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EC144, a Synthetic Inhibitor of Heat Shock Protein 90, Blocks Innate and Adaptive Immune Responses in Models of Inflammation and Autoimmunity

Theodore J. Yun,* Erin K. Harning,* Keith Giza,† Dania Rabah,‡ Ping Li,§ Joseph W. Arndt,¶ David Luchetti,† Marco A. Biamonte,§ Jiandong Shi,¶ Karen Lundgren,‖ Anthony Manning,# and Marilyn R. Kehry*

Heat shock protein 90 (Hsp90) is a molecular chaperone involved in folding and stabilizing multiple intracellular proteins that have roles in cell activation and proliferation. Many Hsp90 client proteins in tumor cells are mutated or overexpressed oncogenic proteins driving cancer cell growth, leading to the acceptance of Hsp90 as a potential therapeutic target for cancer. Because several signal transduction molecules that are dependent on Hsp90 function are also involved in activation of innate and adaptive cells of the immune system, we investigated the mechanism by which inhibiting Hsp90 leads to therapeutic efficacy in rodent models of inflammation and autoimmunity. EC144, a synthetic Hsp90 inhibitor, blocked LPS-induced TLR4 signaling in RAW 264.7 cells by inhibiting activation of ERK1/2, MEK1/2, JNK, and p38 MAPK but not NF-κB. Ex vivo LPS-stimulated CD11b+ peritoneal exudate cells from EC144-treated mice were blocked from phosphorylating tumor progression locus 2, MEK1/2, and ERK1/2. Consequently, EC144-treated mice were resistant to LPS administration and had suppressed systemic TNF-α release. Inhibiting Hsp90 also blocked in vitro CD4+ T cell proliferation in mouse and human MLRs. In vivo, semITHERAPEUTIC administration of EC144 blocked disease development in rat collagen-induced arthritis by suppressing the inflammatory response. In a mouse collagen-induced arthritis model, EC144 also suppressed disease development, which correlated with a suppressed Ag-specific Ab response and a block in activation of Ag-specific CD4+ T cells. Our results describe mechanisms by which blocking Hsp90 function may be applicable to treatment of autoimmune diseases involving inflammation and activation of the adaptive immune response. *The Journal of Immunology, 2011, 186: 563–575.

Heat shock protein 90 (Hsp90) is a cytoplasmic molecular chaperone that is involved in maturation and stabilization of a subset of cellular proteins termed “client” proteins (reviewed in Ref. 1). The number of Hsp90 client proteins is extensive, and they range in function from cell cycle control to signal transduction and cell growth regulation (for a curated list, see http://www.picard.ch/downloads/Hsp90interactors.pdf). Inhibitors of ATP binding to Hsp90, such as the microbially derived benzoquinone ansamycin antibiotic geldanamycin, have been used extensively to characterize Hsp90 function and cellular client proteins (2). Recently, novel fully synthetic Hsp90 inhibitors have been produced that have activity comparable to the natural antibiotics yet do not share their pharmacologic liabilities (3). Several compounds are currently in phase II clinical trials for cancer indications (4, 5).

Destabilizing Hsp90 client proteins has been shown to block tumor cell growth by disrupting multiple cellular activation pathways and blocking the cell cycle. A number of key signaling proteins that are Hsp90 clients also play important roles in activation of normal cells involved in innate and adaptive immune responses. Hsp90 inhibition by benzoquinone ansamycins has been shown in vitro to block macrophage proinflammatory responses, and several studies have provided evidence that IL-1R–associated kinase-1 (IRAK1) stability and Raf-1 and MEK1/2 activation are blocked by geldanamycin (6–11). Hsp90 also appears to be critical for maintaining the stability and function of both the newly synthesized and phosphorylated forms of the Src family kinase p56

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Abbreviations used in this paper: 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; AUC, area under the curve; CIA, collagen-induced arthritis; CII, collagen type II; FGF, fibroblast growth factor; Hsp90, heat shock protein 90; IP-10, IFN-γ–inducible protein-10; IRAK1, IL-1R–associated kinase 1; MDC, macrophage-derived chemokine; MGB, minor groove binder; MMA, mycophenolic acid; MMP-9, matrix metalloproteinase-9; PEC, peritoneal exudate cell; SCF, stem cell factor; TIMP-1, tissue inhibitor of metalloproteinase-1; Tpl2, tumor progression locus 2.
response in retinal cells (14), and geldanamycin has been shown to suppress adjuvant-induced arthritis (8). Similarly, Dello Russo et al. (15) demonstrated that 17-AAG could inhibit proinflammatory TLR4 stimulation in vitro and dramatically reduce disease incidence and severity in myelin oligodendrocyte glycoprotein–peptide–induced experimental autoimmune encephalitis. These therapeutic effects of Hsp90 inhibition correlated with reduced T cell responses to the cognate Ag, and Hsp90 inhibition was proposed to ameliorate disease by downmodulating the innate and adaptive immune responses (15).

More recently, a disease relevant rationale for targeting Hsp90 in autoimmunity with a synthetic nonansamycin Hsp90 inhibitor was extended to rheumatoid arthritis by demonstration of efficacy in rat arthritis models (16). However, the underlying mechanism of efficacy of Hsp90 inhibition in these models was not defined on relevant cell types from the animals. Most of the experiments demonstrating inhibition of inflammatory signals were performed in vitro on human cell lines or fibroblasts with implied roles in rat arthritis model pathogenesis, and immune cells were not examined (16).

In this report, we demonstrate the efficacy of EC144, a novel selective synthetic Hsp90 inhibitor, in modulating the innate and adaptive immune responses driving disease in two rodent arthritis models and in an LPS-induced proinflammatory cytotokine production model. Mice dosed with EC144 showed diminished TNF-α production in response to TLR4 stimulation that correlated with inhibition in macrophages of tumor progression locus 2 (Tpl2) phosphorylation. Efficacy of EC144 in a rat arthritis model correlated with inhibition of proinflammatory cytokine mRNAs in the paws. In addition, in a mouse arthritis model, therapeutically administered EC144 demonstrated efficacy and suppressed recall responses of Ag-specific CD4+ T cells to cognate or autoantigen. Thus, this report provides a mechanistic basis to explain the potential disease-inhibitory effects of selectively targeting Hsp90 in models involving dysfunction of innate and adaptive immune responses.

**Materials and Methods**

**Animals**

Female DBA/1 and BALB/c mice (Tacfon Farms, Germantown, NY, or The Jackson Laboratory, Bar Harbor, ME) and female DA rats (Tacfon Farms) were used for the in vivo experiments. All in vivo experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Biogen Idec (San Diego, CA).

**Reagents**

Test compounds EC144 and 17-AAG were synthesized at Biogen Idec. The backbone of EC144 is based on 2-amino-6-chloro-9-(4-iodo-3,5-dimethylpyridin-2-ylmethyl)purine, and its synthesis has been described previously (17). Specific synthesis of EC144 is detailed in issued U.S. Patent No. 7,544,672 (18). For in vitro treatments, EC144, 17-AAG, and mycophenolic acid (MMA) (Sigma-Aldrich, St. Louis, MO) were dissolved in DMSO at a concentration of 10 mM. Abs used in immunoblotting were obtained from Cell Signaling Technology (Beverly, MA) and were specific to the following proteins: phospho-Tpl2 (Ser444), phospho-JNK (Thr183/Thr185), phospho-ERK1/2 (Thr202/Tyr204), phospho-MEK1/2 (Ser217/ Ser221), phospho-p38 MAPK (Thr180/Tyr182, 3D7), ERK1/2 (137F5), Tpl2, p38MAPK, JNK, and MEK1/2. Anti-GAPDH was a rabbit polyclonal IgG (Abcam, Placid, NY) containing Halt Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA) and Halt EDTA (Thermo Scientific). For peritoneal CD11b+ cells, mice were injected with 2 ml 3% thioglycollate (Becton Dickinson, Sparks, MD) on day −4. Beginning on day −2, mice were dosed once daily for 3 d with EC144 (10 mg/kg). Three hours after the last dose, mice were euthanized by CO2 asphyxiation. Peritoneal exudate cells (PECs) were isolated by lavage, and CD11b+ cells were purified using MACS with a Mouse CD11b+ MicroBeads positive selection kit (Miltenyi Biotec, Auburn, CA). CD11b+ PECs were suspended in warm medium and treated with IL-10 (5 ng/ml) for 30 min. Cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer containing Halt Protease Inhibitor Cocktail and Halt EDTA.

Protein concentrations of cell lysates were determined by Bradford assay (Bio-Rad). Samples were adjusted to 1 mg/ml in lysis buffer. NuPage SDS Sample Buffer (Invitrogen, Carlsbad, CA) and NuPage Reducing Sample Buffer (Invitrogen) were added, and samples were heated to 100°C for 10 min and then stored frozen at −80°C. For immunoblots, samples were thawed, heated to 100°C, and electrophoresed on a NuPage 4–12% SDS polyacrylamide gel (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes (Invitrogen) for immunoblotting. Optimal dilutions of primary Abs were determined for individual Ab lots as indicated in the manufacturer’s instructions. Binding of the secondary Ab (HRP-conjugated goat anti-rabbit IgG, 1:2000 in 5% BSA) was revealed by either Supersignal West Pico or Femto Chemiluminescent Substrate System (Pierce, Rockford, IL). To strip the membranes of bound Ab, membranes were incubated in Restore Western blot Stripping Buffer (Thermo Scientific), rinsed, and blocked with 5% BSA (Sigma Aldrich). Stripped membranes were immunoblotted with Ab to GAPDH as above.

**MLRs**

For the mouse MLR, DBA/1 and BALB/c mice were euthanized by CO2 asphyxiation, and CD4+ cells or Thy-1+ cells were isolated from DBA/1 or BALB/c splenocytes, respectively, using MACS (Miltenyi Biotec). BALB/c Thy-1+ splenocytes were irradiated (2000 rad) using a [35S]source (MDS
Nordin) and combined with DBA/1 CD4+ cells at a ratio of 1:5 (responders:stimulators). MLR culture was for 5 d at 37˚C in DMEM supplemented with 2-ME (Sigma-Aldrich), 4 mM l-thymidine (Invitrogen), 10 mM sodium pyruvate (Invitrogen), 1X MEM nonessential amino acids (Invitrogen), 10 mM HEPES (Invitrogen), 1X antimycotic antibiotic (Invitrogen), and 10% FCS (Hyclone Laboratories).

For each human MLR experiment, blood from two in-house donors (150 ml/donor) was collected into heparinized vacuum tubes (BD Biosciences, San Jose, CA). All human blood was obtained with donor’s informed written consent, and experiments with human blood were conducted under procedures established by the Biogen Idec Occupational Health and Safety Board. PBMCs were isolated on Histopaque (Sigma-Aldrich), and CD4+ cells were separated from both donors using MACS Human CD4+ T cell Isolation Kit II (Miltenyi Biotec). Non-CD4+ cells were irradiated (2000 rad). CD4+ cells from one donor (7.5 X 10^6) were combined with non-CD4+ cells of the other donor at a ratio of 1:3 (responders:stimulators) in round-bottom 96-well plates (Costar, Cambridge, MA). MLR culture was for 5 d at 37˚C in X-Vivo medium (BioWhittaker, Walkersville, MD) and supplemented with 4 mM l-thymidine, 1 mM sodium pyruvate, 1X MEM nonessential amino acids, 10 mM HEPES, 1X antimycotic antibiotic, and 10% FCS (Hyclone Laboratories).

For mouse and human MLRs 18 h prior to harvest, [3H]thymidine (American Radiolabeled Chemicals, St. Louis, MO) was added to the culture (1 µCi/well). Cells were harvested using a Packard Filtermate 196 cell harvester (PerkinElmer, Wellesley, MA) onto UniFilter GFC plates (PerkinElmer). Scintillation fluid (50 µl) was added to the filters, and [3H]thymidine incorporated into DNA was quantified using a TopCount NXT (PerkinElmer).

LPS induction of systemic TNF-α
Mice were dosed by oral gavage with EC144 in two gavages given 3 h apart. Three hours after the second gavage, mice were injected i.p. with LPS (200 µg/kg). One hour later, blood was collected by facial vein puncture in BD Microtainer Serum Separation Tubes (BD Biosciences). Serum were frozen at −80˚C and assayed for TNF-α as above by ELISA.

Pharmacokinetic studies in DBA/1 mice
Female DBA/1 mice were administered a single dose of EC144 (5 mg/kg) by oral gavage (n = 3). Blood, spleen, and lymph nodes were obtained via terminal collection at 0.25, 0.5, 1, 3, 5, 7, 16, and 24 h after dosing. Plasma samples were treated with 3 volumes of acetonitrile to precipitate proteins. Spleens and lymph nodes were homogenized in water containing 0.1% formic acid using a tissue homogenizer (Covaris, Woburn, MA), and the homogenate was extracted with an equal volume of acetonitrile containing 0.1% formic acid. The supernatant fraction was collected and analyzed by ESI-MS by liquid chromatography-tandem mass spectrometry. All pharmacokinetic analyses were performed using noncompartmental methods with WinNonlin V5.2 (Pharsight, Sunnyvale, CA).

Collagen-induced arthritis models
Mouse collagen-induced arthritis. Female DBA/1 LacI mice were sensitized with pristane (400 µl i.p.; Sigma-Aldrich) to maximize severity and incidence of disease (20). Three weeks later, mice were immunized intradermally with chicken collagen type II (CII) (Chondrex, Redmond, WA); 100 µl 1:1 emulsion of 4 mg/ml chicken CII in 0.05 N acetic acid/CFA) in both pinnae and at one site in the abdominal skin. Twenty-one days postimmunization, mice received a second injection of chicken CII (100 µg PBS, i.p.). EC144 administration was by oral gavage beginning on the day of the second immunization.

Rat collagen-induced arthritis. Female DA rats were immunized intradermally with rat collagen type II (CII) (Chondrex; 250 µl 1:1 emulsion of 2 mg/ml rat CII in 0.05 N acetic acid/CFA) in both pinnae and at one site in the abdominal skin. Ten days postimmunization, rats received a second injection of chicken CII (100 µg CII in PBS, i.p.). EC144 administration was by oral gavage beginning on the day of the second immunization.

Clinical severity of collagen-induced arthritis (CIA) was assessed by monitoring inflammation on all four paws, applying a scale ranging from 0 to 4. Each paw was graded as follows: 0, normal; 1, mild but definite redness and swelling of the ankle or wrist, or redness and swelling of any severity for one or two digits; 2, moderate to severe redness and swelling of the ankle or wrist, or more than two digits; 3, redness and swelling (pronounced edema) of the entire paw; and 4, maximally inflamed limb with involvement of multiple joints. The sum of the four individual scores was the arthritis index, with a maximal possible score of 16 for each animal. The mean arthritis index was calculated over an experimental group.

Anti-CII Ab ELISA
Flat-bottom 96-well microtiter plates (MaxiSorp; Nunc, Naperville, IL) were coated with ELISA grade CII from the appropriate species (Chondrex; 10 µg/ml overnight at 4˚C) and blocked with 2% nonfat dry milk. Sera samples were diluted in PBS and 0.1% Tween 20 and incubated in the bioanalyzer washed wells (30 min at 37˚C). Plates were washed three times, and biotin-anti-ration or anti-mouse isotype-specific secondary mAbs (BD Pharmingen, San Diego, CA) were added to wells (30 min at 37˚C). Bound secondary Abs were detected by streptavidin-HRP and ABTS substrate. Absorbance was read at 405 nm with a SpectraMax spectrophotometer (MDS Analytical Technologies, Sunnyvale, CA).

Histology
Histological quantification on joints from arthritic mice was performed 40 d after the initial CIA/CFA immunization. Limbs were collected and fixed in 10% neutral-buffered formalin for 48 h, rinsed in running water, and transferred to a decalcifying solution (20% formic acid buffered with 10% sodium citrate) for 5 d. Samples were rinsed in running water, transferred to 70% ethanol, and processed into paraffin blocks. Processed tissues were H&E stained and microscopically assessed by a veterinary pathologist for the presence of inflammation, cartilage change, bone change, and synovial alteration. Pathology for each parameter was scored on a scale of 0–3.

Ex vivo Ag recall assay
Mice were pristane primed, immunized, and boosted as described above under mouse CIA. Starting on the day of the second immunization (day 21), mice were dosed every other day with EC144 by oral gavage. On d 27 mice were sacrificed and splenic CD4+ T cells isolated by MACS (responder). Stimulator cells were isolated from spleens of naïve DBA/1 mice by MACS depletion of Thy1+ cells. Cells were plated at a responder to stimulator ratio of 1:5. Mouse or chicken CII was added to each culture at the indicated concentrations. Anti-CD40 mAb (BD Biosciences) and diallylated anti-CD154-PE (BD Biosciences) were added to each culture (20 µg/ml each) and incubated overnight at 37˚C. Anti-CD154 was included in the cultures to stabilize CD154 expression on the surface of recently activated CD4+ cells (21–23). Cells were stained with anti-CD69-PE-TC, anti-CD154-PE, anti–B220-allophycocyanin, anti–CD11b-allophycocyanin, 7-aminocoumarin D, anti–CD25-PE-Cy7, and anti–CD4-allophycocyanin-Cy7 (all from BD Biosciences) and analyzed by flow cytometry using a FACSCanto (BD Biosciences). Data were collected and analyzed using FACSDiva software (BD Biosciences).

Real-time PCR for cytokines in rat paws
Nineteen days after primary immunization, rats were euthanized, and whole rat paws were quick frozen in liquid nitrogen and stored at −80˚C. For RNA preparation, paws were pulverized in a liquid nitrogen bath with a mortar and pestle, transferred into 0.06 ml of RNasefree PBS, and further processed as described above.

Forward and reverse oligonucleotide primers and TaqMan minor groove binder (MGB) probes were designed using Primer Express v2.0. (Applied Biosystems) as follows: GAPDH, 5’-CCTGGGAGAATCCGCAAGTAT-3’ (forward), 5’-TCTGGCCGCTGCTT-3’ (reverse), and 5’-ATGACATCAAGAGGTG-3’ (probe); IL-1β, 5’-TGACAGCAAGGGAAAGATIAAGG-3’ (forward), 5’-CTCATCTGAGAGCGGAGTCTC-3’ (reverse), and 5’-TCTGGCTTCCAAGGCCCCT-3’ (probe); TNF-α, 5’-CCCGA- AAAGCAGAAGAACCA-3’ (forward), 5’-GCCTGGCCGCTGCTT-3’ (reverse), and 5’-CCCGAGGCTGCTT-3’ (probe); and IL-6, 5’-CCCGG -CAGGGAAGAAGTCTA-3’ (forward), 5’-GCCGAGGAAACCTCTATGCGTTC-3’ (reverse), and 5’-CTCATCTGAGAGCGGAGTCTC-3’ (probe). TaqMan MGB probes contained a 5’-covalently linked fluorescent reporter dye (FAM) and an MGB/nonfluorescent quencher covalently linked to the 3’ end. Gel-purified oligonucleotide standard templates were purchased from BioSource International (Camarillo, CA). Desalted primers and HPLC-purified probes were from Applied Biosystems. A primer and probe set for GAPDH was used as a normalizing control.
Thermal cycling was performed using quadruplicate PCRs mixed in a 96-well plate (20 µl) in TaqMan Universal Master Mix (Applied Biosystems) and transferred to a 384-well optical plate. Thermal cycling was in a 7900HT thermal cycler (Applied Biosystems) under the following conditions: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s and 60°C for 60 s. Fluorescence emission was collected every 7 s for the length of the run. Relative transcript quantities were determined for each sample by comparison with an oligonucleotide standard curve using Sequence Detection Software (Applied Biosystems) and normalized to a GAPDH control.

Results

EC144 is a potent and selective synthetic Hsp90 inhibitor

EC144 is a novel synthetic Hsp90 inhibitor with the chemical structure shown in Fig. 1A. An x-ray crystallographic structure of EC144 bound to the N-terminal domain of human Hsp90β was determined at 2.2 Å resolution (PDB ID code 3NMQ) and verifies that the inhibitor binds to the ATP binding site of Hsp90 (Fig. 1B) (24). A network of hydrogen-bonding interactions involving the pyrrolopyrimidine ring of EC144 with Asp95, Thr119, and conserved water molecules is a characteristic feature shared by other Hsp90 modulators that have been reviewed recently (25). In contrast to these commonly observed hydrophilic interactions, the methoxypyridine at the right side of EC144 buries into a hydrophobic pocket composed of Leu104, Phe138, Tyr139, and Trp162.

EC144 was tested for inhibitory activity against a panel of 274 human protein kinases that bind and hydrolyze ATP. No significant inhibition of activity of any kinases was observed using 10 µM EC144 (Supplemental Table I).

Using a fluorescence polarization competition assay to measure binding affinities for EC144 to Hsp90α (4), the Ki value for EC144 was determined to be 0.2 nM. The Ki values for the binding of EC144 to the Hsp90 paralogs Grp94 (endoplasmic reticulum localized) and TNF-related activation protein-1 (mitochondria localized) were determined to be 255 and 61 nM, respectively, further demonstrating the selectivity of EC144 for cytoplasmic Hsp90 (Supplemental Table II).

HER2, a member of the epidermal growth factor receptor family that is involved in cellular growth signaling, is a client protein of Hsp90 that is highly sensitive to Hsp90 inhibition (26). Blocking Hsp90 in the MCF-7 breast cancer cell line results in rapid HER2 degradation and is a convenient assay for determining cellular potency of Hsp90 inhibitors (4, 27). MCF-7 cells were treated with various concentrations of the Hsp90 inhibitors EC144 and BIIB021 or the geldanamycin analog 17-AAG for 16 h, and HER2 expression was quantified by flow cytometry. The effective concentrations required to induce a 50% inhibitory effect (EC50) were determined to be 6.5 and 12.6 nM for EC144 and 17-AAG, respectively (Supplemental Fig. 1). In comparison, the EC50 value for BIIB021 in this assay was on average 38 nM (4) (Supplemental Fig. 1). Thus, in MCF-7 cells, EC144 inhibited Hsp90 and induced HER2 degradation with an equal or greater potency as compared with benzoquinone ansamycin-derived Hsp90 inhibitors.

In preliminary studies with EC144 numerous potential Hsp90 client proteins in normal immune cells were explored, and the clients most sensitive to Hsp90 inhibition were found to be those involved in the TCR and TLR signaling pathways (http://www.picard.ch/downloads/Hsp90interactors.pdf). Further studies focused on these aspects of in vitro and in vivo Hsp90 inhibition.

EC144 inhibits TLR4 signaling in RAW 264.7 by blocking MAPK activation

The anti-inflammatory effects of geldanamycin on LPS-stimulated TLR4 signaling have been previously noted in the mouse macrophage-like cell lines J774 and RAW 264.7 as an inhibition of TNF-α and IL-6 production (6, 9–11). To confirm this effect with EC144 and to study the mechanism by which Hsp90 inhibition blocks the TLR4 signaling pathway, RAW 264.7 cells were treated with various concentrations of EC144 and stimulated with LPS.

EC144 treatment resulted in a dose-dependent inhibition in LPS-stimulated production of the proinflammatory cytokines TNF-α and IL-6, with EC50 values of ~20 nM (Fig. 2). To more broadly survey the effects of Hsp90 inhibition on LPS-induced cytokine production, a multiplex assay measuring 58 individual cytokines and chemokines was performed. Of the 58 proteins analyzed, 25 showed a measurable increase in supernatants from LPS-stimulated RAW 264.7 cells as compared with unstimulated cells, and all but one of these increases were inhibited by EC144 (Table I, Supplemental Fig. 2). Maximal inhibition of cytokine and
Because LPS signaling activates both the MAPK and NF-κB pathways, it was possible that Hsp90 inhibition by EC144 blocked activation of these pathways. In macrophages, the MAPKs, ERK1/2, JNK1/2, and p38 MAPK, are all activated by LPS, and inhibition of any one of these MAPKs results in attenuation of TNF-α induction (28). To determine effects of EC144 on LPS-induced MAPK activation, RAW 264.7 cells were treated for 4 h with various concentrations of EC144 and stimulated with LPS for 30 min, a time previously determined to be optimal for detecting maximally phospho-ERK1/2, JNK1/2, and p38 MAPK (data not shown). Treatment of RAW 264.7 cells with EC144 resulted in a dose-dependent decrease of phospho-ERK1/2 and phospho-JNK1/2 (Fig. 3A). Levels of phospho-p38 MAPK were also mildly decreased by EC144 treatment but not to the same extent as phospho-ERK1/2 or phospho-JNK1/2 (Fig. 3A, left panel). Parallel immunoblot analysis revealed no significant changes in total ERK1/2, JNK1/2, or p38 MAPK protein levels (Fig. 3A, right panel). To further characterize the strong dose-dependent inhibition of ERK1/2 phosphorylation by EC144, the phosphorylation of MEK1/2, the MAP2K upstream of ERK1/2, was examined. EC144 treatment markedly decreased MEK1/2 phosphorylation in a dose-dependent manner but did not alter levels of total MEK1/2 (Fig. 3A). These results demonstrate that in RAW 264.7 cells Hsp90 inhibition blocks LPS-induced activation of the MAPK pathways that are required for LPS-induced TNF-α production.

LPS stimulation of macrophages also activates the NF-κB pathway, and one of the transcriptional targets of NF-κB is TNF-α. Because previous studies have reported inhibitory effects of geldanamycin-related compounds on NF-κB activation (29), NF-κB p50 binding activity was analyzed in nuclear extracts from RAW 264.7 cells incubated with various concentrations of EC144 and stimulated with LPS (10 ng/ml) for 8 h. Cytokine concentrations in the culture supernatants were quantified using the RodentMAP multiplex cytokine array technology by Rules Based Medicine based on a standard curve. The EC_{50} values were calculated from the EC144 dose-response curves as the EC144 concentrations required to induce 50% inhibition of maximal cytokine production.

**Table 1. Hsp90 inhibition in RAW 264.7 cells blocks production of multiple LPS-induced soluble factors by RodentMAP analysis**

<table>
<thead>
<tr>
<th>Soluble Factor</th>
<th>EC_{50}(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>17.4</td>
</tr>
<tr>
<td>FGF basic</td>
<td>45.4</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>14.7</td>
</tr>
<tr>
<td>M-CSF</td>
<td>21.1</td>
</tr>
<tr>
<td>MMP-9</td>
<td>22.2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>20.1</td>
</tr>
<tr>
<td>IL-1α</td>
<td>29.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>20.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>19.7</td>
</tr>
<tr>
<td>IL-11</td>
<td>40.0</td>
</tr>
<tr>
<td>LIF</td>
<td>17.8</td>
</tr>
<tr>
<td>Oncostatin M</td>
<td>39.4</td>
</tr>
<tr>
<td>SCF</td>
<td>42.5</td>
</tr>
<tr>
<td>Tissue factor</td>
<td>23.1</td>
</tr>
<tr>
<td>IP-10</td>
<td>19.8</td>
</tr>
<tr>
<td>MDC</td>
<td>12.7</td>
</tr>
<tr>
<td>MCP-1</td>
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</tr>
<tr>
<td>MCP-3</td>
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<tr>
<td>MIP-2</td>
<td>19.7</td>
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<tr>
<td>MIP-1α</td>
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<tr>
<td>MIP-3β</td>
<td>21.7</td>
</tr>
<tr>
<td>RANTES</td>
<td>23.0</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>25.2</td>
</tr>
</tbody>
</table>

RAW 264.7 cells were incubated with various concentrations of EC144 and stimulated with LPS for 4 h and stimulated with LPS (10 ng/ml) for 8 h. Cytokine concentrations in the culture supernatants were quantified using the RodentMAP multiplex cytokine array technology by Rules Based Medicine based on a standard curve. The EC_{50} values were calculated from the EC144 dose-response curves as the EC144 concentrations required to induce 50% inhibition of maximal cytokine production.

FIGURE 2. Inhibition of Hsp90 with EC144 blocks LPS-induced production of the proinflammatory cytokines TNF-α and IL-6 by RAW 264.7 cells. A, RAW 264.7 cells were incubated with EC144 for 4 h and stimulated with LPS (10 ng/ml) for 4 h. TNF-α concentrations in the culture supernatants were quantified by ELISA. A time course to determine an optimal time for treating RAW 264.7 cells with EC144 prior to LPS stimulation was performed for 0, 15 min, 30 min, 1 h, 2 h, 3 h, or 4 h. After 1 h of exposure, >90% of the LPS-induced TNF-α was inhibited, and by 4 h, >95% of TNF-α was inhibited. B, RAW 264.7 cells were incubated with EC144 for 4 h and stimulated with LPS (10 ng/ml) for 8 h. IL-6 concentrations in the supernatants were quantified by ELISA. Data are representative of at least two independent experiments.

Chemokine induction was observed at EC144 concentrations ≥13.7 nM, with EC_{50} values for the proteins analyzed ranging between 12.7 and 45.4 nM (Table I). These results demonstrate that in RAW 264.7 cells EC144 generally down modulates LPS signaling and decreases production of many LPS-induced proteins.

It has been previously reported that exposure of various cell types to geldanamycin resulted in surface CD14 downmodulation (6, 9, 14). Because CD14 is a coreceptor for LPS binding to TLR4, modulation of CD14 by Hsp90 inhibition was a possible mechanism for the EC144-induced general decrease in the LPS response. We therefore examined by flow cytometry the surface expression of CD14 and TLR4 on RAW 264.7 cells after 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, or 4 h of exposure to EC144 and found expression of these receptors was not altered at any time point (data not shown). Thus, inhibition of LPS-induced TNF-α and IL-6 production in RAW 264.7 cells by 4-h incubation with EC144 occurred through a mechanism that did not involve downmodulation of receptors for LPS.

Because LPS signaling activates both the MAPK and NF-κB pathways, it was possible that Hsp90 inhibition by EC144 blocked activation of these pathways. In macrophages, the MAPKs, ERK1/2, JNK1/2, and p38 MAPK, are all activated by LPS, and inhibition of any one of these MAPKs results in attenuation of TNF-α induction (28). To determine effects of EC144 on LPS-induced MAPK activation, RAW 264.7 cells were treated for 4 h with various concentrations of EC144 and stimulated with LPS for 30 min, a time previously determined to be optimal for detecting maximally phospho-ERK1/2, JNK1/2, and p38 MAPK (data not shown). Treatment of RAW 264.7 cells with EC144 resulted in a dose-dependent decrease of phospho-ERK1/2 and phospho-JNK1/2 (Fig. 3A). Levels of phospho-p38 MAPK were also mildly decreased by EC144 treatment but not to the same extent as phospho-ERK1/2 or phospho-JNK1/2 (Fig. 3A, left panel). Parallel immunoblot analysis revealed no significant changes in total ERK1/2, JNK1/2, or p38 MAPK protein levels (Fig. 3A, right panel). To further characterize the strong dose-dependent inhibition of ERK1/2 phosphorylation by EC144, the phosphorylation of MEK1/2, the MAP2K upstream of ERK1/2, was examined. EC144 treatment markedly decreased MEK1/2 phosphorylation in a dose-dependent manner but did not alter levels of total MEK1/2 (Fig. 3A). These results demonstrate that in RAW 264.7 cells Hsp90 inhibition blocks LPS-induced activation of the MAPK pathways that are required for LPS-induced TNF-α production.

LPS stimulation of macrophages also activates the NF-κB pathway, and one of the transcriptional targets of NF-κB is TNF-α. Because previous studies have reported inhibitory effects of geldanamycin-related compounds on NF-κB activation (29), NF-κB p50 binding activity was analyzed in nuclear extracts from
RAW 264.7 cells treated with 10 or 1 μM EC144 for 4 h and stimulated with LPS. No decrease in NF-kB p50 binding activity relative to DMSO-treated, LPS-stimulated control cells was found (Fig. 3B). Therefore, in RAW 264.7 cells, Hsp90 inhibition appears to not alter LPS-dependent activation of NF-kB p50.

EC144 is active in vivo and inhibits LPS-induction of TNF-α

In vivo Hsp90 inhibition with 17-AAG in a rat LPS-induced uveitis model has been shown to suppress multiple manifestations of LPS treatment, including retinal leukocyte adhesion, cytokine production, and PI3K activity (14). To determine whether the selective small-molecule Hsp90 inhibitor EC144 could block LPS-induced responses in vivo, EC144 was tested in a mouse LPS shock model that generates an acute systemic inflammatory response dependent on TNF-α (28, 30). The EC144 dosing regimen was based on the in vivo pharmacokinetics of EC144 (Fig. 4A). Plasma analyses after a single orally administered dose of 5 mg/kg EC144 in DBA/1 mice revealed good exposure at or above 10 times the EC50 for ~6 h. Interestingly, EC144 was well distributed to spleen and lymph nodes with exposure in these tissues ~2.5- to 4-fold higher than that in plasma. (Fig. 4A, Table II). In the LPS shock model, mice administered various doses of EC144 by oral gavage showed attenuation of serum TNF-α induction in response to LPS. The response to EC144 was dose dependent with 2.5, 5, and 10 mg/kg doses producing a significant decrease in serum TNF-α induction (Fig. 4B). All EC144 doses were well-tolerated by the DBA/1 mice. Thus, in vivo Hsp90 inhibition also abrogates LPS-induced TNF-α production.

EC144 blocks TLR4 signaling in primary CD11b+ PECs by inhibiting activation of Tpl2, MEK1/2, and ERK1/2

To further investigate the biochemical mechanism by which in vivo administration of an Hsp90 inhibitor decreased LPS-induced TNF-α, CD11b+ PECs were isolated from mice that received three daily doses of EC144. Cells were stimulated in vitro for 30 min with LPS, and cell lysates were analyzed by immunoblotting for phospho-p38 MAPK, JNK1/2, MEK1/2, and ERK1/2. LPS-stimulated CD11b+ PECs from mice dosed with EC144 had dramatically lower levels of phospho-MEK1/2 and phospho-ERK1/2 as compared with LPS-stimulated cells isolated from vehicle-treated mice (Fig. 5A). In contrast to results with the RAW 264.7 cell line (Fig. 3A), inhibiting Hsp90 in normal cells did not modulate JNK1/2 or p38 MAPK phosphorylation (Fig. 5A). Parallel immunoblots analyzing total levels of each signaling protein did not reveal any differences between cells from EC144 or vehicle-treated mice (Fig. 5A). Thus, in normal CD11b+ PECs from EC144-dosed mice, there appears to be a downmodulation in activation of the MEK1/2 to ERK1/2 signaling pathway induced by LPS.

The MAP3K Tpl2 has been shown to be upstream of MEK1/2 and ERK1/2 and is activated by LPS in vivo (28). A critical role for the Tpl2/MEK1/2/ERK1/2 signaling pathway in LPS-induced
was not detected in the absence of LPS stimulation, and all CD11b+ T cells from mice administered 2.5, 5, or 10 mg/kg EC144 was significant by Student’s test. From EC144-treated animals (Fig. 5B). In repeated MLR assays, EC144 was consistently more potent than MMA, a reversible inhibitor of inosine-5'-monophosphate dehydrogenase in the de novo purine biosynthesis pathway that is essential for lymphocyte proliferation (31). EC144 was additionally ~2-fold more potent than 17-AAG, which had an EC50 of ~24 nM (Fig. 6A). In an MLR using mouse CD4+ responder cells purified from DBA/1 mice with BALB/c stimulators, EC144 inhibited CD4+ T cell proliferation with an EC50 comparable to its potency on human cells (5 nM; Fig. 6B). Similar to the effects on human CD4+ cells, in mouse CD4+ cells, EC144 was consistently more potent than MMA or 17-AAG (Fig. 6B).

**Therapeutic treatment with EC144 ameliorates disease in a rat CIA model and blocks proinflammatory cytokine production**

In collagen-induced and spontaneous rodent arthritis models, a role has been reported for proinflammatory TLR4 signaling and TNF-α in disease severity and pathogenesis (32, 33). The ability of EC144 to suppress TNF-α induced by an in vivo inflammatory stimulus through TLR4 (Fig. 4B) suggested that EC144 should suppress disease development in a rat model of CIA. Initial pharmacokinetic studies in diseased rats established an optimal suspension formulation resulting in a 5-h plasma half-life and high distribution of EC144 to lymphoid tissues (~10-fold higher in spleen and lymph nodes as compared with plasma; data not shown). Rats immunized with rat CII in IFA and boosted on day 10 with rat CII in PBS were dosed starting on day 12 with EC144 on either a daily or every-third-day schedule. Within each dosing schedule, two different doses of EC144 were used to give equivalent cumulative compound exposure. Animals dosed with the higher EC144 doses (1.5 mg/kg daily or 5 mg/kg every third day) developed little or no disease symptoms, whereas animals dosed with the lower EC144 doses (0.5 mg/kg daily or 1.5 mg/kg every third day) developed disease indistinguishable from vehicle-treated animals (Fig. 7A). At the end of the study, circulating levels of anti-rat CII IgG2a and IgG2b Abs were measured, and no significant differences were found in any of the EC144 dose groups compared with levels detected in vehicle-treated control rats (Fig. 7B). Levels of the proinflammatory cytokine mRNAs known to be induced in this model, TNF-α, IL-1β, and IL-6 (33), were quantified in paws of naive rats and rats administered vehicle or the efficacious EC144 dose of 5 mg/kg every third day. EC144 treatment completely abrogated expression of mRNAs of these proinflammatory cytokines, and transcript levels were comparable to those measured in paws of naive rats (Fig. 7C).

**EC144 decreases disease severity in mouse CIA and inhibits the memory CD4+ T cell response**

In contrast to the rat CIA model, where disease rapidly develops into a severe inflammatory illness (33), the pathogenesis in mouse CIA models develops more slowly, over a ~21-d time course, and has been described to be mediated by both collagen-specific CD4+ T cells and Abs specific for CII (34, 35). Because EC144 effectively blocked CD4+ T cell responses in MLRs (Fig. 6), it was therefore also of interest to test the effects of Hsp90 inhibition by EC144 in a mouse CIA model. Mice immunized with chicken CII

**Inhibition of CD4+ T cell activation by EC144**

In T cells, it has been shown previously that Hsp90 inhibition by benzoquinone ansamycins can block TCR-mediated activation by destabilizing p56lck (12, 13). To verify that Hsp90 inhibition with EC144 could block T cell activation, human primary CD4+ cells were incubated with EC144 in an allogeneic MLR. EC144 potently inhibited human T cell proliferation in the MLR, with an EC50 between 12 and 15 nM (Fig. 6A). In repeated MLR assays, EC144 was consistently more potent than MMA, a reversible inhibitor of inosine-5’-monophosphate dehydrogenase in the de novo purine biosynthesis pathway that is essential for lymphocyte proliferation (31). EC144 was additionally ~2-fold more potent than 17-AAG, which had an EC50 of ~24 nM (Fig. 6A). In an MLR using mouse CD4+ responder cells purified from DBA/1 mice with BALB/c stimulators, EC144 inhibited CD4+ T cell proliferation with an EC50 comparable to its potency on human cells (5 nM; Fig. 6B). Similar to the effects on human CD4+ cells, in mouse CD4+ cells, EC144 was consistently more potent than MMA or 17-AAG (Fig. 6B).

**FIGURE 4.** In vivo EC144 has favorable pharmacokinetics and inhibits LPS-induced systemic TNF-α release. A, A single dose of EC144 suspension in vehicle (5 mg/kg) was administered by oral gavage to DBA/1 mice (n = 3). Plasma, spleens, and lymph nodes were obtained via terminal collection at 0.25, 0.5, 1, 3, 5, 7, 16, and 24 h after dose. Spleens and lymph nodes were homogenized and extracted as described under Materials and Methods. EC144 levels were quantified using mass spectrometry and converted to concentrations based on volume (plasma) or weight (tissues). B, EC144 as a suspension in vehicle was administered to female DBA/1 mice (seven per group) at the indicated doses by oral gavage. LPS (200 μg/kg) was injected i.p., and mice were bled 1 h later. PBS and LPS only groups were composed of three mice per group; the untreated DBA/1 control group was composed of two mice. Levels of serum TNF-α were quantified by ELISA. The decrease in systemic TNF-α from mice administered 2.5, 5, or 10 mg/kg EC144 was significant by a Student t test (p < 0.005). Data are representative of three independent experiments.

TNF-α production has been demonstrated in Tpl2-deficient mice, which are resistant to LPS shock, primarily because of their inability to activate ERK1/2 (28). Although total Tpl2 protein was weakly detected in RAW 264.7 cells treated with LPS, we were not able to detect phosphorylated Tpl2 (data not shown), suggesting that this pathway is altered in the cell line as compared with normal cells. Phosphorylated Tpl2 was, however, readily detectable and upregulated in lysates of LPS-stimulated primary CD11b+ PECs from vehicle-treated mice (Fig. 5B). In contrast, phosphorylated Tpl2 was not detected in LPS-stimulated CD11b+ PECs isolated from EC144-treated animals (Fig. 5B). Phosphorylated Tpl2 was not detected in the absence of LPS stimulation, and all CD11b+ PEC lysates had similar levels of total Tpl2 (Fig. 5B). These results demonstrate that inhibiting Hsp90 in vivo with EC144 blocked activation of the Tpl2/MEK1/2/ERK1/2 pathway in LPS-stimulated CD11b+ PECs.
in CFA and boosted on day 21 with chicken CII in PBS were dosed with EC144 on an every-other-day schedule, beginning on the day of the CII boost. Significant inhibition of arthritis scores was observed in mice dosed every other day with 8 or 10 mg/kg EC144 (Fig. 8A). Both of these doses of EC144 also reduced the disease incidence from 90 to 60% by day 42 (data not shown) and reduced disease severity, on average, from a clinical score of 12 to 4 (Fig. 8A). EC144 was well tolerated on the every-other-day dosing regimen, and there were no major differences in mean body weights between mice dosed with EC144 or vehicle-treated mice (Supplemental Fig. 3). Efficacy was also observed in mice dosed with EC144 at 5 mg/kg every other day, although this dose was less efficacious in inhibiting arthritis development than the higher doses (Fig. 8A). Representative histology sections of hind paws of EC144-treated mice and vehicle control-treated mice at the end of the study are illustrated in Fig. 8B. Mice that received EC144 at 10 mg/kg every other day showed minimal joint inflammation, and an

**FIGURE 5.** CD11b⁺ PECs from mice treated with EC144 show decreased phosphorylation of Tpl2, MEK1/2, and ERK1/2 in response to LPS. A, Mice were primed with thioglycollate and dosed daily with EC144 (10 mg/kg) by oral gavage for 3 d. Three hours after the last dose, the PECs were harvested by lavage, and CD11b⁺ cells were purified by MACS. Cells were stimulated with LPS (10 ng/ml) for 30 min, lysed, and clarified cell lysates subjected to immunoblotting for the phosphorylated forms of ERK1/2, MEK1/2, JNK1/2, and p38 MAPK (left panel). Parallel membranes were probed with mAbs specific for total ERK1/2, MEK1/2, JNK1/2, and p38 MAPK (right panel). To verify gel loading, each membrane was stripped and reprobed for GAPDH. B, Cell lysates of CD11b⁺ PECs from EC144-dosed mice isolated and LPS stimulated as in A were immunoblotted for phosphorylated Tpl2 (left panel) or total Tpl2 (right panel). To verify gel loading, each membrane was stripped and reprobed for GAPDH. Data are representative of at least three independent experiments.

**FIGURE 6.** EC144 blocks CD4⁺ T cell proliferation in human and mouse MLRs. A, Human CD4⁺ T cells purified from PBMCs were cocultured with irradiated non-CD4⁺ cells from a different donor at a responder:stimulator ratio of 1:3 in the presence of varying concentrations of EC144, MMA, or 17-AAG. After 5 d, proliferation was measured by [³H]thymidine incorporation into DNA. Data are representative of seven independent experiments using different blood donors. B, Splenic CD4⁺ T cells purified from DBA/1 mice were cocultured with Thy1.2-depleted splenocytes from BALB/c mice at a responder: stimulator ratio of 1:10 in the presence of varying concentrations of EC144, MMA, or 17-AAG. After 5 d, proliferation was measured by [³H]thymidine incorporation into DNA.

### Table II. Pharmacokinetic analysis of EC144 in female DBA/1 mice after a single oral dose

<table>
<thead>
<tr>
<th>Specimen</th>
<th>T&lt;sub&gt;MAX&lt;/sub&gt; &lt;sup&gt;a&lt;/sup&gt; (h)</th>
<th>C&lt;sub&gt;MAX&lt;/sub&gt; &lt;sup&gt;b&lt;/sup&gt; (ng/ml)</th>
<th>AUC&lt;sub&gt;SP&lt;/sub&gt; &lt;sup&gt;c&lt;/sup&gt; (ng × h/ml)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>AUC &gt; 10 × EC&lt;sub&gt;50&lt;/sub&gt; (ng × h/ml)</th>
<th>Duration &gt; 10 × EC&lt;sub&gt;50&lt;/sub&gt; (h)</th>
<th>Tissue/Plasma AUC</th>
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</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1</td>
<td>835.3</td>
<td>2135.6</td>
<td>1.5</td>
<td>1494.0</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>0.5</td>
<td>2144.3</td>
<td>5208.9</td>
<td>1.7</td>
<td>4294.7</td>
<td>7.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.5</td>
<td>1886.7</td>
<td>8544.3</td>
<td>2.8</td>
<td>6523.3</td>
<td>7.0</td>
<td>4.0</td>
</tr>
</tbody>
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Blood, spleen, and lymph nodes were obtained from female DBA/1 mice (n = 3/time point) administered a single oral dose of EC144 (5 mg/kg) at 0.25, 0.5, 1, 3, 5, 7, 16, and 24 h after dosing. AUC > 10 × EC<sub>50</sub> represents where the level of compound exposure is greater than ×10 the EC<sub>50</sub> value. Duration > 10 × EC<sub>50</sub> represents the amount of time after the single dose where the level of compound is >10 times the EC<sub>50</sub> value. Extracted plasma and tissue samples were analyzed for EC144 by liquid chromatography-tandem mass spectroscopy, and pharmacokinetic analyses were performed using noncompartmental methods with WinNonlin V.5 (Pharsight).

<sup>a</sup>Time after dosing to reach maximal concentration in plasma or tissue.

<sup>b</sup>Maximal concentration attained in plasma or tissue.

<sup>c</sup>Area under the curve (AUC) extrapolated to infinity.
absence of cartilage and synovial lining damage and bone erosion, closely resembling joints from naive, nondiseased mice. In contrast, joints from mice that were administered vehicle had inflammation with infiltrating cells, erosion of cartilage, and marked changes in bone and synovial lining (Fig. 8B, 8C).

At the end of the study, circulating levels of anti-chicken CII IgG1, IgG2a, and IgG2b Abs were measured. Mice dosed every other day with 5, 8, or 10 mg/kg EC144 had a trend toward lower levels of collagen-specific Abs, relative to levels detected in vehicle-treated control mice; however, only the anti-chicken CII IgG1 and IgG2a levels in mice dosed with 8 mg/kg EC144 were significantly decreased (p < 0.05; Fig. 8A). The decreased anti-chicken CII Ab levels in the 8 mg/kg treatment group remained at the level in animals that did not receive the CII boost on day 21 (Fig. 9A).

To further elucidate the mechanism of arthritis disease inhibition by EC144, the ex vivo CD4+ T cell Ag recall response of mice immunized and treated with EC144 as in the CIA model (10 mg/kg every other day starting at the day of the boost) was tested. Chicken CII immunization generates T cells reactive to the immunogen as well as cross-reactive T cells recognizing mouse CII. Therefore, splenic CD4+ cells were prepared 6 d after the CII boost and stimulated with either chicken or mouse CII presented by syngeneic Thy1-depleted splenocytes from unimmunized mice. To directly quantify the activated CD4 cells that responded to Ag, expression of CD154 was measured. T cells from mice that were dosed with EC144 had an Ag recall response to both chicken and mouse CII that was similar to CD4+ T cells purified from naive, unimmunized mice (Fig. 9B). In contrast, CD4+ T cells purified from mice administered vehicle had a significantly higher per-
percentage of activated CD4+ T cells responding to either chicken or mouse CII in the in vitro recall response assay (Fig. 9B).

**Discussion**

Previous studies have reported therapeutic benefit of targeting Hsp90 in the rodent inflammatory autoimmune disease models uveitis, experimental autoimmune encephalitis, and rat arthritis, using both ansamycin-derived inhibitors and a fully synthetic Hsp90 inhibitor (8, 14–16). In this report, we demonstrate that the synthetic Hsp90 inhibitor EC144 is potent and selective and is efficacious in an inflammatory mouse model of endotoxin shock and an autoimmune mouse CIA model. We also confirm efficacy in a rat CIA model and provide a principal mechanistic basis for the efficacy of EC144 in each of these disease models. Thus, a highly selective Hsp90 inhibitor could modulate both the innate and adaptive arms of the immune system to effectively suppress autoimmune and inflammatory diseases.

The potent inhibition of MAPK activation through TLR4 with EC144 accounts for the ability of Hsp90 inhibition to decrease proinflammatory cytokines, such as TNF-α and IL-6. Two major pathways of TLR4 signal transduction have been revealed genetically: one that is mediated via the adaptor molecule MyD88 and the other by the MAP3K Tpl2 (28, 36). In macrophages, ERK1/2

![FIGURE 8. EC144 suppresses mouse CIA disease severity.](http://www.jimmunol.org/)

A, Pristane-primed female DBA/1 mice immunized and boosted with chicken CII as described in *Materials and Methods* were dosed every other day with EC144 or vehicle by oral gavage beginning on the day of the boost (day 21). Clinical symptoms were monitored and scored every other day. Each point indicates the mean ± SEM arthritis scores for 12–14 mice/group. B, Representative images of H&E-stained sections of hind paws from mice treated with either vehicle (left panel) or EC144, 10 mg/kg every other day (right panel). Histological sections were scored for inflammation (black arrows), boney changes (white arrow), and synovial alterations (hatched arrow). Original magnification ×4, inset original magnification ×0.6. Black boxes in the inset indicate area magnified in the main image. C, Inflammation, cartilage change, boney change, and synovial lining alteration were scored on H&E stained slides on a scale of 0–3. Mean score ± SEM for four mice in the vehicle group (open bars) or EC144 treatment group 10 mg/kg every other day (filled bars).
FIGURE 9. EC144 decreases anti-chicken CII Ab and blocks T cell activation in the adaptive memory response. A, Serum samples collected from mice at the end of study in Fig. 8 (day 40) were analyzed by ELISA for mouse IgG1, IgG2a, or IgG2b Abs specific for chicken CII. Ab levels were quantified relative to a standard of pooled sera from diseased mice. Each bar is the mean ± SEM values for 12–14 mice/group. B, Pristane-primed female DBA/1 mice immunized and boosted with chicken CII were dosed as in A with EC144 (10 mg/kg every other day) by oral gavage. On day 27, mice were sacrificed, and purified splenic CD4+ T cells from each mouse were cocultured with Thy1.2-depleted splenocytes from naive DBA/1 mice in the presence of various concentrations of Ag (chicken CII or mouse CII) and anti–CD154-PE. Anti-CD154 was included in the cultures to stabilize CD154 expression on the surface of recently activated CD4+ cells (21–23). After overnight incubation, cells were analyzed by flow cytometry for expression of the activation markers CD25, CD69, and CD154. The percentages of CD154+ cells of the live CD4+CD25+CD69+ gated cells are shown. Each point is the mean ± SEM for five mice per group. Data are representative of three independent experiments.

activation by LPS is primarily mediated by the Tpl2/MEK1/2/ERK1/2 pathway, resulting in induction of the nuclear to cytoplasmic transport of TNF-α mRNA (28) and early systemic release of TNF-α. Tpl2-deficient mice are resistant to LPS-induced shock, primarily due to an inability to activate ERK1/2 (28). Despite these defects, macrophages from Tpl2 deficient mice are capable of activating the NF-κB pathway in response to LPS (28). Our findings that Hsp90 inhibition in macrophages blocks LPS-induced activation of Tpl2, MEK1/2, and ERK1/2 with abrogation of systemic TNF-α release are fully consistent with findings in Tpl2-deficient mice and suggest that the primary anti-inflammatory mechanism for EC144 in these studies is an inhibitory effect on Tpl2 activation. Because total Tpl2 was not altered as a result of Hsp90 inhibition, it appears unlikely that the Tpl2 protein is an Hsp90 client and instead suggests that Hsp90 may participate in stabilizing phospho-Tpl2, similar to the Hsp90-dependent stabilization of activated p56lck.

Hsp90 inhibition in primary ex vivo CD11b+ PECs, in contrast to the RAW 264.7 cell line, did not block p38 MAPK or JNK activation. Although we could not detect phospho-Tpl2 in RAW 264.7 cells, it was clearly detectable and inhibited by EC144 in LPS-stimulated CD11b+ PECs. These differences in the effects of Hsp90 inhibition on the MAPK signaling pathways between RAW 264.7 cells, derived from mouse macrophages transformed by Abelson leukemia virus (37), and primary macrophages have been observed in previous studies (28) and are likely due to alterations in the TLR4 signaling pathways during transformation.

Stimulation of the MyD88 pathway in macrophages by LPS leads to IRAK1 activation, association with TRAF6, and subsequent MAPK and NF-κB activation. We did not find any effects of EC144 on LPS-induced nuclear NF-κB p50, which was surprising because IRAK1 has been demonstrated to be an Hsp90 client protein (7). The differences between our findings and the literature on IRAK1 could result from the use of different cell types or different treatment conditions with geldanamycin. In macrophages, the effects of high concentrations of geldanamycin and long treatment times used by De Nardo et al. (7) are likely to result in modulation of other Hsp90 client proteins.

Interestingly, the IκB kinase IKKβ appears to be required for activation of Tpl2 by cleaving and inactivating Tpl2-associated p105, the precursor to NF-κB p50 (28, 38). Because Tpl2 phosphorylation in normal macrophages was sensitive to Hsp90 inhibition in the absence of effects on NF-κB, this suggests that the primary effects we observed on MAPK inhibition were through the MAP3K Tpl2 and not on the MyD88 pathway.

Understanding the significant effects of Hsp90 inhibition on TLR4 signaling pathways and T cell proliferation suggested several avenues for exploring the mechanism of efficacy of EC144 in rat and mouse arthritis models. The rat CIA model used in these studies is a rapidly developing and severely inflammatory disease characterized by proinflammatory cytokine production and neutrophil infiltration in the joints (33) and upregulation of macrophage and dendritic cell-specific genes (39). Minimal B cell activation and Ab production have been noted in this model (33, 39). The dramatic efficacy of EC144 in this model confirmed a previous report describing efficacy of a different synthetic Hsp90 inhibitor in two rat arthritis models (16). We also demonstrated a correlation of EC144 efficacy with abrogation of the expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in paws from treated rats, consistent with the mechanisms that drive disease in this model.

In contrast to rat CIA, mouse CIA models have historically been characterized as mediated by both collagen-specific CD4+ T cells and Abs specific for CII (34, 35). Recent evidence also suggests that inhibition of TLR4 signaling in mouse CIA reduces disease severity without modulating the autoantibody response to endogenous mouse CII (32). The dose-dependent efficacy of EC144 in a mouse CIA model is consistent with our finding that EC144 inhibited T cell proliferation in human and mouse MLR assays and are supported by previous studies that demonstrate inhibiting Hsp90 with geldanamycin destabilizes activated p56lck and blocks T cell activation through the TCR pathway (12, 13). Some reduction in the levels of circulating anti-CII Abs was observed in mice treated with EC144; however, this was not well correlated with reduction in paw inflammation and may just reflect the profound block in CD4+ T cell recall response noted above. Thus, it is...
likely that the ability of EC144 to block adaptive T cell responses and innate TLR4 signaling contribute to the mechanism of efficacy for Hsp90 inhibition in mouse CIA.

Targeting Hsp90 in cancer has been of interest because Hsp90 function is critical for stabilizing multiple mutated or overexpressed client proteins that are involved in tumor cell growth. In this study, we have provided mechanistic evidence supporting Hsp90 as a potential therapeutic target in autoimmune disease. In CD4+ T cells and macrophages, cell types believed to mediate autoimmune disease pathogenesis and activation of key proteins involved in cellular signaling via the TCR and TLR4 pathways, autoinflammatory disease pathogenesis and activation of key proteins Hsp90 inhibitor compounds as therapies for treating autoimmune disease.

In CD4+ T cells and macrophages, cell types believed to mediate this study, we have provided mechanistic evidence supporting Hsp90 inhibition in mouse CIA.

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Disclosures
All authors are current or former employees and stockholders of Biogen Idec. Biogen Idec has filed a patent application related to EC144.

References

