Prevention of autoimmune disease by induction of tolerance to Toll-like receptor 7

Tomoko Hayashi[a], Christine S. Gray[a], Michael Chan[a], Rommel I. Tawatao[a], Lisa Ronacher[b], Maureen A. McGargill[c], Sandip K. Datta[d], Dennis A. Carson[a,1], and Maripat Corr[b,1]

[a]Moores Cancer Center, and [b]Department Medicine, University California at San Diego, La Jolla, CA 92093; [c]Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105; and [d]National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD 20892

Contributed by Dennis A. Carson, December 23, 2008 (sent for review December 1, 2008)

Activation of Toll-like receptors (TLR) contributes to the initiation and maintenance of chronic inflammation in autoimmune diseases, yet repeated exposure to a TLR agonist can induce hyporesponsiveness to subsequent TLR stimulation. Here, we used a synthetic TLR7 agonist, 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (SM360320, 1V136) to study TLR7 induced attenuation of inflammatory responses and its application to autoimmune diseases. Repeated low dose administration of this TLR7 agonist induced hyporesponsiveness or tolerance to TLR2, -7, and -9 activators and limited the course of neural inflammation in an experimental allergic encephalomyelitis model. The hyporesponsiveness did not depend on T or B lymphocytes, but did require bone marrow derived cells. In addition, TLR7 tolerance reduced inflammation in a passive antibody mediated arthritis model. TLR7 tolerance did not cause global immunosuppression, because susceptibility to Listeria monocytogenes infection was not altered. The mechanism of TLR7 tolerance involved the up-regulation of 2 inhibitors of TLR signaling: Interleukin 1 Receptor Associated Kinase (IRAK) M, and Src homology 2 domain-containing inositol polyphosphate phosphatase (SHIP)-1. These findings suggest that induction of TLR7 tolerance might be a new therapeutic approach to subdue inflammation in autoimmune diseases.

arthritis | encephalomyelitis | synthetic agonist

Toll-like receptors (TLRs) recognize and are activated by microbial lipids, nucleic acids and proteins leading to rapid stimulation of the innate immune system. However, TLRs also interact with endogenous ligands released by necrotic cells and this process can intensify autoimmune diseases such as rheumatoid arthritis (RA) (1), systemic lupus erythematosus (SLE) (2) and multiple sclerosis (MS) (3). Myeloid differentiation primary response gene 88 (MyD88) is 1 of 2 proximal adapter proteins mediating TLR signal transduction, the other being TIR-domain-containing adapter-inducing IFN-β (TRIF). MyD88 deficient mice do not develop experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis (4), and are resistant to antibody transferred arthritis (5). These results suggest that innate immune mechanisms contribute to inflammation in these models. The discovery that synthetic molecules can bind specific TLRs has generated enthusiasm for the development of novel therapeutics for diseases that involve innate immunity. Some success has been achieved with oligonucleotide antagonists of TLRs 7 and 9 (6, 7).

Although a single administration of a TLR agonist can generate a profound "sickness" response, due to cytokine release (8), repeated exposure to low doses of TLR agonists reduce subsequent inflammatory responses to the same or other TLR agonists (9, 10). Classically, low doses of the TLR4 agonist LPS, desensitize the host to subsequent endotoxin stimulation, a phenomenon known as endotoxin tolerance (11, 12). This desensitization property has been described with other TLR agonists (13) and will be referred to as tolerance in this study.

Recently, TLR7 has been implicated in autoantibody-mediated diseases (14–16). In a murine model of SLE, the genetic loss of TLR7 ameliorated disease, decreased lymphocyte activation and reduced serum IgG (16). These findings prompted us to hypothesize that repeated stimulation with a synthetic TLR7 agonist, at well tolerated low doses (10, 17, 18), might also restrain autoimmune inflammation.

TLR7 recognizes naturally occurring single strand (ss) RNA and synthetic low molecular weight ligands, including imidazoquinolines, and purine-like molecules (19–23). Among the latter, 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (SM360320; designated here as 1V136), has been shown to be a potent and specific TLR7 agonist (23). In the present study, we tested whether repeated low doses of this TLR7 ligand could induce refractoriness, or tolerance, to a TLR ligand challenge. Furthermore, we examined the ability of TLR tolerance induced by chronic, low-dose TLR7 stimulation to alter the course of inflammation in a T cell-dependent multiple sclerosis model and in an autoantibody-induced arthritis model. In both cases the induced TLR7 tolerance significantly reduced autoimmune inflammation.

Results

Repeated Low Doses of a TLR7 Agonist (1V136) Induce Tolerance to a Subsequent Challenge. We described the anti-viral and mucosal protective properties of a TLR7 agonist, 1V136 (8, 19). Here, graded doses of 1V136 were injected i.v. into wild type mice, to determine the maximum dosage that did not induce detectable serum levels of TNFα, and IL-6 after 2 h. A 250-nmol dose of 1V136 was required to elevate serum cytokine levels (Fig. 1*A*). Lower doses of 2, 10, and 50 nmol were ineffective at cytokine induction. However, mice that received 50 nmol of 1V136 daily i.v. for 3 days (pretreatment) and then were challenged intranasally (i.n.) with 500 nmol of 1V136 24 h after the last injection (Fig. 1*B*) were nearly completely refractory to the pharmacologic dose ($P < 0.01$, Fig. 1*C*).

Macrophages are important mediators of primary host defenses and release substantial amounts of IL-6 and TNFα when stimulated via TLRs (24). We investigated whether TLR7-mediated activation of Mitogen-Activated Protein Kinases (MAPK) and/or NF-κB is suppressed in murine bone marrow derived macrophages (BMDMs) preexposed to 1V136. A single dose of 1V136 strongly induced activation of all 3 MAPKs [JNK, extracellular signal-regulated kinases (ERK), and p38], and degradation of IκBα (Fig. 1*D*). However, after overnight incubation, the activation of JNK was completely inhibited, whereas

Author contributions: T.H., M.A.M., S.K.D., D.A.C., and M. Corr designed research; T.H., C.S.G., M. Chan, R.I.T., L.R., M.A.M., S.K.D., and M. Corr performed research; T.H., D.A.C., and M. Corr analyzed data; and T.H., D.A.C., and M. Corr wrote the paper.

The authors declare no conflict of interest.

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[1]To whom correspondence may be addressed. E-mail: mpcorr@ucsd.edu or dcarson@ucsd.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0813037106/DCSupplemental.

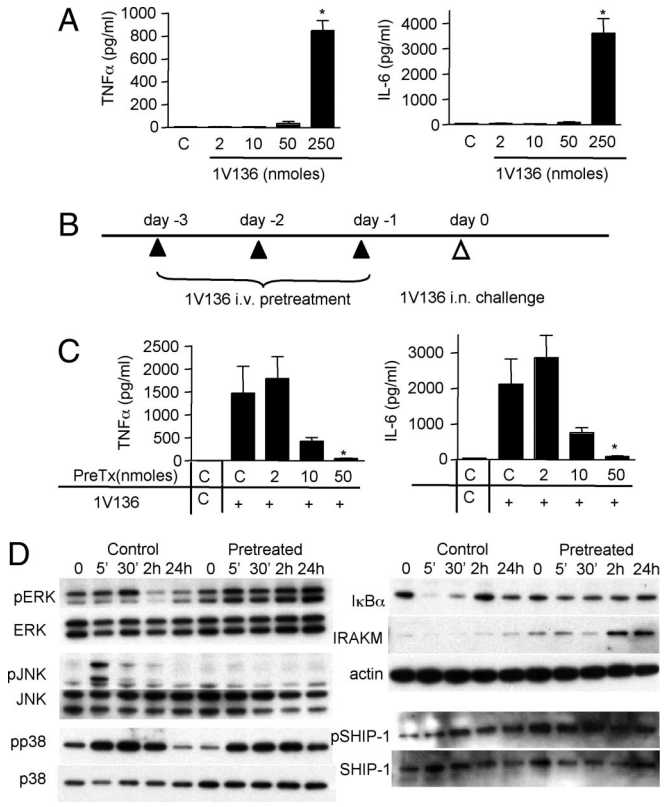

Fig. 1. Induction of TLR7 ligand tolerance. Daily administration of a low dose TLR7 agonist (1V136) tolerizes to a subsequent challenge with the same agonist. (*A*) C57BL/6 mice (10 per group) received 0, 10, 50 and 250 nmol of 1V136 i.v. and sera were collected after 2 h. (*B*) In vivo tolerization protocol: C57BL/6 mice (6 per group) received daily i.v. injections of graded doses 1V136 for 3 days (pretreatment). The mice were then challenged with 500 nmol of 1V136 i.n. 24 h after the last i.v. injection, and sera were collected 2 h later. (*C*) The sera from mice (8 per group) injected with the indicated doses of 1V136 were collected 2 h after i.n. challenge with 1V136. All sera above were tested for TNFα and IL-6 levels by Luminex bead assay. Data shown are means ± SEM and are representative of 3 independent experiments. *, $P < 0.05$ compared with control. (*D*) BMDMs were treated with media or 100 nM 1V136 for 24 h and then stimulated with 100 nM 1V136. The cells were lysed at the indicated intervals and the protein separated by SDS PAGE and transferred to membranes. The relative levels of whole cell lysate IkBα and phospho-44/42 ERK, phospho-JNK, phospho-p38, IRAKM, and phospho-SHIP-1 were assessed by immunoblotting. Shown are representative blots of 3–5 experiments.

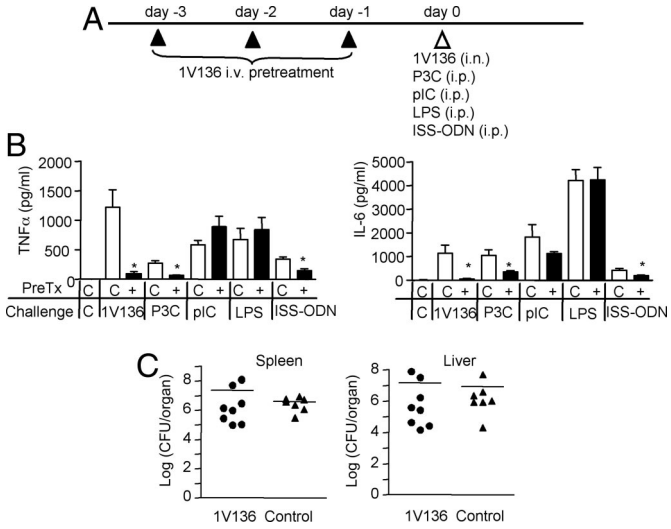

Fig. 2. Induction of cross-tolerance by 1V136. (*A*) In vivo cross tolerization protocol: C57BL/6 mice received i.v. injections of 50 nmol of 1V136 for 3 days. Twenty-four hours after the last injection, mice received 1V136 (i.n. 500 nmol), Pam3Cys (i.p. 25 μg), pIC (i.p. 25 μg per mouse), LPS (i.p. 2 μg per mouse) or ISS-ODN (i.p. 20 μg per mouse). Two hours after challenge the sera were collected. (*B*) Daily injections of 1V136 tolerized mice to challenges with MyD88-dependent TLR2 and TLR9 agonists. Eight to 10 mice per group were treated as indicated and the serum levels of TNFα and IL-6 were measured by multiplex bead assay. Data shown are means ± SEM of 2 independent experiments. *, $P < 0.01$, by unpaired Student's *t* test compared with the vehicle treated group. (*C*) Pretreatment with 1V136 does not impair host defense to *L. monocytogenes* infection. C57BL/6 mice ($n = 8$) were injected with 50 nmol of 1V136 on days -3 to −1 and then infected with 5×10^5 live *L. monocytogenes* i.p. on day 0. Forty-eight hours after infection, CFU in the spleens and livers were determined. Data shown are means ± SEM and are representative of 2 independent experiments. There was no significant difference between 1V136 treated and control (vehicle) treated groups by unpaired Student's *t* test ($P > 0.05$).

there was less effect on p38 and there was no increase in pERK, although the basal levels were higher. TLR refractoriness has been attributed to several mechanisms, including activation of phosphatases like Src homology 2 domain-containing inositol polyphosphate phosphatase (SHIP)-1, and to limiting Interleukin 1 Receptor Associated Kinase (IRAK)-1 activity through an increase in its antagonist IRAKM (25, 26). Overnight exposure to 1V136 increased the phosphorylation status of SHIP-1 and increased the level of IRAKM, suggesting that there are multiple active regulatory mechanisms involved in TLR7 tolerance induction.

TLR7-Induced Tolerance Resulted in Hyporesponsiveness to Other MyD88-Dependent TLR Agonists. Cross tolerance among different TLRs, which share the MyD88 signaling pathway, has been reported (27–29). Hence, we tested whether TLR7 agonist pretreatment could induce in vivo tolerance to other TLR agonists. Groups of mice received daily i.v. injections of 50 nmol of 1V136 for 3 days (pretreatment), and then were challenged with i.n. 1V136 (500 nmol), i.p. Pam3Cys (a TLR2 agonist, 25 μg per mouse), i.p. pIC (a TLR3 agonist, 25 μg per mouse), i.p. LPS (a TLR4 agonist, 2 μg per mouse), or i.p. ISS-ODN (a TLR9 agonist, 20 μg per mouse) (Fig. 2*A*). Pretreatment with 1V136 significantly limited the levels of TNFα and IL-6 released in the sera of Pam3Cys-treated or ISS-ODN treated mice, but not in pIC- treated or LPS-treated mice (Fig. 2*B*).

TLR7 Tolerance Does Not Affect Susceptibility to Listeria Infection. Because low doses of 1V136 induced refractoriness to TLR2 and TLR9 agonists (Fig. 2*A* and *B*), we questioned whether this cross tolerance would increase susceptibility to bacterial infection. As a representative model, mice were pretreated with 150 nmol of 1V136 for 3 days and then infected with *Listeria monocytogenes*. Pretreatment with 1V136 did not change the numbers of bacterial colony forming units in the spleens and livers 48 h after infection (Fig. 2*C*).

Daily Injection of 1V136 Suppresses Inflammation in EAE. Although pretreatment with low doses of 1V136 diminished cytokine release it was unclear if the anti-inflammatory effect would be maintained over a longer time period and if it could alter the course of autoimmune disease. To examine the effect of TLR7 tolerance in an autoimmune model, we immunized animals with a myelin peptide and adjuvants, to induce spinal inflammation in an EAE model that is used to test drugs for the treatment of multiple sclerosis. The mice that received 1V136 from day 6 onward developed significantly less clinical disease and had fewer inflammatory cells in their spinal cords, when compared with controls (Fig. 3).

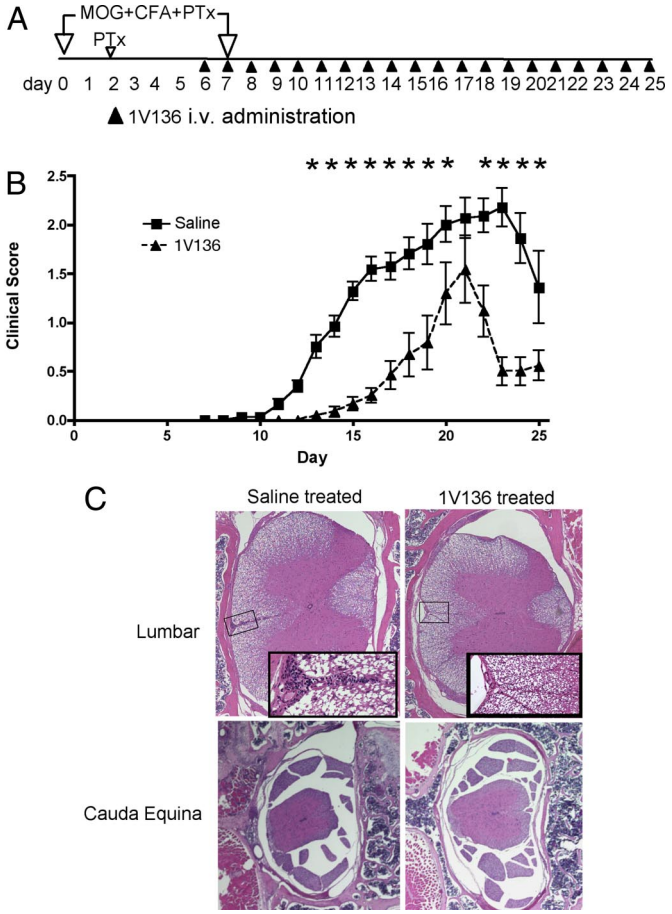

Fig. 3. 1V136 ameliorates EAE disease severity. (*A*) EAE induction and 1V136 administration protocol: C57BL/6 mice were immunized with 125 μg of MOG_{35-55} in CFA with 352 ng of Bordetella pertussis toxin (Ptx) i.p. on day 0 and Ptx again on Day 2. On day 7, the mice were boosted with 125 μg of MOG_{35-55} emulsified in CFA, followed by 1 injection of 325 ng of Ptx. Mice received 50 nmol of 1V136 i.v. daily from day 6 onward. (*B*) Repeated 1V136 injection reduces manifestations of EAE. Mice were immunized with MOG and Ptx as above and divided into saline ($n = 21$) and 1V136 treatment groups ($n = 13$). The mice were scored for disease daily and the data were pooled from 3 independent experiments. 1V136 treated mice had significantly reduced disease induction. *, $P < 0.05$ by 2-way ANOVA with Bonferroni post hoc testing. (*C*) Representative lumbar spinal cord sections of saline- and 1V136-treated animals with EAE. On day 27, mice were killed and lumbar spines were harvested, and prepared for histological evaluation. In the saline treated mice inflammatory infiltrates are seen in the subarachnoid and subdural spaces, but not in the 1V136 treated mice. (Magnification: 100×; *Insets*, 400×.)

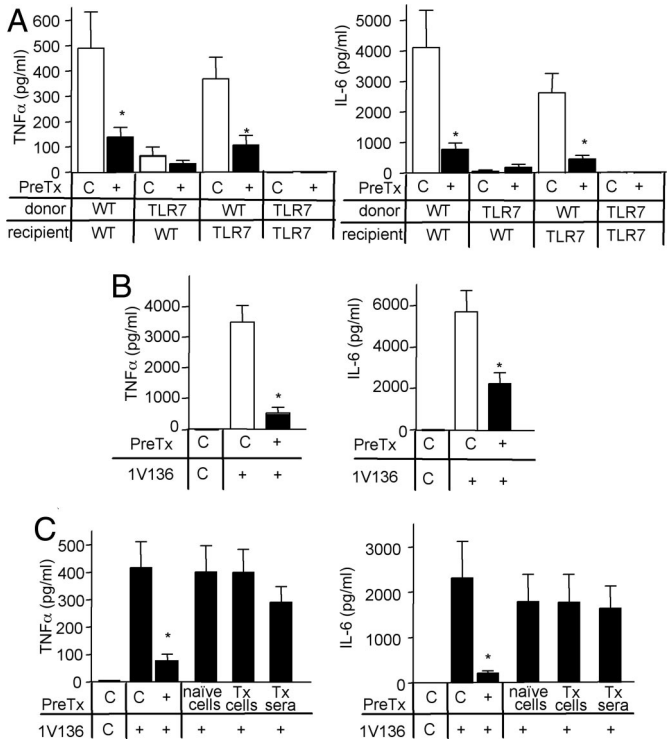

Fig. 4. Haematopoietic cells, but not T or B cells, are necessary for TLR7 tolerance induction. (*A*) C57BL/6 (10–12 per group) mice were lethally irradiated and injected with donor bone marrow cells as indicated. Six weeks after reconstitution, each group of chimeras were divided into 2 groups and pretreated with 50 nmol of 1V136 i.v. daily for 3 days. Twenty-four hours after the last injection, mice were injected with 500 nmol of 1V136. Two hours after injection, sera were collected and the levels of TNFα and IL-6 were measured by Luminex multiplex bead cytokine assay. Data shown are means ± SEM pooled from 2 independent experiments. *, $P < 0.01$, by unpaired Student's *t* test. (*B*) TLR7 tolerance can be induced in Rag1$^{-/-}$ mice. Rag1$^{-/-}$ mice (5 per group) were pretreated with daily i.v. injections of vehicle control (C) or 50 nmol of 1V136 for 3 days. Twenty-four hours after the last injection, mice received 500 nmol of 1V136. Two hours after injection, sera were collected and the levels of TNFα and IL-6 were measured by Luminex multiplex bead cytokine assay. 1V136 induced higher levels of TNFα in Rag1$^{-/-}$ mice, compared with WT mice ($P = 0.001$). Data shown are mean ± SEM of representative of 2 independent experiments. *, $P < 0.001$, by 1-way ANOVA followed by Dunnett's post hoc test, was significant. (*C*) C57BL/6 mice (5 per group) received daily i.v. injections of vehicle control or 50 nmol of 1V136 for 3 days (pretreatment). Mice were killed 4 h after the last injection. Sera and splenocytes were collected and transferred to naïve recipient C57BL/6 mice. Recipient mice (5 per group) were challenged with 500 nmol of 1V136 i.n. 16 h after transfer. Two hour sera samples were assayed for cytokine levels by Luminex multiplex bead assay. Data shown are means ± SEM, representative of 2 independent experiments. *, $P < 0.01$, by 1-way ANOVA followed by Dunnett's post hoc test.

Bone Marrow Derived Cells, but Not T or B Lymphocytes, Are Sufficient to Induce TLR7 Tolerance. As EAE is a complex model, we next questioned which cell types are responsible for TLR7 tolerance. To assess the role of bone marrow-derived cells, chimeras were made by irradiating C57BL/6 and TLR7$^{-/-}$ recipients and reconstituting them with TLR7$^{-/-}$ and C57BL/6 donor bone marrow, respectively. After 8 weeks, the chimeras received daily injections of 50 nmol of 1V136 or PBS for 3 days (pretreatment) and were then administered i.n. 1V136 (500 nmol). Pretreatment with 1V136 significantly reduced the levels of TNFα and IL-6 induced by subsequent 1V136 administration in the irradiated wild type mice reconstituted with wild type (WT) bone marrow cells (WT → WT) and after WT bone marrow reconstitution of TLR7-deficient mice (WT → TLR7$^{-/-}$) (Fig. 4*A*). In contrast, TLR7 $^{-/-}$ bone marrow reconstitution of irradiated WT mice (TLR7$^{-/-}$ → WT) or irradiated TLR7$^{-/-}$ (TLR7$^{-/-}$ → TLR7$^{-/-}$) enabled only minimum cytokine release in serum after TLR challenge (Fig. 4*A*).

To further study the involvement of T and B lymphocytes, we used Rag1$^{-/-}$ mice that are devoid of these cells, using the same tolerizing and challenge protocol. Pretreatment with 1V136 significantly inhibited the response to subsequent 1V136 administration (Fig. 4*B*). These results indicated that bone marrow derived cells, but not T or B cells, were sufficient to induce TLR7 tolerance.

To explore if TLR7 tolerance could be transferred by humoral factors or splenic cell populations, we adoptively transferred splenocytes or serum from 1V136 tolerized mice into naïve mice. Neither serum nor splenocytes from tolerized mice transferred the phenotype of TLR7 tolerance to naïve mice (Fig. 4*C*),

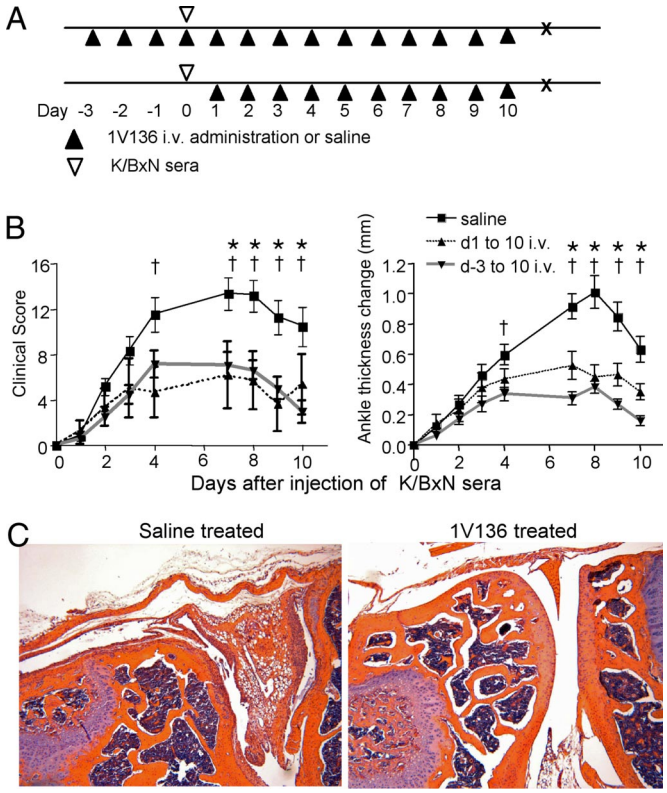

Fig. 5. Repeated injections of 1V136 suppresses induction of inflammatory arthritis. (*A* and *B*) Treatment regimens: 150 μL of pooled K/BxN sera was injected on day 0. Saline or 1V136 was injected iv daily on days −3 to 10 or days 1–10. (*B*) Ankle thickness was measured daily and the mice were clinically scored for joint swelling. Data shown are mean ± SEM of 2 independent experiments. * and †, $P < 0.05$ (days 1–10 and days −3 to day 10, respectively), by 2-way ANOVA followed by Bonferroni post hoc test. (*C*) Representative histology of knee joints of 1V136 treated (day −3 to 10) and saline treated mice. Mice were killed on day 11 and knee joints were fixed, decalcified, and prepared for histologic analysis. (Magnification, ×100.)

suggesting that the tolerized cells must be directly treated with drug.

Daily Injection of a TLR7 Agonist Suppresses Antibody Induced Joint Inflammation. The K/BxN serum transfer model of arthritis is mediated by IgG antibody. We tested 3 different regimens of TLR7 tolerance induction in this system (Fig. 5*A* and Fig. S1). Daily i.v. administration of 1V136 significantly reduced the overall joint inflammation score, and paw swelling, from day 4 to 10 ($P < 0.05$) (Fig. 5*B* and Fig. S1). The reductions seen in the day −3 to 10, or day 1 to 10 treatment regimens were similar, and more robust than observed in the day −3 to −1 pretreatment regimen. To more closely mimic a human situation, 1V136 was orally administered by gavage at 150 nmol per day, which we established as the oral dose required to suppress systemic TNFα and IL-6 release on challenge. This regimen reduced paw swelling, albeit at a more modest level than after i.v. administration (Fig. S1). Histological analysis revealed that daily injections of 1V136 suppressed inflammatory cell infiltration and inhibited joint erosions ($P < 0.02$) (Table 1).

Table 1. Histological scores

	n	Saline-treated	1V136-treated	*P*
Inflamation	6	2.2 ± 0.4	1.2 ± 0.5	0.02
Erosion	6	1.5 ± 0.2	3.0 ± 0.4	0.02

The joints were histologically scored. The 1V136 treated mice had significantly less inflammatory infiltrates and erosions ($P < 0.02$ by unpaired Student's *t* test).

Discussion

During inflammation, there is release of cellular elements such as chromatin and heat shock proteins, and components of the extracellular matrix, which can potentially stimulate TLRs, causing release of cytokines and perpetuation of tissue injury (30). A low level of response to TLR ligands has been proposed to be an adaptive response to chronic exposure of innate immune cells to commensal flora in the gut. The partial refractoriness may protect the host from endotoxin and other TLR ligands. Induction of TLR tolerance may be of clinical utility insofar as induction of TLR7 tolerance in malignant B cells sensitizes them to cytotoxic agents (31). We attempted to use this observation as a means to dampen the continual stimulation of inflammation by endogenous TLR ligands, which may occur in autoimmune diseases. Indeed, repeated low dose stimulation with a synthetic TLR7 agonist attenuated the onset and course of disease in 2 separate inflammatory models of multiple sclerosis and rheumatoid arthritis, respectively.

TLR-induced cytokine production requires the adapter proteins MyD88 and/or TRIF (32). With the exception of TLR3 and TLR4, all TLRs that have been characterized to date signal exclusively through the MyD88-dependent pathway (32). TLR4 is unique in that it activates both the MyD88 and TRIF signaling cascades (33). Agonists that stimulate through MyD88 can reduce the response to other TLRs that obligately require this protein, without affecting TLRs that can use the alternative TRIF pathway (33). Our results showed that the TLR7 agonist 1V136 induced cross tolerance to TLR2 and TLR9 activators, which exclusively use the MyD88-dependent pathway. However, the response to TLR3 and TLR4 stimulation was preserved, presumably via the TRIF pathway.

Experiments with bone marrow chimeras demonstrated that TLR7 is essential not only for 1V136 induced cytokine release, but also for the development of refractoriness to subsequent TLR activation. Adaptive immune cells were not required for this effect, insofar as T and B cell deficient $Rag^{-/-}$ mice were still tolerized. The 1V136 tolerizing effect was also not transferable with splenocytes or sera, suggesting that refractoriness requires direct contact with the drug, and is not mediated by suppressive factors in blood.

Several molecular pathways may be involved in TLR tolerance induction. The tolerized macrophages did not degrade IκBα and did not activate JNK or increase pERK. In addition our data suggest that IRAKM and SHIP-1 play key roles. IRAKM is an inhibitor of IRAK1 (34), a proximal protein kinase in TLR signaling, whereas SHIP-1 is a phosphatase, which may regulate elements further in the cascade. SHIP-1 is also essential for LPS induced tolerance (25). It is likely that the cross tolerance induced by low dose TLR7 stimulation involves the same complex set of mechanisms that restrain excessive TLR activation in mucosal and in associated lymphoid tissues.

In summary, we have demonstrated that induction of TLR7 tolerance in vivo by daily injection of non-toxic doses of a synthetic low molecular weight TLR7 ligand can prevent inflammation of the joint and nervous systems. Although TLR7 tolerance also induced refractoriness to other MyD88-dependent TLRs, it did not alter susceptibility to bacterial infection. Thus, the anti-inflammatory state induced by this regimen might provide a new opportunity for successful treatment of chronic inflammatory diseases.

Materials and Methods

Mice. C57BL/6 mice were purchased from Charles River Laboratories. Rag1$^{-/-}$ were purchased from The Jackson Laboratory. TLR7$^{-/-}$ mice were a gift from S. Akira (Osaka University, Osaka, Japan) and backcrossed for 10 generations onto the C57BL/6 background. KRN transgenic mice were a kind gift of D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and the Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France) (35) and were maintained on a C57BL/6 background (K/B). Arthritic mice were obtained by crossing K/B with NOD/Lt (N) animals (K/BxN). Mice were bred and maintained under standard conditions in the University of California, San Diego Animal Facility. All animal protocols receive prior approval by the institutional review board.

Induction of TLR7 Tolerance. C57BL/6 mice were pretreated with 50 nmol of 1V136 i.v. for 3 days (days −3 to −1). The mice were then treated i.n. or i.p. with 500 nmol of 1V136, or i.p. with (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys$_4$-OH, trihydrochloride (Pam$_3$Cys, 25 μg per mouse, EMC Microcollections GmbH, Tübingen, Germany), polyinosinic-polycytidylic acid (pIC, 25 μg per mouse, Sigma–Aldrich), LPS (*Escherichia coli* 026:B6, 2 μg per mouse, Sigma–Aldrich), or immunostimulatory sequence oligodeoxynucleotide (ISS-ODN 1018, 20 μg per mouse, Trilink Biotechnologies, San Diego, CA). Saline was used as the vehicle control. Sera samples were collected 2 h after TLR agonist challenge and cytokine levels were measured by Luminex multiplex bead cytokine assay (Millipore). The lower limits of detection for TNFα and IL-6 detection were 20 pg/mL and 10 pg/mL, respectively.

Adoptive Transfer. C57BL/6 mice received daily i.v. injections of 50 nmol of 1V136 for 3 days (pretreatment). Mice were killed 4 h after the last injection and serum and splenocytes were collected. Splenocytes (4×10^7 per mouse) and sera (300 μL per mouse) were transferred to naïve C57BL/6 mice. Recipient mice were then challenged with i.n. 500 nmol of 1V136 16 h after transfer. Serum samples were collected 2 h after intranasal challenge of 1V136 and the levels of cytokine were measured by Luminex multiplex bead cytokine assay.

Bone Marrow Chimeras. Bone marrow cells from the femurs and tibia of donor mice ($5–8 \times 10^6$) were injected i.v. into lethally irradiated recipient mice (900 cGy). To verify chimerism with >90% bone marrow engraftment, we used quantitative PCR on peripheral blood cells and compared it to a standard curve as reported (36). Genomic DNA was isolated from peripheral leukocytes using a DNAeasy kit (Qiagen) and quantified by spectrometer. The DNA from each mouse was serially titrated from 200 ng per well to 1.6 ng per well. The DNA was amplified with primers specific to WT, TLR7$^{-/-}$ and involucrin for 30 cycles. Standard curves with WT or TLR7 DNA were established with 1:2 serial dilutions. The ΔCt values using involucrin as the standardizing gene were compared with the standards to determine the percentage WT vs. TLR7$^{-/-}$ genomic DNA.

Immunoblot. Bone marrow derived macrophages were prepared from the femora and tibia of mice as described in ref. 20. After 7 days in culture macrophages were replated in 6-well plates at $1 - 2 \times 10^6$ cells per well and tested on day 10. After removal of medium, cells were disrupted in lysis buffer and proteins were electrophoresed under reducing conditions and transferred to a membrane. Antibodies to IRAKM, phospho-SHIP-1, phospho-JNK, phospho-ERK, phospho-p38 (Cell Signaling) and IκBα (Santa Cruz) were used. Horseradish peroxidase-conjugated anti-IgG (Santa Cruz) was used as the secondary antibody. The membranes were developed using a chemiluminescence system (ECL detection reagent: Amersham Life Science).

Experimental Allergic Encephalomyelitis. Mice were immunized with 125 μg of myelin oligodendrocyte glycoprotein (MOG)$_{35–55}$ (Genemed Synthesis) emulsified in complete Freund's adjuvant containing 0.4 mg of H37Ra *Mycobacterium tuberculosis* (Chondrex) in each hind flank. The mice also received 325 ng of *Bordetella pertussis* toxin (Ptx) (List Biological) i.p. immediately after immunization and again on day 2. On day 7, the mice were boosted with 125 μg of MOG$_{35–55}$ emulsified in CFA, followed by an injection of 325 ng Ptx. Mice received 50 nmol of 1V136 i.v. daily from day 6 onward. The mice were scored for disease using the following scale: 0, no signs of disease; 0.5, altered gait and/or hunched appearance; 1, limp tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, complete hind limb paralysis and partial forelimb paralysis. Mice with a score of 4 were euthanized. On day 27, mice were killed and lumbar spines were harvested, and decalcified with Cal-ExII Fixative/Decalcifier (Fisher Chemical). Sections were stained with hematoxylin and eosin (Moores Cancer Center Histology Core Facility).

K/BxN Serum Transfer. Arthritic adult K/BxN mice were bled and the sera were pooled. Recipient mice were injected with 150 μL of sera i.p. on day 0. Mice were treated with 150 nmol of 1V136 i.g. and 50 nmol of i.v. daily, because these doses gave biologically comparable effects. Each mouse was scored on a scale of 0–4 per paw. Ankle thickness was measured with a caliper (Manostat) (37). Whole knee joints and hind paws were fixed in 10% formalin, decalcified, trimmed, and embedded. Sections were prepared from the tissue blocks and stained with hematoxylin and eosin (HistoTox). Inflammation and erosion were scored as described in ref. 38.

Listeria Infection. *Listeria monocytogenes* strain 10403s was a gift from E. Pamer (Memorial Sloan-Kettering, New York). Mice were infected with 5×10^5 live *L. monocytogenes* i.p. on day 0 as described in ref. 39. Forty-eight hours after infection, colony-forming units (CFU) in the spleens and livers were determined.

Statistical Analysis. Data are presented as mean ± SEM. Student's *t* test was used for comparisons between 2 groups and ANOVA for repeated measures or multiple groups with Dunnett's or Bonferroni's post hoc test using PRISM version 4.0b (GraphPad Software, San Diego, California).

ACKNOWLEDGMENTS. This work was funded in part by National Institutes of Health Grants 5 U01 AI056453 and CA119335, the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases, and the Arthritis Foundation.

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