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Honokiol, a Natural Plant Product, Inhibits Inflammatory Signals and Alleviates Inflammatory Arthritis¹

Melissa E. Munroe,* Jack L. Arbiser,[§] and Gail A. Bishop^{2*†‡}

Honokiol (HNK), a phenolic compound isolated and purified from magnolia, has been found to have a number of pharmacologic benefits, including anti-angiogenic and anti-inflammatory properties. HNK has long been used in traditional Asian medicine without toxic side effects. We and others have extensively studied signaling to B cells by CD40 and its Epstein Barr viral mimic, latent membrane protein 1 (LMP1), which has been implicated in exacerbation of chronic autoimmune disease. We asked whether HNK could inhibit CD40 and LMP1 inflammatory signaling mechanisms. In vivo, HNK stabilized the severity of symptomatic collagen-induced arthritis in both CD40-LMP1 transgenic mice and their congenic C57BL/6 counterparts. Ex vivo studies, including collagen-specific serum Ab and Ag recall responses, as well as CD40 or LMP1-mediated activation of splenic B cells, supported the anti-inflammatory effects of HNK. In mouse B cell lines expressing the human CD40-LMP1 chimeric receptor, CD40- and LMP1-mediated NF- κ B and AP-1 activation were abrogated in a dose-dependent manner, with a concomitant decrease in TNF- α and IL-6. These promising findings suggest that the nontoxic anti-inflammatory properties of HNK could be valuable for blocking the autoimmune response. *The Journal of Immunology*, 2007, 179: 753–763.

Rheumatoid arthritis (RA)³ is considered the most common systemic autoimmune disease (1). Improved survival in patients with RA and other rheumatic diseases such as systemic lupus erythematosus, in part due to better treatment regimens, has unmasked additional comorbidities and mortalities. A number of conditions are associated with the chronic inflammation and elevated levels of TNF- α and IL-6 seen in RA, including heart disease (2) and cancer (3). Although the gastrointestinal tract is one of the most common sites of such cancer induction, a similar mechanism can also be found in plasma cells, leading to lymphoproliferative changes, lymphomas, and myelomas.

Both T and B lymphocytes contribute to the pathogenesis of RA (4, 5), including the presence of collagen-specific Th1 (6), Th17 cells (7), B cells, and Ab production (8). CD40, a member of the TNFR superfamily, is a key costimulatory molecule in T-B cell interactions (9), promoting the up-regulation of inflammatory cytokines such as TNF- α and IL-6 (10) and autoantibody production (11). A possible link between RA and the role of chronic inflammation in the development of additional comorbidities is the reactivation of EBV, which latently infects a proportion of memory B cells in >90% of the world's population (12). Among the EBV-encoded proteins implicated in viral pathogenesis, considerable at-

tention has focused upon latent membrane protein 1 (LMP1). Of the nine EBV genes expressed as proteins in EBV-transformed cells, LMP1 is the best characterized, and is the only EBV-encoded gene product capable of transforming cells in vitro (13) and in vivo (14), resulting in the potential for lymphoproliferative changes and malignancy. In addition to its established role in the pathogenesis of B cell lymphoma and other malignancies, EBV infection may be linked to exacerbation of various human autoimmune diseases, including RA and systemic lupus erythematosus (15).

Studies in our laboratory have shown that LMP1 closely mimics CD40, including activation of signaling cascades, up-regulation of costimulatory and adhesion molecules, and Ab secretion (16). However, LMP1 signals in a dysregulated manner in contrast to the tightly regulated activation of CD40 (17). The six transmembrane domains of LMP1 spontaneously aggregate and oligomerize within the plasma membrane, resulting in ligand-independent constitutive activation. To circumvent the continuous activation of LMP1, we have developed a system to study LMP1-mediated B cell activation via a chimeric molecule with the extracellular and transmembrane portions of CD40 and the cytoplasmic tail of LMP1 (17). These studies include analysis of mice bearing the transgene for the mouse (m) CD40-LMP1 chimeric molecule, with the extracellular and transmembrane domains of mCD40 and the cytoplasmic tail of LMP1. The transgene is driven by a MHC class II promoter, so it is expressed in all hemopoietic compartments that express CD40. These mice have been bred onto the C57BL/6 CD40^{-/-} background so that only the mCD40-LMP1 transgene (and not endogenous CD40) is expressed in those cells that would normally express CD40 (17). Initial functional assessments of these transgenic (Tg) mice (17) indicate that while these mice contain normal lymphocyte subsets and respond appropriately to immunization, they also spontaneously produce autoantibodies and have elevated levels of serum IL-6, making them attractive candidates for studies evaluating the development of rheumatic disease.

The mouse collagen-induced arthritis (CIA) model has many pathologic and immunologic parallels to RA (18) and provides a stable, predictable model for evaluating the therapeutic potential of novel compounds for the treatment of RA and other chronic inflammatory conditions. This model has been used successfully in the development

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; LMP, latent membrane protein; Tg, transgenic; m, mouse; CIA, collagen-induced arthritis; HNK, honokiol; h, human; WT, wild type; CII, type II chicken collagen.

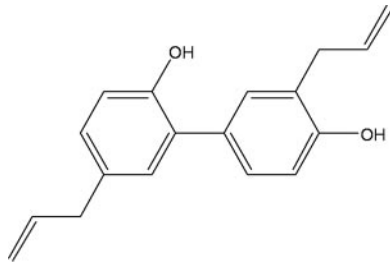


FIGURE 1. Chemical structure of HNK.

of TNF- α blockade therapy (19), which is now used clinically with great success (20). However, a potential pitfall in using humanized Abs or chimeric molecules to block TNF- α is the development of a neutralizing Ab response with repeated treatments (21). This has prompted the development and evaluation of small m.w. compounds capable of inhibiting inflammatory mediators such as TNF- α and IL-6.

Honokiol (HNK) (Fig. 1) is a small organic molecule purified from *Magnolia* species that has been well-tolerated in models of heart disease (22) and cancer (23). It has also been used without noticeable side effects for many years in traditional Asian medicine (24). In vitro studies in macrophages (25) and neutrophils (26) suggest an anti-inflammatory role for HNK. However, to date, there have been no studies on the effect of HNK on lymphocyte-mediated inflammation or its sequelae. In this study, we evaluated the ability of HNK to alter symptomatic CIA in mice and also assessed its effects on specific CD40- or LMP1-mediated activation events in B cells. HNK treatment stabilized CIA pathology in both wild-type (WT) and mCD40-LMP1 Tg mice. In addition to alleviated paw swelling, HNK treatment led to an alteration in serum Ab levels and a change in isotype proportions, a profile further supported by a decrease in the proinflammatory response in Ag-recall cultures of regional lymph nodes. CD40- or LMP1-mediated activation of B cells from HNK-treated CIA mice induced altered proinflammatory cytokine profiles compared with CIA mice that did not receive HNK. Treatment of mouse B cell lines with HNK in vitro recapitulated the cytokine profile seen in primary mouse B cells with a concomitant dose-dependent decrease in CD40- and LMP1-mediated NF- κ B and AP-1 activation. These findings reveal a prominent anti-inflammatory role for HNK, potentially in both the cognitive phase of the immune response, as well as the effector phase, by inhibiting cytokines that lead to chronic inflammation and additional pathology.

Materials and Methods

Reagents and Abs

HNK was isolated from the stem bark of *Magnolia abovata* thumb and *Magnolia officinalis* rhed using HPLC techniques, as previously described (27). Twenty-percent intralipid was purchased from Sigma-Aldrich. Recombinant mouse IFN- γ , TNF- α , IL-6, IL-10, and IL-4 were purchased from PeproTech. Streptavidin-HRP was purchased from Jackson ImmunoResearch Laboratories. ELISA TMB peroxidase substrate was purchased from KPL.

The G28-5 (anti-human (h) CD40, mouse IgG1), 1C10 (anti-mCD40, rat IgG2a), 72-2 (rat IgG2a isotype control), and SXCI (anti-mouse IL-10 ELISA Ab) hybridomas were obtained from the American Type Culture Collection (ATCC). MOPC-31c (mouse IgG1 isotype control) was obtained from Sigma-Aldrich. Anti-mouse IFN- γ (coating and biotinylated), IL-6 (coating and biotinylated), TNF- α (coating and biotinylated), IL-4 (coating), and IL-10 (biotinylated) ELISA Abs were purchased from eBioscience. Anti-mouse IL-4 (biotinylated) ELISA Ab was purchased from Caltag Laboratories. Anti-mouse Ig isotype Abs for ELISA (coating, biotinylated, and standards) were purchased from Southern Biotechnology Associates. BioSource Multiplex buffers and Abs to mouse IL-2, IL-12 (p40/p70), IL-17, IL-4, IL-5, IL-6, and TNF- α were purchased from Invitrogen Life Technologies.

Mice/CIA induction

Female C57BL/6 mice were purchased at 5–8 wk of age from the National Cancer Institute. Female mice Tg for the molecule mCD40-LMP1, driven by the MHC class II E α promoter, were developed and bred onto the C57BL/6 CD40^{-/-} background at the University of Iowa, as previously described (17). All mice were housed in a specific pathogen-free barrier facility with restricted access, and all procedures were performed as approved by the University of Iowa Animal Care and Use Committee.

CIA was induced based on the methods of Campbell et al. (28). Briefly, mice were either left naive, immunized in the tail s.c. with 100 μ g of type II chicken collagen (CII; Sigma-Aldrich) dissolved in 10 mM acetic acid and emulsified in IFA (Sigma-Aldrich) containing 5 mg/ml H37 RA heat-killed mycobacteria (Difco Laboratories; CFA), or immunized with 10 mM acetic acid emulsified in CFA. Mice received either vehicle control or HNK i.p. (3 mg/ml/day HNK diluted in 20% intralipid) starting at day 21 postimmunization.

Mice were monitored for limb erythema and swelling; each paw (four measurements per mouse) was given a clinical score (0–4) and measured with calipers two to three times per week (no. 4026F; Mitutoyo), as previously described (28). Clinical score measurements were assigned as follows: 0, no erythema/swelling; 1, erythema/mild swelling confined to mid-foot (tarsals) or ankle joint, or swelling of individual digits; 2, erythema/moderate swelling extending from ankle to mid-foot; 3, erythema/moderate swelling extending from ankle to metatarsal joints; 4, erythema and severe swelling encompassing ankle, foot, and digits.

On day 70 postimmunization, before euthanasia, mice were bled by retro-orbital puncture under CO₂ anesthesia for serum collection. Hind paws from euthanized mice were skinned and decalcified in EM-400 (Sur-gipath) before paraffin embedment, cut into 7- μ m serial sections from tissue blocks, and stained using H&E by the University of Iowa Central Microscopy Research Facility. Histology results were interpreted using an Olympus BX-51 Light Microscope fitted with a SPOT RT KE 3-shot color camera and accompanying SPOT imaging software from Diagnostic Instruments, located at the University of Iowa Central Microscopy Research Facility.

Lymph node (LN) cell culture

Single-cell suspensions of inguinal and para-aortic LNs from mice were cultured in Click's medium containing 1% nutridoma-SP (Roche), 10 μ M 2-ME, and antibiotics, in the presence of indicated amounts of heat-denatured CII. CII-specific proliferation was determined in 72 h 96-well cultures by pulsing with 1 μ Ci/well [³H]TdR (GE Healthcare/Amersham Biosciences) at 48 h and cpm was determined by liquid scintillation 24 h later. Culture supernatants were collected at optimal culture times for cytokine analysis: IL-2, IL-6, TNF- α , IL-10, and IL-12 (p40/p70) were collected at 48 h; IFN- γ , IL-17, IL-4, and IL-5 were collected at 72 h.

B cell isolation

B cells were isolated from mouse spleens 70 days postimmunization. Briefly, spleens from euthanized mice were teased apart with forceps, erythrocytes lysed in ACK buffer, and remaining cells were stained with anti-CD43 MACS beads and run over a negative selection column per manufacturer's protocol (Miltenyi Biotec). Primary mouse B cells (>95% B220⁺ by flow cytometry) were cultured in RPMI 1640 containing 10% FCS (HyClone), 10 μ M 2-ME, and antibiotics.

Cell lines

The mouse B cell lines M12.4.1, CH12.LX, and their stable transfectants expressing the chimeric molecule hCD40-LMP1 (29), have been previously described. These subclones are referred to as M12.hCD40-LMP1 and CH12.hCD40-LMP1. Cell lines were maintained in RPMI 1640 containing 10% FCS, 10 μ M 2-ME, and antibiotics. Hi5 insect cells expressing CD154 (mouse or human) have been described and characterized previously (30). These cells grow at 26°C, rapidly die to form membrane fragments at 37°C, and therefore do not overgrow cell cultures.

NF- κ B/AP-1 dual-luciferase reporter assays

M12.hCD40-LMP1 cells (1.5 \times 10⁷) were transiently transfected with 10 μ g of 4 \times NF- κ B or 40 μ g of 7 \times AP-1 luciferase reporter plasmid (31) and 1 μ g of *Renilla* luciferase vector (pRL-null; Promega) by electroporation. Cells were rested on ice for 15 min, then stimulated (2 \times 10⁶ cells/ml) for 6 (NF- κ B) or 24 (AP-1) h with 10 μ g/ml anti-CD40 or isotype control, or Hi5 cells (at a ratio of 1 Hi5 cell:4 B cells) expressing WT baculovirus, mCD154, or hCD154. After stimulation, cells were pelleted, lysed, and assayed for relative luciferase activity (NF- κ B, NFAT, or AP-1; *Renilla*) per manufacturer's protocol (Promega) using a Turner Designs 20/20 luminometer, with settings of 2 s delay followed by 10 s read.

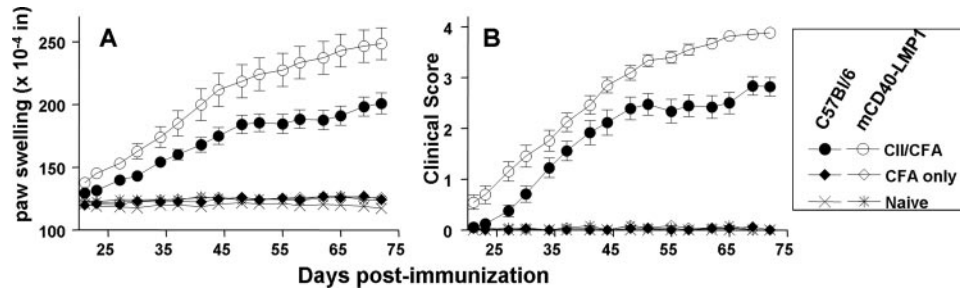


FIGURE 2. Development of CIA in C57BL/6 and mCD40-LMP1 Tg mice. Female C57BL/6 and mCD40-LMP1 Tg mice were either left naive or immunized with 100 μ g of CII/CFA or CFA only as described in *Materials and Methods*. Paw swelling (A) and clinical scores (B) were determined for each paw every 3–4 days from days 21 to 70 postimmunization. C57BL/6: $n = 48$ for CII/CFA, $n = 44$ for naive and CFA only groups; mCD40-LMP1 Tg: $n = 48$ for CII/CFA, $n = 44$ for naive and CFA only groups. Statistical analysis: $p < 0.0001$ naive/CFA only vs CII/CFA for both strains (paw swelling and clinical score); $p < 0.01$ C57BL/6 vs mCD40-LMP1 CII/CFA groups (paw swelling and clinical score).

Cytokine ELISA/Multiplex

Primary mouse B cells or M12.hCD40-LMP1 or CH12.hCD40-LMP1 cells were stimulated at optimal, empirically derived time points with culture medium or Hi5 insect cells (at a ratio of 1 Hi5 cell: 4 B cells) expressing WT baculovirus, mCD154 (stimulating mCD40), or hCD154 (stimulating hCD40-LMP1). Cytokine concentrations in culture supernatants were determined by ELISA, using cytokine-specific coating Abs and biotinylated detection Abs. Streptavidin-HRP binding to biotinylated detection Abs was visualized with TMB substrate and the reaction was stopped with 0.18 M H_2SO_4 . Plates were read at 450 nm by a SpectraMax²⁵⁰ Reader (Molecular Devices). Data were analyzed with SoftMax Pro software (Molecular Devices); unknowns were compared with a standard curve containing at least five to seven dilution points of the relevant recombinant cytokine on each assay plate. In all cases, the coefficient of determination for the standard curve (r^2) was >0.98 . Samples were diluted to fall within the standard values. Cytokine concentrations in supernatants from LN cell cultures were

assayed as above or by using the BioSource Multiplex system (Invitrogen Life Technologies) per manufacturer's protocol. Samples were analyzed using a Bio-Rad Bio-plex 200 instrument and multiplex software; unknowns were compared with a standard curve containing at least five to seven dilution points of the relevant recombinant cytokine on each assay plate. In all cases, the coefficient of determination for the standard curve (r^2) was >0.98 . Samples were diluted to fall within the standard values.

Isotype and CII-specific Ig ELISA

Assays for total (32) and anti-CII (33) isotype-specific Abs in mouse sera were performed by ELISA, using isotype-specific coating Abs (total Ig assay), 5 μ g/ml soluble CII (CII-specific Ig assay), biotinylated detection Abs, isotype standards, and *p*-nitrophenyl phosphate substrate according to the protocol provided by Southern Biotech. The reaction was stopped with 5% EDTA. Plates were read at 405 nm by a SpectraMax²⁵⁰ Reader. Data were analyzed with SoftMax Pro software (Molecular Devices); unknowns

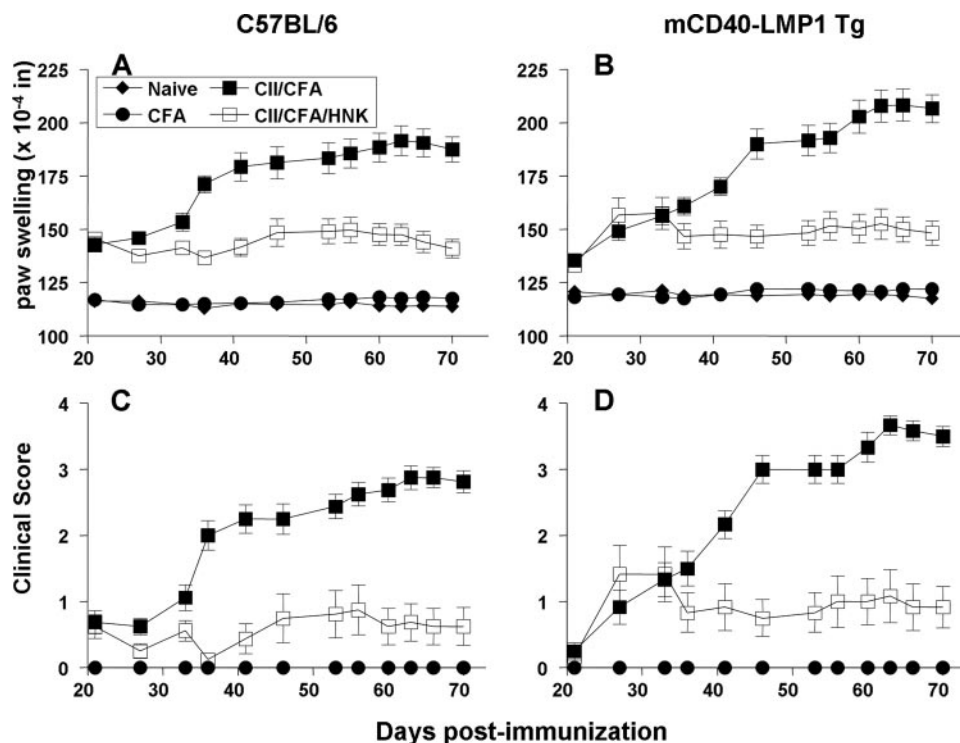


FIGURE 3. Effect of HNK treatment on symptomatic CIA in C57BL/6 and mCD40-LMP1 Tg mice. Female C57BL/6 (A and C) and mCD40-LMP1 Tg (B and D) mice were either left naive or immunized with 100 μ g of CII/CFA or CFA only as described in *Materials and Methods*. Some of the mice receiving CII/CFA were injected i.p. with 3 mg/mouse/day HNK suspended in 20% intralipid, starting at day 21 postimmunization. Paw swelling (A and B) and clinical scores (C and D) were determined for each paw every 3–4 days from days 21 to 70 postimmunization. C57BL/6: $n = 16$ for each group; mCD40-LMP1 Tg: $n = 12$ for CII/CFA and CII/CFA/HNK groups, $n = 8$ for naive and CFA only groups. Statistical analysis: $p < 0.0001$ naive/CFA only vs CII/CFA or HNK/CII/CFA for both strains (paw swelling and clinical score); $p = 0.05$ C57BL/6 vs mCD40-LMP1 CII/CFA groups overall, $p \leq 0.001$ days 41–70 postimmunization (paw swelling and clinical score); $p = 0.01$ CII/CFA vs HNK/CII/CFA (both strains, paw swelling and clinical score).

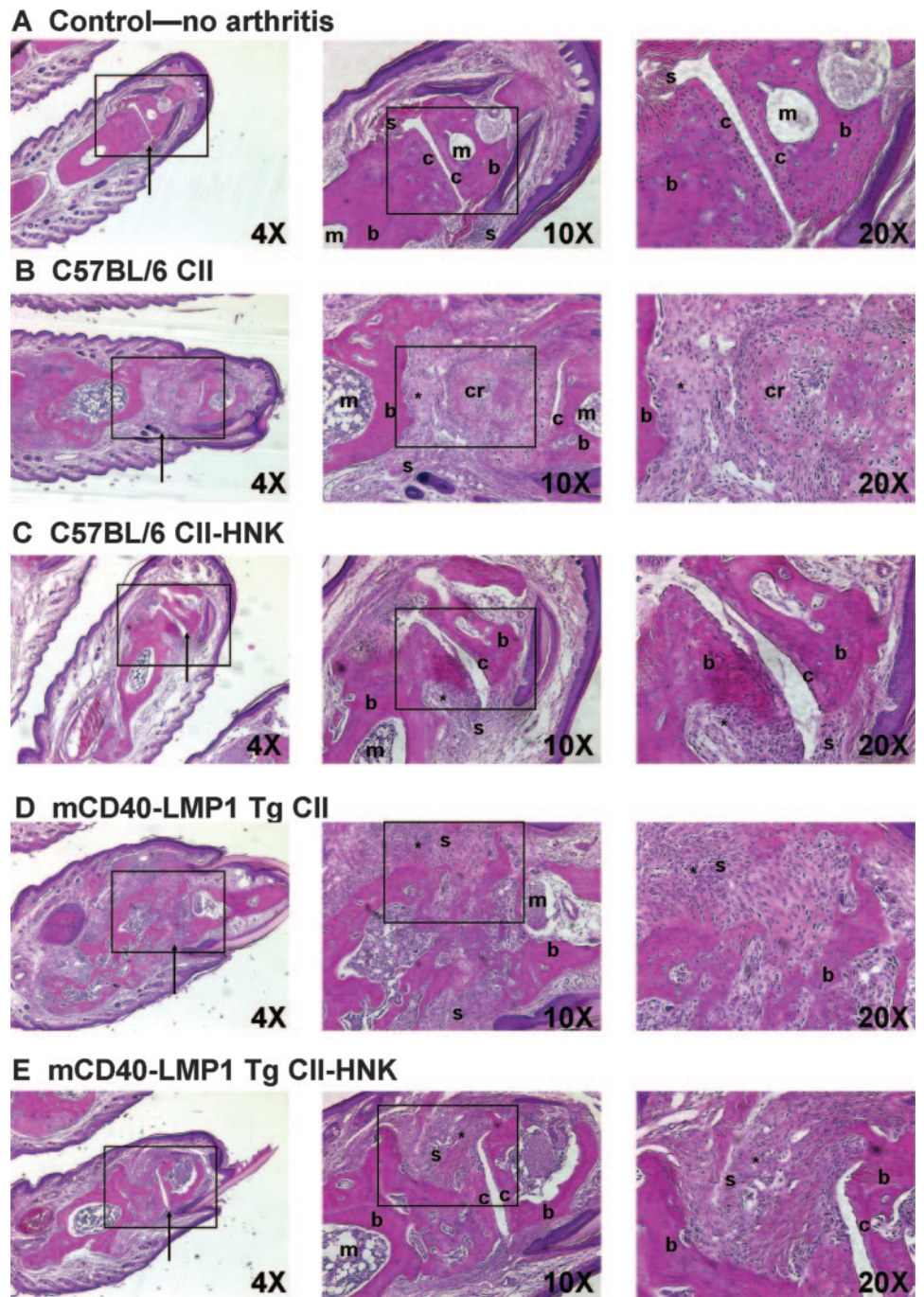


FIGURE 4. Effect of HNK on CIA-mediated inflammation. H&E-stained tissue sections from the hind paws of a control mouse (representing naive or CFA only C57BL/6 and mCD40-LMP1 Tg mice) (A), a C57BL/6 mouse immunized with CII/CFA (B), a C57BL/6 mouse immunized with CII/CFA and treated with HNK (C), a mCD40-LMP1 Tg mouse immunized with CII/CFA (D), and a mCD40-LMP1 Tg mouse immunized with CII/CFA and treated with HNK (E). Paws were analyzed on day 70 postimmunization. Each section was presented at $\times 4$, $\times 10$, and $\times 20$ original magnification, with the magnified field boxed. For orientation: b, bone; c, cartilage; s, synovium; m, marrow; cr, cartilage remodeling; *, representative site of inflammation.

were compared with a standard curve containing at least five to seven dilution points of the relevant recombinant cytokine on each assay plate. In all cases, the coefficient of determination for the standard curve (r^2) was >0.98 . Samples were diluted to fall within the standard values.

Statistical analyses

Analyses were performed with GraphPad InStat software. A two-tailed paired Student's *t* test was used to determine significance between groups in CIA experiments and for cytokine ELISA and luciferase reporter assays.

Results

Effect of HNK treatment on CIA

CIA is a well-described experimental model of inflammatory RA that is easily attainable in C57BL/6 mice (28) and is dependent on CD40-mediated B cell activation (33). Our previous findings in-

dicate that the EBV protein LMP1 mimics CD40 in a dysregulated manner (16) and that mice expressing the mCD40-LMP1 transgene (on a C57BL/6, CD40-deficient background) have elevated serum autoantibodies, as well as IL-6 and TNF- α (17). We therefore asked to what extent mCD40-LMP1 Tg mice developed CIA compared with their WT C57BL/6 counterparts (Fig. 2). Both C57BL/6 and mCD40-LMP1 Tg mice immunized with CII/CFA developed significant paw swelling and increased clinical scores over the course of 70 days compared with CFA only and naive controls. Immunization of mCD40-LMP1 Tg with CII/CFA resulted in 40% greater paw swelling than their C57BL/6 counterparts through day 70 postimmunization.

Previous studies suggest that HNK is a potential anti-inflammatory agent that is well tolerated in nonarthritic disease models (22, 23, 34). Most patients with RA or other inflammatory

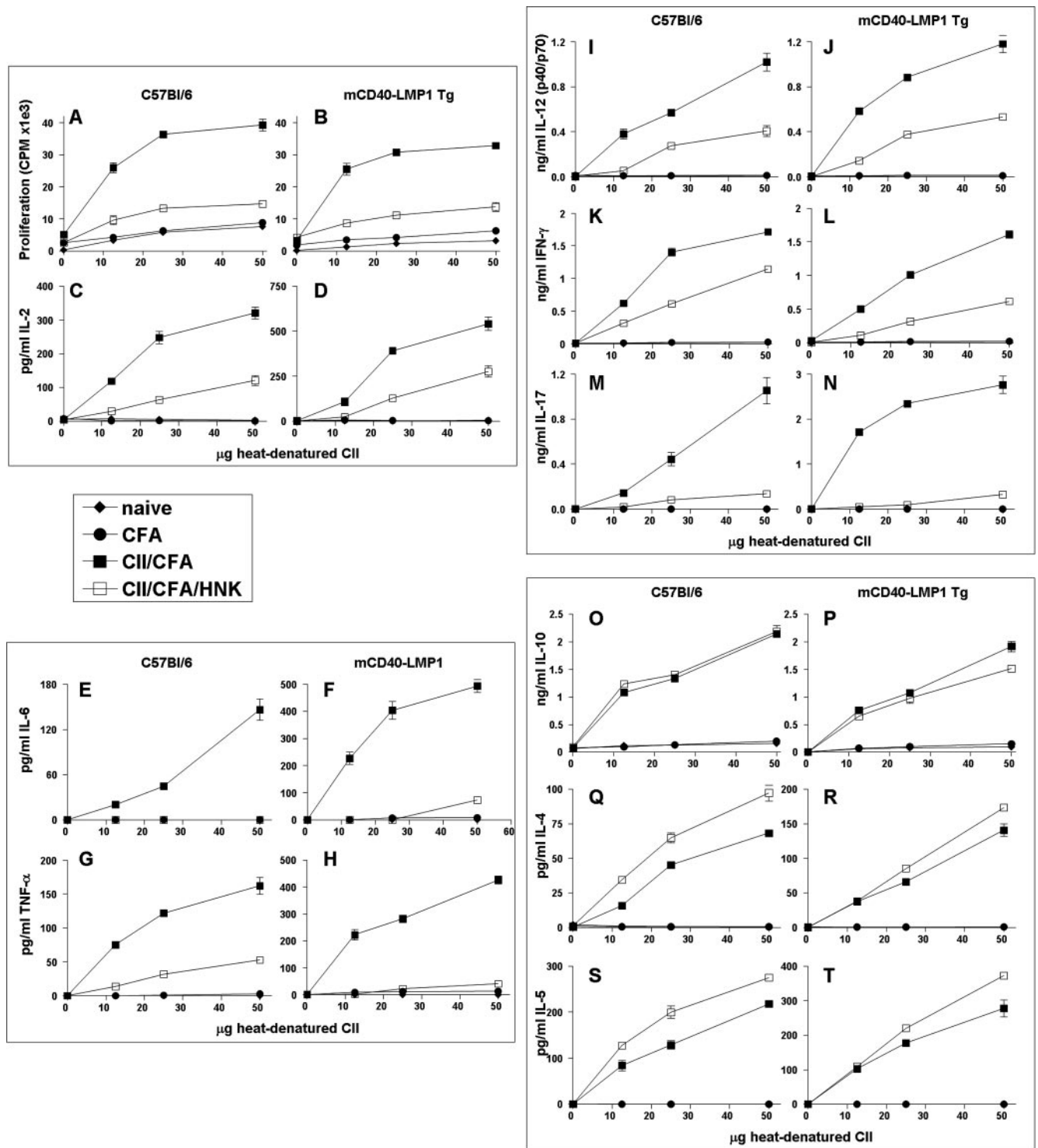


FIGURE 5. HNK effects on CII-specific response in lymph node cultures. Single-cell suspensions of inguinal and para-aortic lymph nodes from female C57BL/6 or mCD40-LMP1 Tg mice (4 e5 cells/well) were cultured with heat-denatured CII and assessed for proliferation (^3H) incorporation, cpm) and cytokine production (by ELISA), as described in *Materials and Methods*. Data points represent mean \pm SEM of duplicate wells from two experiments using pooled lymph nodes. Mice were assessed 70 days postimmunization with CII/CFA or CFA only (or naive). Some mice received HNK (3 mg/day) from days 21 to 70 postimmunization (CII/CFA/HNK). Statistical analysis: $p < 0.001$ CFA only/naive mice vs CII/CFA (both strains for proliferation and all cytokines assayed; $p \leq 0.001$ CII/CFA vs CII/CFA/HNK for proliferation in A and B and cytokines tested in C–N; $p \leq 0.05$ for cytokines tested in Q–T; N.S. for cytokines tested in O and P).

autoimmune diseases initially present clinically with already evident signs and symptoms. We therefore asked whether HNK would have an effect on the severity of CIA in C57BL/6 and mCD40-LMP1 Tg mice if daily injections (3 mg/ml/mouse) were given starting at day 21 postimmunization, when CIA is already

present in both strains of mice (Fig. 3). Overall, HNK-treated CII/CFA mice had at least 25% less paw swelling and 50–66% lower clinical scores compared with mice that received CII/CFA alone. Although HNK had a significant effect on CIA in C57BL/6 mice by day 36 ($p = 0.01$), mCD40-LMP1 Tg mice with CIA required

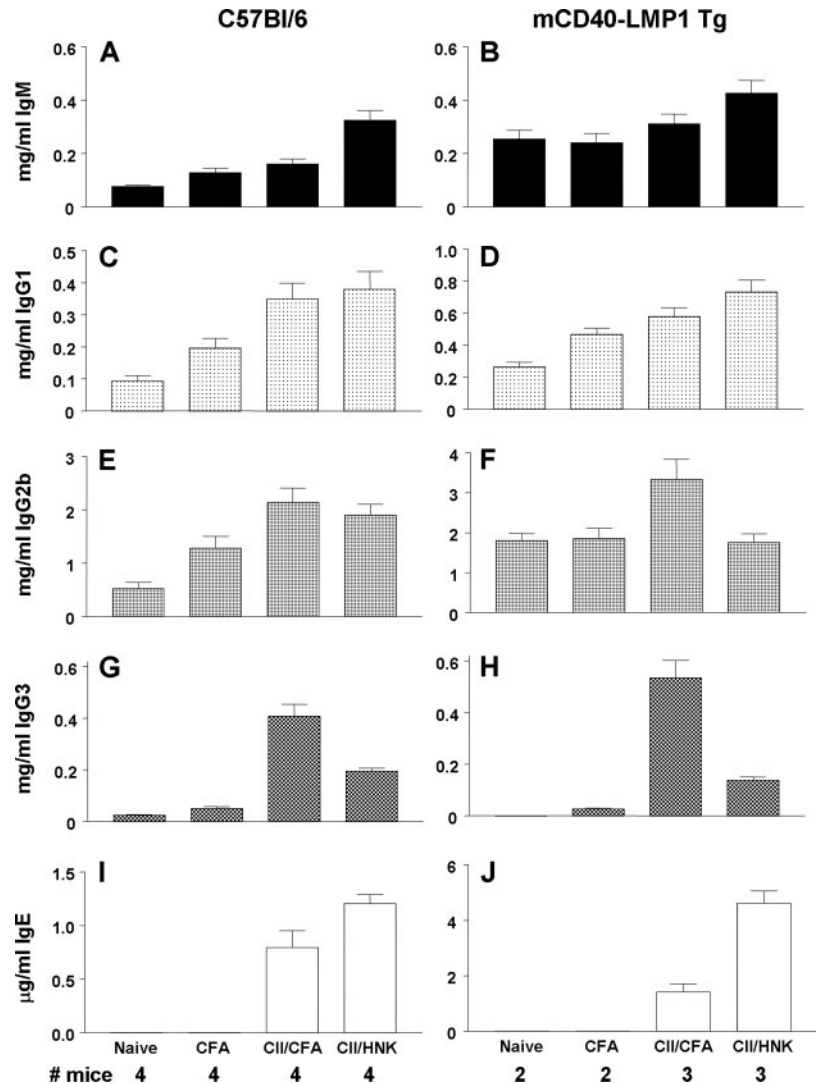


FIGURE 6. Total serum Ig isotypes in CIA mice treated with HNK. Sera were obtained from female C57BL/6 (A, C, E, G, and I) or mCD40-LMP1 Tg (B, D, F, H, and J) mice 70 days postimmunization with CII/CFA or CFA only (or naive). Some mice received HNK (3 mg/day) from days 21 to 70 postimmunization (CII/HNK). Total levels of Ig isotypes were determined by ELISA, as described in *Materials and Methods* (IgM, A–B; IgG1, C–D; IgG2b, E–F; IgG3, G–H; IgE, I–J). Each bar represents the mean \pm SEM of triplicate wells of sera from individual mice that were serially diluted from 1/16,000 to 1/256,000. Statistical analysis: CII/CFA vs HNK/CII/CFA: IgG2b ($p = 0.01$ mCD40-LMP1 Tg; IgG3 ($p < 0.001$ both strains); IgE ($p = 0.03$ C57BL/6; $p < 0.0001$ mCD40-LMP1 Tg).

10 more days of daily HNK treatment to see an optimal effect ($p = 0.03$). Although both C57BL/6 and mCD40-LMP1 Tg mice continued to have paw swelling and positive clinical scores through day 70 postimmunization compared with CFA only and naive controls, HNK treatment clearly stabilized already symptomatic CIA, and in the case of hCD40LMP1 mice, actually modestly reduced the clinical score starting at 30–40 days of treatment.

We used histological analysis of hind paws to confirm the above clinical assessments (Fig. 4). Both C57BL/6 and mCD40-LMP1 Tg mice that remained naive or received only CFA had normal joint architecture, with a distinct joint space (arrow), and no inflammatory infiltrates, consistent with a lack of inflammatory arthritis (Fig. 4A). However, paws from C57BL/6 mice receiving CII/CFA had significant disruption of the joint space (arrow), with inflammatory infiltration (macrophages, neutrophils, and lymphocytes) and obvious remodeling of articular cartilage (Fig. 4B). Inflammatory infiltration and subsequent indiscriminant remodeling of the joint space (arrow) was even more pronounced in mCD40-LMP1 Tg mice (Fig. 4D), with significant bone erosion. HNK was able to alleviate some, but not all, of the inflammatory infiltration and joint remodeling in mice that received CII/CFA (Fig. 4, C and E), corresponding to the paw swelling results seen in Fig. 3. However, unlike mice receiving CII/CFA without HNK (Fig. 4, B and D), where the joint space is completely pervaded with inflamma-

tion, both C57BL/6 (Fig. 4C) and mCD40-LMP1 Tg (Fig. 4E) mice receiving HNK had preserved joint spaces, despite residual inflammation.

Effect of HNK on CII recall response

The above data demonstrate the ability of HNK to alleviate, although not eliminate, CIA after paw swelling was already evident. To see whether HNK altered CII-specific responses, we cultured cells from inguinal and para-aortic lymph nodes of C57BL/6 and mCD40-LMP1 Tg mice in the presence of increasing amounts of heat-denatured CII (Fig. 5). Ag recall LN cultures from C57BL/6 and mCD40-LMP1 Tg mice immunized with CII/CFA were comparable with respect to proliferation (Fig. 5, A and B) and cytokine production (Fig. 5, C–T), compared with naive and CFA-only immunized mice. Mice treated with HNK continued to display a T cell-proliferative (Fig. 5, A and B) and IL-2 (Fig. 5, C and D) response to CII; however, Ag recall cultures demonstrated 3- to 4-fold lower response than cells from CII/CFA-immunized mice. Ag-specific proinflammatory IL-6 (Fig. 5, E and F) and TNF- α (Fig. 5, G and H) responses were all but ablated with HNK treatment. The proinflammatory cytokine, IFN- γ (Fig. 5, K and L), as well as IL-12 (Fig. 5, I and J), normally produced by APCs to drive IFN- γ production, were reduced 2- to 4-fold with in cultures from HNK-treated mice. Of recent importance to CIA studies, IL-17

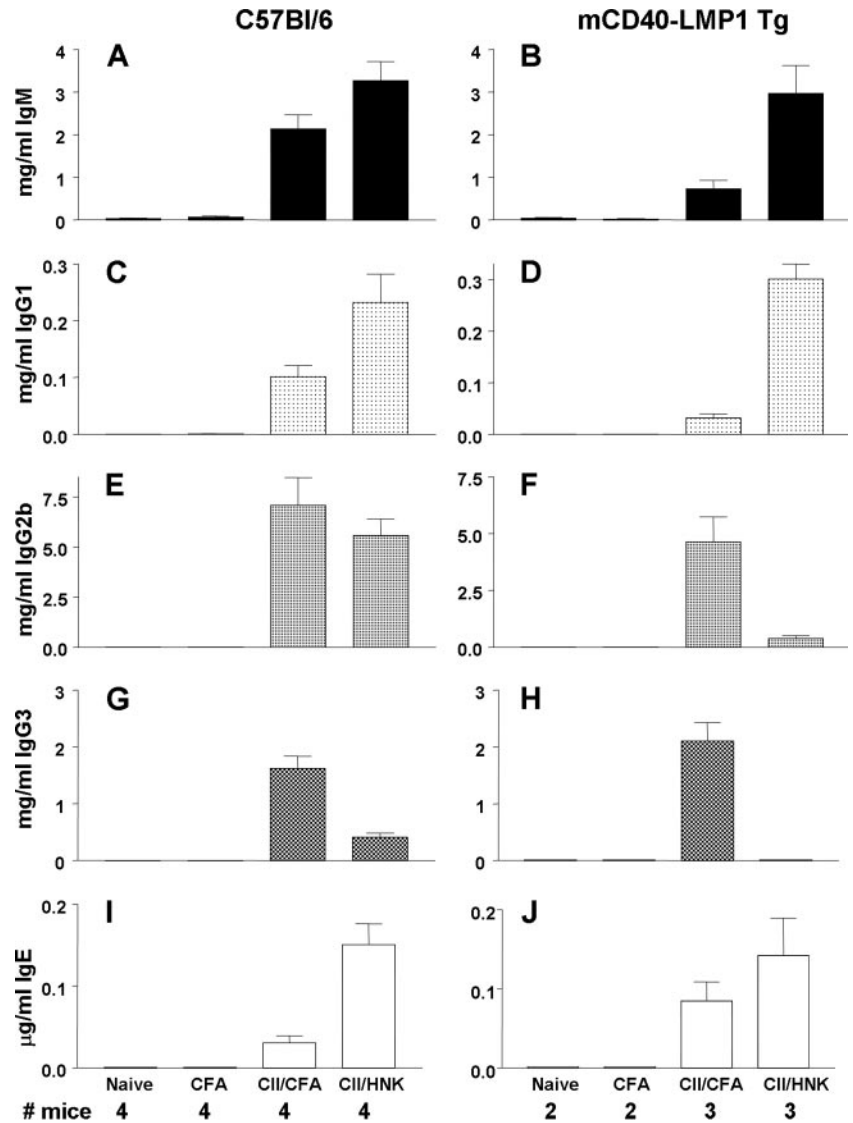


FIGURE 7. CII-specific serum Ig isotypes in CIA mice treated with HNK. Sera were obtained from female C57BL/6 (A, C, E, G, and I) or mCD40-LMP1 Tg mice (B, D, F, H, and J) 70 days postimmunization with CII/CFA or CFA only (or naive). Some mice received HNK (3 mg/day) from days 21 to 70 postimmunization (CII/HNK). Levels of CII-specific Ig isotypes were determined by ELISA, as described in *Materials and Methods* (IgM, A–B; IgG1, C–D; IgG2b, E–F; IgG3, G–H; IgE, I–J). Each bar represents the mean \pm SEM of triplicate wells of sera from individual mice that were serially diluted from 1/1,000 to 1/16,000. Statistical analysis: CII/CFA vs HNK/CII/CFA: IgM ($p = 0.05$ C57BL/6, $p = 0.002$ mCD40-LMP1 Tg); IgG1 ($p = 0.02$ C57BL/6, $p < 0.0001$ mCD40-LMP1 Tg); IgG2b ($p = 0.001$ mCD40-LMP1 Tg); IgG3 ($p < 0.0001$ both strains); IgE ($p < 0.001$ both strains).

(Fig. 5, M and N), greatly enhanced in cultures from mice receiving CII/CFA, was almost completely eliminated in cultures from HNK-treated mice. However, not all CII-specific responses were negatively affected by HNK treatment, as seen by the undiminished levels of IL-10 (Fig. 5, O and P), IL-4 (Fig. 5, Q and R), and IL-5 (Fig. 5, S and T).

Alteration of serum Ab isotype distribution by HNK treatment

B cells and the Abs they produce are critical to the pathogenesis of CIA (35), as well as to human RA, and certain Ig isotypes have been implicated as being particularly pathogenic (35, 36). We analyzed sera from C57BL/6 and mCD40-LMP1 Tg mice to determine the isotype profile of both total (Fig. 6) and CII-specific (Fig. 7) Abs. HNK-treated C57BL/6 and mCD40-LMP1 Tg mice had $\geq 30\%$ higher levels of total serum IgM (Fig. 6, A and B) and IgE (Fig. 6, I and J) Abs, with only slightly higher levels of serum IgG1 (Fig. 6, C and D), compared with CII/CFA mice. However, HNK-treated mice had a $\geq 50\%$ decrease in IgG3 (Fig. 6, G and H) and lower levels of total serum IgG2b (Fig. 6, E and F; C57BL/6 mice do not make IgG2a). This was particularly evident in mCD40-LMP1 Tg mice, which demonstrated a 40% decrease in levels of IgG2b Ab. When evaluating the presence of anti-CII-specific Abs (Fig. 7), we observed that these Abs were only present in the sera of mice immunized with CII/CFA. Again, we observed

a 30% increase in CII-specific IgM (Fig. 7, A and B) and IgE (Fig. 7, I and J) Abs, with double the amount of IgG1 (Fig. 7, C and D) in the sera of HNK-treated mice from both mouse strains. The levels of IgG2b (Fig. 7, E and F) and IgG3 (Fig. 7, G and H) were decreased in response to HNK treatment, especially in mCD40-LMP1 Tg mice. These Ab isotypes correspond to the pathogenic IgG1 and IgG3 Abs in human disease (37). The distribution trend (IgG2b/IgG3 vs IgG1/IgE) of Ab isotype production observed with HNK treatment parallels what was observed in the Ag recall culture responses in Fig. 5: decreased levels of proinflammatory IL-6, TNF- α , IL-12, IFN- γ , and IL-17, with unaffected levels of IL-10, IL-4, and IL-5.

Anti-inflammatory effects HNK on CD40- and LMP1-mediated B cell activation

B cells contribute to the development of CIA by secreting both Abs and proinflammatory cytokines, including TNF- α and IL-6, in response to CD40 (10). Given the stabilization of CIA (Figs. 3 and 4) and altered total and CII-specific Ab response in HNK-treated mice (Figs. 6 and 7), we hypothesized that HNK would also affect CD40- and LMP1-mediated proinflammatory cytokine production in B cells. Splenic B cells were isolated from C57BL/6 and mCD40-LMP1 Tg mice previously immunized with CII/CFA \pm HNK treatment, and were then stimulated with Hi5 insect cells

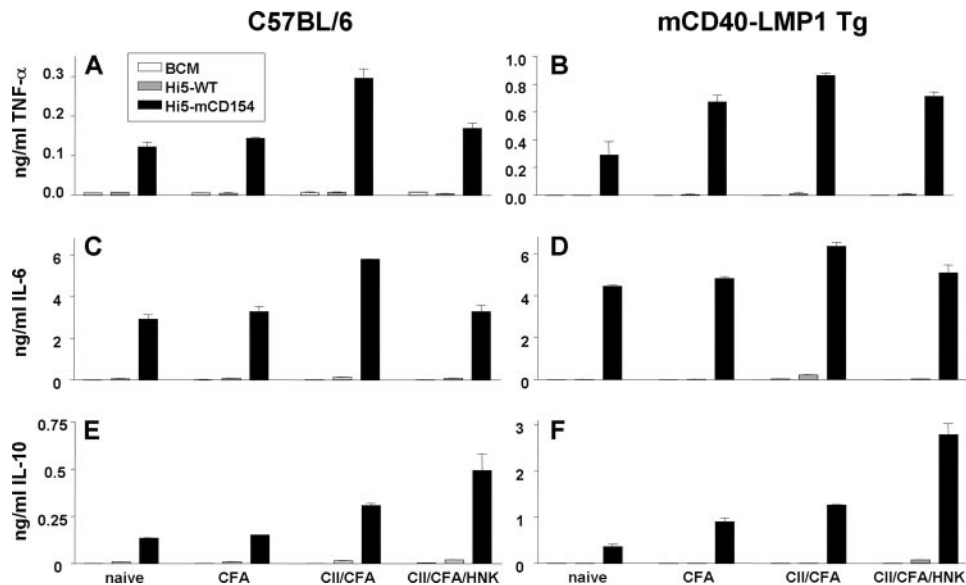


FIGURE 8. CD40 and LMP1-mediated cytokine response in primary B cells in CIA mice with or without HNK treatment. Splenic B cells were isolated from female C57BL/6 (A, C, and E) or mCD40-LMP1 Tg (B, D, and F) mice 70 days postimmunization with CII/CFA or CFA only (or naive). Some mice received HNK (3 mg/day) from days 21 to 70 postimmunization (CII/CFA/HNK). Negatively selected splenic B cells (4 e5/well TNF- α and IL-10; 1 e5/well IL-6) were cocultured in medium alone (BCM), or with Hi5 insect cells (1 e5/well TNF- α and IL-10; 2.5 e4/well IL-6) infected with baculovirus (WT) expressing mouse CD154, the ligand for CD40. Cytokines in culture supernatants was assessed by ELISA at 24 h (TNF- α) or 48 h (IL-6 and IL-10). Data points represent mean \pm SEM of duplicate wells from two experiments using B cells from individual mice, except for C57BL/6 naive and CFA only mice, where two spleens per pool were used. C57BL/6: $n = 4$ for each group; mCD40-LMP1 Tg: $n = 4$ for CII/CFA and CII/CFA/HNK groups, $n = 2$ for naive and CFA only groups. Statistical analysis: CFA only/naive vs CII/CFA: TNF- α ($p \leq 0.03$ both strains); IL-6 ($p \leq 0.01$ both strains); IL-10 ($p < 0.02$ both strains); CII/CFA vs HNK/CII/CFA: TNF- α ($p \leq 0.05$ both strains); IL-6 ($p \leq 0.05$ both strains); IL-10 ($p \leq 0.05$ both strains).

expressing CD154, the ligand for CD40 (Fig. 8). C57BL/6 and mCD40-LMP1 Tg B cells from mice receiving CII/CFA had significantly higher levels of TNF- α (Fig. 8, A and B), IL-6 (Fig. 8, C and D) and IL-10 (Fig. 8, E and F), compared with naive and

CFA only controls. This was particularly evident in C57BL/6 mice, where CD40-mediated cytokine production in B cells from CFA-only and naive controls was much lower than that of mCD40-LMP1 Tg mice. B cells from mice which received HNK

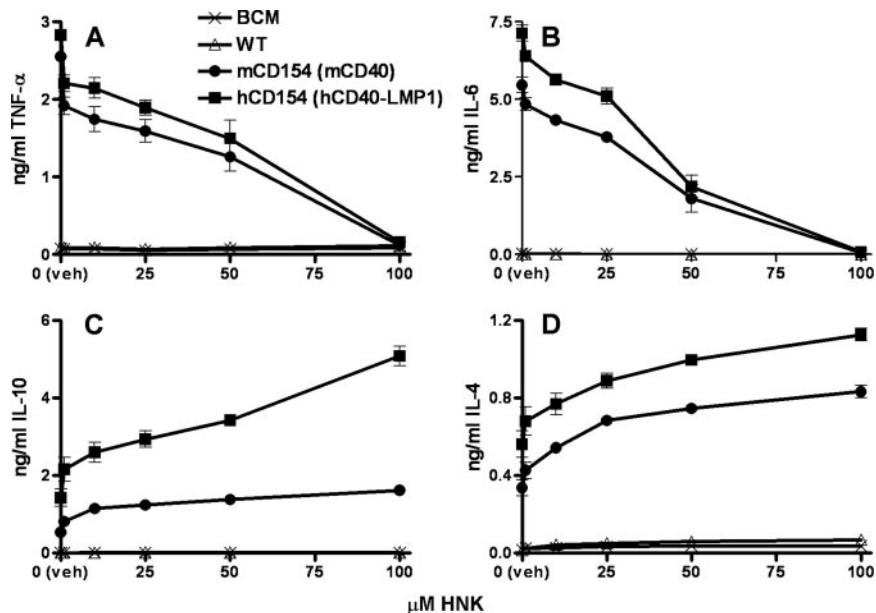
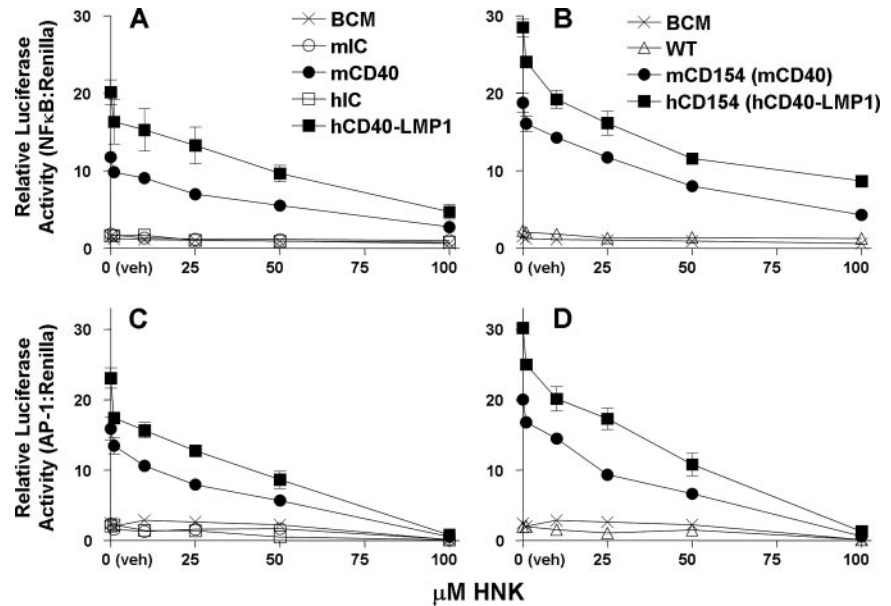


FIGURE 9. In vitro HNK treatment effects on CD40 and LMP1-mediated cytokine response in mouse B cell lines. CH12.hCD40-LMP1 (TNF- α (A) and IL-4 (D), 4 e5/well; IL-6 (B), 1 e5/well) or M12.hCD40-LMP1 (IL-10 (C), 4 e5/well) cells were cocultured in medium alone (BCM), or with Hi5 insect cells (1 e5/well TNF- α , IL-4, and IL-10; 2.5 e4/well IL-6) infected with baculovirus (WT) expressing CD154, the ligand for CD40. Cytokines in culture supernatants were assessed by ELISA at 4 h (TNF- α), 24 h (IL-4 and IL-10), or 48 h (IL-6). Data points represent mean \pm SEM of triplicate wells. Similar results were obtained in a second experiment. Statistical analysis: BCM/WT vs mCD40 or hCD40-LMP1: $p < 0.001$ for all cytokines tested); vehicle only vs HNK treatment: mCD40: TNF- α ($p < 0.001$ all HNK doses); IL-6 ($p = 0.04$ 1 mM HNK, $p \leq 0.001$ 10–100 mM HNK); IL-10 ($p = 0.01$ 1 mM HNK, $p < 0.001$ 10–100 mM HNK); IL-4 ($p < 0.001$ 10–100 mM HNK); hCD40-LMP1: TNF- α ($p < 0.001$ all HNK doses), IL-6 ($p = 0.01$ 1 mM HNK, $p < 0.001$ 10–100 mM HNK); IL-10 ($p = 0.04$ 1 mM HNK, $p < 0.01$ 10–100 mM HNK); IL-4 ($p < 0.05$ 10 mM HNK, $p < 0.001$ 25–100 mM HNK).

FIGURE 10. In vitro HNK treatment effects on CD40 and LMP1-mediated activation of NF- κ B and AP-1. M12.hCD40-LMP1 cells were transiently transfected with 4 \times NF- κ B-luciferase (A and B) or 7 \times AP-1-luciferase (C and D) and *Renilla*-luciferase reporter plasmids, rested on ice for 30 min, then stimulated for 6 (NF- κ B) or 24 (AP-1) h. Cells (2e6/ml) were stimulated either in the presence of medium alone (BCM) or 10 μ g/ml anti-mouse (mCD40) or human (hCD40-LMP1) CD40 (or relative isotype controls), A and C. Alternatively, cells were stimulated with Hi5 insect cells (4 e5/ml) expressing either (WT) baculovirus or CD154, the ligand for CD40, B and D. Relative luciferase activity (NF- κ B or AP-1:*Renilla*) of stimulus vs control was calculated as the mean \pm SEM of duplicate samples from two independent experiments. Statistical analysis: control (BCM, IC, or WT) vs mCD40 or hCD40-LMP1 stimulation: $p < 0.001$ for both NF- κ B and AP-1; HNK treatment significantly inhibited ($p < 0.0001$) both NF- κ B and AP-1.



treatment showed lower CD40- or LMP1-mediated TNF- α (Fig. 8, A and B) and IL-6 (Fig. 8, C and D), with IL-10 responses (Fig. 8, E and F) that were at least twice as high as mice receiving only CII/CFA.

Signaling via NF- κ B and AP-1 is required for optimal production of TNF- α (38) and IL-6 (31), so we next tested whether the HNK-mediated diminution of these cytokines in the Ag recall response (Fig. 5) and B cell activation by CD40 or LMP1 (Fig. 8) was due to an alteration in either of these transcription factors. To this end, we used mouse B cell lines, M12.4.1 and CH12.LX, that stably express the chimeric molecule (human) hCD40-LMP1 (29). These cells, as in the mCD40-LMP1 Tg mouse, allow us to evaluate LMP1-mediated signaling without constitutive expression of LMP1 and with endogenous expression of mCD40 as an internal control. Data in Fig. 9 demonstrate that stimulation of mouse B cell lines by mCD40 or hCD40-LMP1 results in production of elevated amounts of TNF- α (Fig. 9A), IL-6 (Fig. 9B), IL-10 (Fig. 9C), and IL-4 (Fig. 9D), compared with controls. As seen in ex vivo stimulation of B cells from mice treated with HNK (Fig. 8), in vitro treatment of mouse B cell lines with HNK altered these cytokines in a dose-dependent manner. HNK treatment inhibited CD40- and LMP1-mediated activation of TNF- α and IL-6, with a positive effect on IL-10 and IL-4 production. The dose-dependent decrease in TNF- α and IL-6 production was not due to a toxicity of HNK on cell cultures, as evidenced by IL-10 and IL-4 production, as well as a lack of significant apoptosis (<5% death via propidium iodide staining with 100 μ M HNK in culture at all time points assayed; data not shown). We then used these cells to evaluate the effect of HNK on CD40- and LMP1-mediated NF- κ B and AP-1 activation using reporter gene assays (Fig. 10). Both mCD40 and hCD40-LMP1 were able to activate NF- κ B (Fig. 10, A and B) and AP-1 (Fig. 10, C and D), whether stimulating with agonistic Ab (Fig. 10, A and C) or Hi5 insect cells which express CD154 (Fig. 10, B and D), compared with controls. Again, there was a dose-dependent decrease in both NF- κ B and AP-1 activation by either mCD40 or hCD40-LMP1 in the presence of HNK. Although AP-1 luciferase activity was completely abrogated by 100 μ M HNK, this was not the case for NF- κ B, which, although significantly inhibited, was still present. These data demonstrate that HNK mediated anti-inflammatory effects at the transcriptional level.

Discussion

There is a pressing need for safe and effective antirheumatic agents that have minimal side effects. Long-used standard treatments, including methotrexate and steroid drugs, have well-known and significant toxic side effects. Anti-TNF- α therapy has provided some relief of symptoms and respite from these effects. However, like other broad anti-immune agents, there are still side effects, including increased susceptibility to infection (39). Data presented in this study indicate that HNK treatment stabilized already symptomatic CIA (Figs. 3 and 4) at a dose (34) that was well tolerated over 7 wk of daily injections and falls within pharmacokinetic range (23). Despite comparatively augmented B cell activation by LMP1, HNK also effectively exerted its anti-inflammatory effects on LMP1-mediated inflammation. These findings are particularly encouraging in light of studies whereby EBV proteins, including LMP1 (40), are implicated not only in the pathogenesis of arthritis (41), but in the development of lymphoproliferative and oncogenic disease secondary to arthritis (42).

Treatment with HNK ex vivo or in vitro altered known mediators of CIA. Specifically, treatment induced a distinct shift of CII-specific recall (Fig. 5) and Ab isotype responses (Figs. 6 and 7) from a response characterized by the proinflammatory cytokines IFN- γ and IL-17 toward a response dominated by cytokines such as IL-10, IL-4, and IL-5. CD40-mediated B cell activation, a key component of CIA pathogenesis (33), was inhibited by HNK treatment, and TNF- α and IL-6 were diminished in a dose-dependent manner, without decreasing IL-4 or IL-10 secretion (Figs. 8 and 9). The support of IL-4 and IL-10 production by HNK is particularly beneficial given the established anti-inflammatory effects of these two cytokines (43, 44).

Although HNK treatment stabilized CIA in both C57BL/6 and mCD40-LMP1 Tg mice, the arthritis was not completely abrogated. Unlike mice immunized with CII/CFA, where the joint space was completely obliterated with inflammation, the joint space of HNK-treated mice was seemingly preserved, despite residual inflammation in the synovium (Fig. 4). This may be due to a reduction in IL-17 with HNK treatment, a cytokine which has been shown to lead directly to bone erosion and joint damage (45). Although levels of IL-17 were almost completely abrogated in Ag recall cultures from HNK-treated mice (Fig. 5, M and N), levels of

IL-12 (Fig. 5, *I* and *J*) and IFN- γ (Fig. 5, *K* and *L*) were inhibited, but remaining amounts of cytokine were detected. This continued basal Ag-specific response was also evident via proliferation (Fig. 5, *A* and *B*) and IL-2 production (Fig. 5, *C* and *D*), factors which may have contributed to continued although reduced synovial inflammation in HNK-treated mice (Figs. 3 and 4).

Given the importance of B cells in CIA pathogenesis, incomplete abrogation of TNF- α and IL-6 production, particularly with LMP1-mediated activation (Fig. 8) may have also contributed to residual inflammation. Furthermore, B cells treated with HNK in vivo (Fig. 8), or in vitro (Fig. 9), made more CD40- or LMP1-mediated IL-10 and IL-4, cytokines which supported CII-specific Ab production and isotype switching (46), as demonstrated in Figs. 6 and 7. Although increased levels of these cytokines could actually lead to the elimination of CIA, this is not the case, either through direct administration (47) or HNK treatment. It is possible that once the proinflammatory response is established, resulting in joint damage, cytokines such as IL-4 and IL-10 can only inhibit further joint destruction, or alternatively, maintain chronic disease via supported production of CII-specific Abs.

There are multiple mechanisms by which HNK could exert its stabilization of CIA. The ability of HNK to inhibit IL-12, IFN- γ , and IL-17 (Fig. 5), as well as TNF- α and IL-6 (Figs. 5, 8, and 9), is likely to be an important contributor to this process. IFN- γ receptor-deficient mice have decreased CIA with an increase in IL-4 and IL-10 responses (48), similar to that seen with HNK treatment. Also similar to effects of HNK treatment is CIA in IFN- γ -deficient mice, a model in which the Th2 Ab isotype IgG1 plays a significant role in disease pathogenesis (49). The ability of HNK to inhibit IL-17 production (Fig. 5) and preserve the joint space (Fig. 4), is quite similar to what is seen when IL-17 is directly blocked (50). With respect to TNF- α and IL-6, although the TNFRI/IL-6 double knockout mouse has significantly decreased CIA (51), TNFRII, which is the only TNFR present on mouse B cells (52) and plays a significant role in CD40-mediated B cell activation (53), can still mediate disease (54).

It is consistent with the anti-inflammatory effect of HNK on CIA to see the dose-dependent inhibition of the transcriptional activators NF- κ B and AP-1 in B cells (Fig. 9). Although NF- κ B and AP-1 also play roles in isotype switching, the presence of IL-4 can compensate for their inhibition (55). This suggests that the increased amount of IgM seen with HNK treatment may be the result of NF- κ B and AP-1 inhibition, with the presence of IL-4 allowing for some isotype switching, particularly to the IgG1 and IgE isotypes (Figs. 6 and 7). That NF- κ B is not completely inhibited by HNK (Fig. 10, *A* and *B*) may explain why there is still some level of IgG2b and IgG3 seen with HNK treatment in vivo (Figs. 6 and 7).

In addition to alteration of cytokine and Ab production, there is evidence that HNK alters oxidative stress (25, 26). NO and reactive oxygen species have both been shown to contribute to the chronic inflammation of arthritis (56) and the direct alteration in joint cartilage homeostasis (57). One possible common mechanism to explain the effect of HNK on these varied mediators of inflammation is its binding with peripheral γ -aminobutyric acid GABA(A) receptors (58). Peripheral GABA(A) receptors are present on immune cells and have been shown to inhibit lymphocyte activation and lymphocyte-mediated inflammatory disease (59).

HNK has promise as a clinical therapeutic agent. It has been long used in traditional Asian medicine and appears to be safe and effective in mice for models of inflammatory arthritis (present study) and as an antiangiogenic agent in cancer (34). RA has been shown to be a predisposing factor to cancer development, either as

a primary risk factor or secondary to therapy, especially with the use of methotrexate (3). This is particularly problematic in arthritis patients who are EBV positive, as is the majority of the human population, because LMP1 is a known contributor to lymphoma pathogenesis (14). HNK could therefore be a beneficial addition to current therapeutic strategies to not only manage rheumatologic symptoms due to inflammation, but to also reduce the risk of cancer development, particularly in those patients positive for EBV.

Disclosures

The authors have no financial conflict of interest.

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