In this regard, it is encouraging that the antiinflammatory properties of IFN- γ are being increasingly appreciated and studied in both species, leading to the recognition that some of the critical molecular mechanisms are shared, at least as far as can be concluded from cell culture experiments (45). Also promising is that clinical trials of rhIFN- γ in small cohorts of RA patients in the 1980s and 1990s proved it safe, and usually (46–48), but not always (49), somewhat effective. It may be worth revisiting this strategy, or allied therapeutic strategies, especially if it becomes possible to phenotypically or genetically stratify the patients most likely to have a beneficial response.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), B6.SJL (CD45.1⁺ congenic B6 mice), and NOD/Lt (NOD) mice were purchased from The Jackson Laboratory. KRN TCR-transgenic mice, which were described elsewhere (50), were maintained on the B6 genetic background (K/B6). Crossing K/B6 animals with NOD mice generated arthritic K/BxN offspring. H-2^{g7} congenic mice on the B6 background (B6g7) were bred in our animal facility. The various gene-deficient mice are described in the Supplemental materials and methods (available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>). All mouse procedures were conducted in compliance with federal and institutional guidelines.

Reagents. Reagents are described in the Supplemental materials and methods.

Serum-transfer system and evaluation of arthritis. Optimal serum-transferred arthritis was induced by i.p. injection of 100–150 μ l of pooled serum from 8-wk-old K/BxN mice on days 0 and 2, unless otherwise mentioned. Suboptimal arthritis was induced by i.p. injection of 100 μ l K/BxN serum on day 0. Ankle thickness was measured with a caliper (J15 Blet micrometer). Each limb was scored on a scale from 0 (no observable swelling) to 3 (severe inflammation), and the four-limb scores were summed to yield a clinical index (maximum 12 points).

Histology. Ankle sections were stained with hematoxylin and eosin or with CAE plus hematoxylin as a counterstain, as previously described (50, 51). Neutrophils exhibited a red cytoplasmic staining with CAE, which is a marker of the myeloid and mast cell lineages that does not stain monocytes, macrophages, or basophils. Immunohistology methods are detailed in the Supplemental materials and methods, with procedures carried out basically as previously described (15).

Intravital confocal microscopy. Microscopy was performed as recently published (11), and is detailed in the Supplemental materials and methods.

Neutrophil imaging: a laser-scanning, time-lapse intravital microscopy system. The imaging system for neutrophils is described in detail in the Supplemental materials and methods.

ELISAs. Anti-GPI Ab titers were measured as previously described (52). In brief, recombinant mouse GPI was coated on ELISA plates at 5 mg/ml, and diluted mouse sera was added. Subsequently, alkaline phosphatase (AP)-conjugated anti-mouse total IgG, followed by AP-conjugated streptavidin, was applied. After substrate addition, titers were quantitated according to the optimal density (OD) values from an ELISA reader. Serum levels of IL-12 and -23 were measured by an ELISA kit, using the instructions provided by the company (eBioscience). Capture-Ab clones C18.2 (anti-IL-12p35) and G23-8 (anti-IL-23p19) were specific for their respective cytokines. Both detection systems used clone C17.8 as the detection Ab for the p40 subunit shared by the two cytokines. Standard curves were generated using the recombinant IL-12 or -23 provided by the kit.

Flow cytometry. Abs and flow cytometry techniques are described in the Supplemental materials and methods.

Cell culture. An equal ratio of splenocytes from TLR9^{-/-} (CD45.2⁺) and B6.SJL mice (CD45.1⁺) mice were cultured at 2×10^6 /well in 96-well plates with 1 μ M CpG. Cells were harvested after 2 or 4 h and sorted into WT or TLR9^{-/-} DC and NK cell populations based on the CD45 alleles expressed.

NK cell depletion. Polyclonal rabbit anti-asialo-GM1 Ab (Cedarlane Laboratories) was used for in vivo depletion of NK cells. Mice were injected i.p. with 50 μ l of Ab on days 1, 2, 5, and 9 via serum injection or with an equivalent amount of rabbit gamma-globulin (The Jackson Laboratory).

RT-PCR. RNA was isolated from splenocytes via Trizol and reverse-transcribed using oligo dT priming and Superscript polymerase (Invitrogen).

Quantitative RT-PCR was performed on an Mx3000p instrument (Stratagene), using gene-specific fluorogenic assays (TaqMan; Applied Biosystems). Primers and probes are listed in the Supplemental materials and methods. The cytokine transcripts in spleens were quantitated by RT-PCR using hypoxanthine guanine phosphoribosyl transferase mRNA as an internal standard. Fold induction represents relative expression levels of cytokine transcripts from CpG-ODN-treated mice standardized against the level of cytokine transcripts from untreated B6 mice.

Online supplemental material. Fig. S1 shows various signal strengths induced by different CpG-ODNs. Fig. S2 is a FACS plot of both CD8 α -positive and -negative DC population. Fig. S3 shows IL-12 staining in both WT and TLR9 KO DC after ODN1668 stimulation. Fig. S4 shows lack of NK cells in IL-15 KO mice and depletion of NK cells in anti-asialo-GM1-treated mice. Fig. S5 shows that T and B cells are not required for CpG-mediated protection. Fig. S6 demonstrates that IL-2 is not induced by CpG stimulation. Fig. S7 shows CpG-mediated protection is IDO independent. Fig. S8 shows that CpG only induces minor IKDC expansion. Fig. S9 shows that pDC is partially required for CpG-mediated protection. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20070285/DC1>.

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