ORIGINAL RESEARCH

# Modified High-Molecular-Weight Hyaluronan Promotes Allergen-Specific Immune Tolerance

John A. Gebe[1], Koshika Yadava[2,3], Shannon M. Ruppert[2,3], Payton Marshall[3], Paul Hill[4], Ben A. Falk[3], Johanna M. Sweere[2,3], Hongwei Han[1], Gernot Kaber[2], Carlos Medina[2,3], Katalin Mikecz[5], Steven F. Ziegler[1], Swathi Balaji[6], Sundeep G. Keswani[6], Vinicio A. de Jesus Perez[7], Manish J. Butte[3], Kari Nadeau[7], William A. Altemeier[8], Neil Fanger[4], and Paul L. Bollyky[1,2,3]

[1]Benaroya Research Institute, Seattle, Washington; [2]Division of Infectious Diseases and Geographic Medicine, Department of Medicine, [3]Stanford Immunology, and [7]Division of Pulmonary and Critical Care, Stanford University Medical Center, Stanford University School of Medicine, Stanford, California; [4]Virtici, LLC, Seattle, Washington; [5]Department of Orthopedic Surgery, Rush University Medical Center, Chicago, Illinois; [6]Division of Pediatric Surgery, Baylor College of Medicine, Houston, Texas; and [8]Division of Pulmonary and Critical Care Medicine, University of Washington, Seattle, Washington

## Abstract

The extracellular matrix in asthmatic lungs contains abundant low-molecular-weight hyaluronan, and this is known to promote antigen presentation and allergic responses. Conversely, high-molecular-weight hyaluronan (HMW-HA), typical of uninflamed tissues, is known to suppress inflammation. We investigated whether HMW-HA can be adapted to promote tolerance to airway allergens. HMW-HA was thiolated to prevent its catabolism and was tethered to allergens via thiol linkages. This platform, which we call "XHA," delivers antigenic payloads in the context of antiinflammatory costimulation. Allergen/XHA was administered intranasally to mice that had been sensitized previously to these allergens. XHA prevents allergic airway inflammation in mice sensitized previously to either ovalbumin or cockroach proteins. Allergen/XHA treatment reduced inflammatory cell counts, airway hyperresponsiveness, allergen-specific IgE, and T helper type 2 cell cytokine production in comparison with allergen alone. These effects were allergen specific and IL-10 dependent. They were durable for weeks after the last challenge, providing a substantial advantage over the current desensitization protocols. Mechanistically, XHA promoted CD44-dependent inhibition of nuclear factor-κB signaling, diminished dendritic cell maturation, and reduced the induction of allergen-specific CD4 T-helper responses. XHA and other potential strategies that target CD44 are promising alternatives for the treatment of asthma and allergic sinusitis.

**Keywords:** dendritic cell; hyaluronan; tolerance; allergy; T cell

### Clinical Relevance

We describe a novel tolerogen that can be used to induce allergen-specific tolerance in animals sensitized previously to those allergens. These effects were durable for 2 weeks, providing an advantage over current desensitization protocols.

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Correspondence and requests for reprints should be addressed to Paul L. Bollyky, M.D., Ph.D., Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University School of Medicine, Grant Building, 300 Pasteur Drive, Stanford, CA 94305-5107. E-mail: pbollyky@stanford.edu

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Hyaluronan (HA) is an extracellular matrix polysaccharide with well-established roles in pulmonary immunology and pathophysiology that are dependent, in part, on the length of HA polymers (1–4).

Intact, high-molecular-weight hyaluronan (HMW-HA) ($>1 \times 10^6$ Da) predominates in uninjured or healing tissues (5) and is antiinflammatory in a variety of *in vitro* and *in vivo* model systems (6–11). HMW-HA prevents cell growth and differentiation (12), diminishes the production of inflammatory cytokines (13), and impairs phagocytosis (14). Many of these antiinflammatory effects of HMW-HA are mediated via CD44 (15).

Conversely, fragments of low-molecular-weight hyaluronan (LMW-HA), generated through catabolism of HMW-HA (16), predominate in chronically inflamed tissues and are typically proinflammatory (17–19). LMW-HA promotes the activation and maturation of dendritic cells (DC) (20), drives the release of proinflammatory cytokines (21), drives chemokine expression and cell trafficking (18), and promotes proliferation (22). Many of these proinflammatory effects are attributed to interactions with Toll-like receptor (TLR) 2 or TLR4 (22–24). LMW-HA promotes TLR-mediated nuclear translocation of nuclear factor-κB (NF-κB) (17). LMW-HA plays well-established roles in asthma (4, 25–27) and is associated with worse airway disease (11, 28–30). In light of these associations, HA size has been termed a natural biosensor for the state of tissue integrity (31).

Given the antiinflammatory properties of HMW-HA, we hypothesized that it could be used to promote tolerance to airway allergens. Specifically, we asked whether delivery of an allergen, bundled with HMW-HA, could ameliorate disease in a mouse model of allergic airway inflammation.

## Materials and Methods

### Reagents

HMW-HA ($1.5 \times 10^6$ Da) was purchased from Genzyme (Cambridge, MA). XHA was generated using thiol-labeled HA ($5 \times 10^5$ Da) sold as Glycosil by BioTime, Inc. (Alameda, CA). This was mixed together with polyethylene (glycol) diacrylate (PEGDA) crosslinker sold by BioTime Inc. at ratios per the manufacturer's instructions. Phosphate-buffered saline (PBS) was used as the diluent before the addition of PEGDA to achieve a 0.1% hydrogel. This material was made fresh at the beginning of each 5-day intranasal (IN) administration period and was allowed to polymerize fully overnight before use. LMW-HA was generated from these reagents either via overnight digestion with hyaluronidase (HA'ase), as described previously (32) or via sonication for 60 minutes.

### Transgenic Mice

All animals used, including $CD44^{-/-}$ mice (33), were on a BalbC background, with the exception of the IL-$10^{-/-}$ mice, which were on a C57Bl6 background. The animals were bred and maintained at a specific pathogen-free Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility at the Benaroya Research Institute. All experiments were approved by the Benaroya Research Institute Institutional Animal Care and Use Committees (protocol approval number 10116).

### Allergic Airway Inflammation Protocol

Mice were primed intraperitoneally on Days 1 and 8 with 200 μL containing 100 μL alum (InvivoGen Alhydrogel 2%), 100 μL PBS, with 50 μg chicken ovalbumin (OVA) (Sigma A7642–1VL, St. Louis, MO). IN challenge reagents were prepared by mixing in the following order: (*1*) OVA, (*2*) PBS, (*3*) HA when used, and (*4*) PEGDA crosslinker when generating XHA. Challenge reagents sat at room temperature for at least 1 hour to ensure complete crosslinking of HA. XHA and controls were used at a final concentration of 0.1% wt/vol. OVA was used at 25 μL/ml. The mice were IN challenged for 5 consecutive days (Days 21–25), with 50 μL/day of challenge reagent after brief anesthesia with isoflurane. On the day after the last challenge (Day 26), the mice were killed with 2-2-2-Tribromethanol for tissue harvest. Bronchoalveolar lavages (BAL) were performed with $4 \times 1$ ml flushes of the lung with PBS. Before cell counting and flow cytometry, cells were lysed with ACK RBC lysis buffer (5′ 37°C, washed with media or PBS).

### Physiologic Assessment of Airway Hyperreactivity

The mice were anesthetized with pentobarbital (90 mg/kg, i.p.) and intubated with a 20-gauge metal cannula via tracheotomy. Intubated mice were connected to a computer-controlled mechanical ventilator (flexiVent; SCIREQ, Montreal, QC, Canada) with a tidal volume of 10 ml/kg, rate of 180 bpm, and end-expiratory pressure of 3 cm $H_2O$. After induction of neuromuscular paralysis with pancuronium (0.8 mg/kg), a slow inflation to total lung capacity as defined by an end-inspiratory pressure of 30 cm $H_2O$ was repeated twice. Lung resistance and elastance were then measured, using a single-frequency forced oscillation maneuver as described previously (34) at baseline and after progressive aerosolized methacholine challenge with concentrations ranging from 0 to 25 mg/ml.

### Statistical Analyses

Unless otherwise stated, each experiment was performed at least two times. Data were analyzed using Student's *t* test. Standard error is shown. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Statistical significance was considered to be $P < 0.05$.

## Results

### Thiolation and Crosslinking Prevents the Catabolism of HMW-HA

A practical concern with using HMW-HA to promote immune tolerance is the rapid catabolism of HMW-HA *in vivo* by HA'ases (16). We found that this can be overcome via the thiolation of the aldehyde groups on HA disaccharides. Although unadorned HMW-HA was broken down by increasing concentrations of HA'ase, HA that had been thiolated at ∼40% of its residues was highly resistant to enzymatic degradation (Figure 1A).

To further enhance the stability of thiolated HA polymers, we crosslinked them using PEGDA. Although thiol groups will crosslink spontaneously over time through interactions with ambient oxygen, the addition of PEGDA allowed us to standardize the level of crosslinking. We called this crosslinked preparation "XHA."

Chemical crosslinking also increased the effective size of HA. Compared with uncrosslinked HA and terminally digested LMW-HA fragments, XHA barely migrated through the gel (Figure 1B). This is consistent with XHA being crosslinked into a network of polymers. There were no

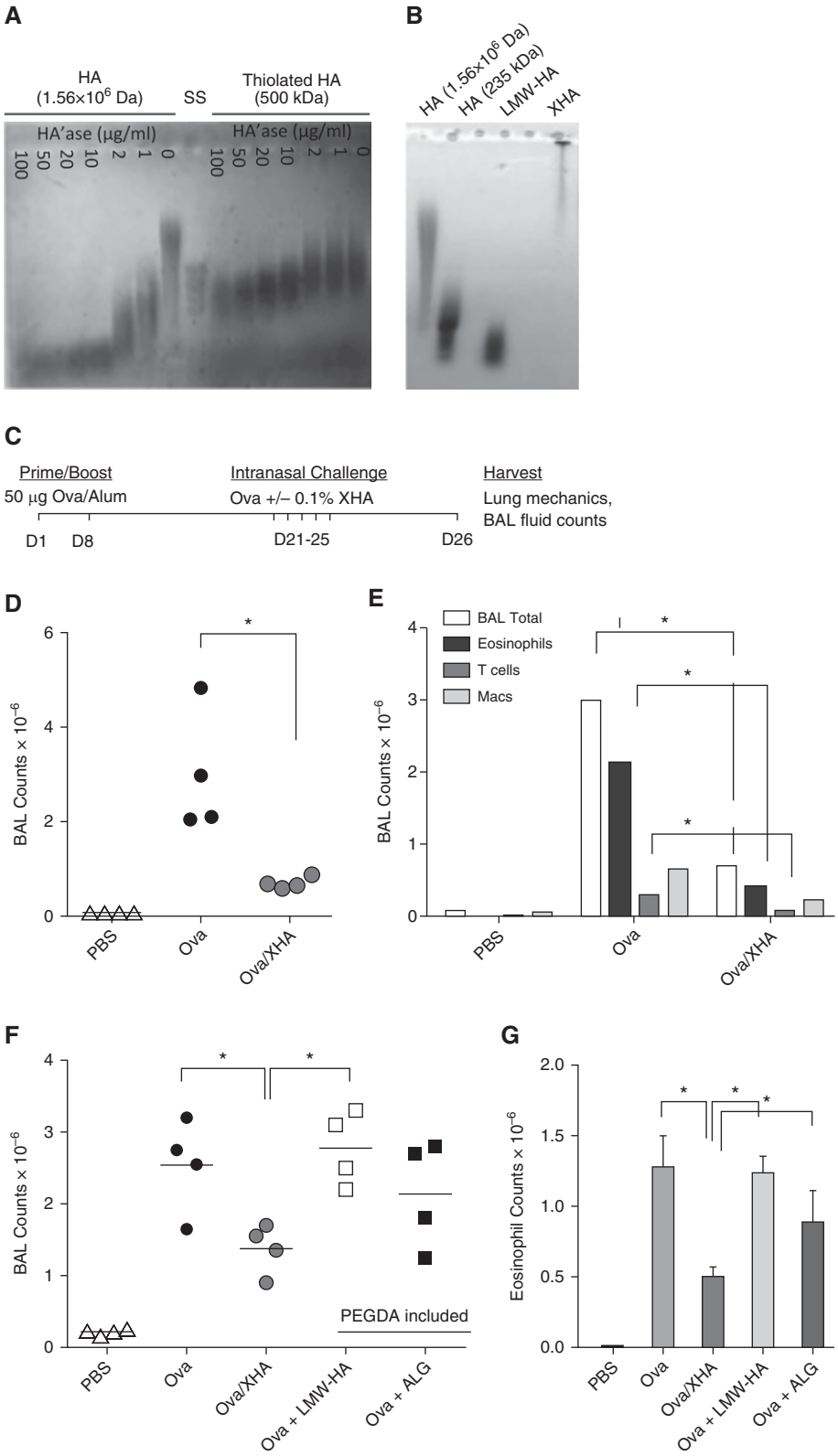


**Figure 1.** Chemically modified high-molecular-weight hyaluronan (HMW-HA) (XHA) coupled with ovalbumin (OVA) (OVA/XHA) prevents allergic airway inflammation in mice previously sensitized to OVA. (*A*) Agarose gel electrophoresis of unmodified 1.56 × 106 443 Da HMW-HA and thiolated, 500 kD HMW-HA treated with increasing concentrations of hyaluronidase (HA'ase). A size standard (SS)

lower bands, suggesting that the whole XHA preparation was involved in this crosslinking. A solution of 0.1% XHA could be readily administered IN, whereas more concentrated preparations were judged to be too viscous. Unless otherwise noted, the concentration of XHA used for all experiments was always 0.1%.

### XHA Inhibits Allergic Airway Inflammation in Previously Sensitized Mice

We investigated the role of XHA in preventing allergic response in an OVA-induced mouse model of allergic airway inflammation (35). In this model, mice were sensitized by intraperitoneal (IP) injection of OVA with alum before OVA challenge on Days 21 through 25. On Day 26, the mice were killed and the development of allergic airway inflammation was evaluated. A schematic of this protocol is shown in Figure 1C.

Pretreatment with XHA on Days 16–20 did not protect the mice from allergic airway inflammation after OVA challenge on Days 21–25 (data not shown). Injection of XHA intraperitoneally, unlike OVA, which was given IN on Days 21–25, also did not protect mice from allergic airway inflammation (data not shown). These data indicated that XHA given at different times or via different routes than those of allergen did not prevent allergic airway inflammation.

We then evaluated the strategy of conjugating the OVA to XHA and delivering these molecules at the same time. Because OVA has four free thiol groups, the preincubation of OVA and thiolated HA can be expected to result in a covalently linked complex, particularly on addition of PEGDA. Using this preparation, the mice were challenged IN with OVA or OVA conjugated to XHA (which we call OVA/XHA) on Days 21 through 25 (Figure 1C). We found that the administration of OVA/XHA prevented airway inflammation in previously sensitized animals (Figure 1D). In more three independent experiments, each involving between four and eight mice per experimental group, this treatment typically led to an approximately threefold decrease in total BAL cell counts. We also observed decreased numbers of T cells ($CD3^+CD4^+$) and eosinophils ($CD45^+CD11c^-$/Siglec $F^+$) (Figure 1E), key mediators of allergic disease.

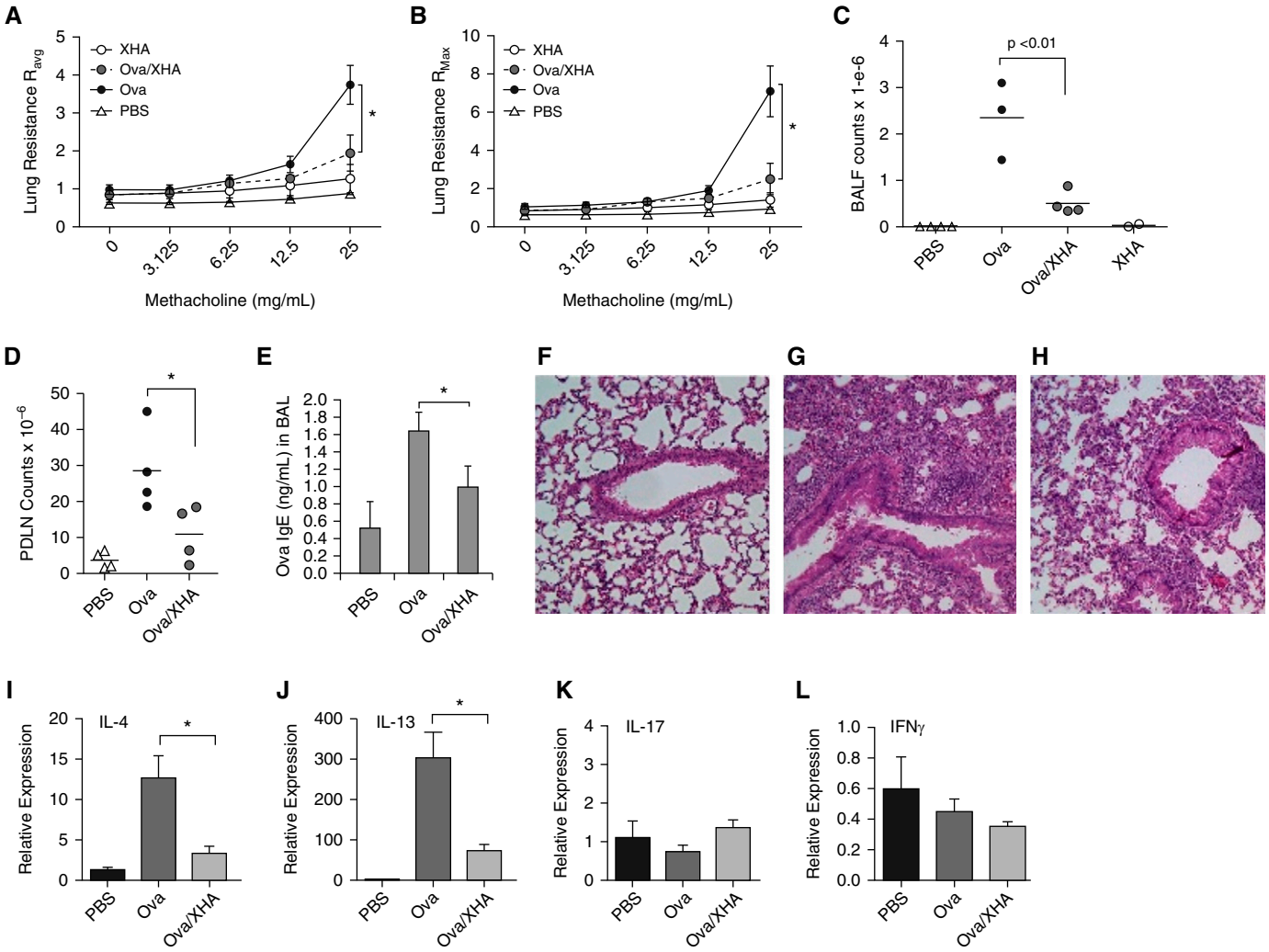


**Figure 2.** OVA/XHA prevents physiologic, histologic, and immunologic manifestations of allergic airway inflammation in mice sensitized previously to OVA. Average (*A*) and maximal (*B*) lung resistance in response to methacholine challenge. (*C*) Total BAL fluid (BALF) cell counts for the same mice as in *A* and *B*. (*D*) Pulmonary draining lymph node (PDLN) cell numbers for mice sensitized with OVA and then subsequently challenged with OVA or OVA/XHA. (*E*) OVA-specific IgE levels in BALF isolated from sensitized mice challenged with OVA or OVA/XHA. Data are for BAL pooled for five mice per group and measured in triplicate. Representative hematoxylin and eosin–stained lung sections for mice treated with (*F*) PBS, (*G*) OVA, or (*H*) OVA/XHA. Cytokine mRNA expression in pulmonary tissue of mice treated with OVA or OVA/XHA, specifically (*I*) IL-4, (*J*) IL-13, (*K*) IL-17, and (*L*) IFN-γ, all normalized to 18S expression. Tissues from five animals were pooled for this experiment and measured in triplicate. $^*P < 0.05$ by a Student's *t* test. SE is shown.

### HA Fragments and Unmodified HA Do Not Suppress Allergic Airway Inflammation

We sought to define the properties of XHA that are critical to suppressing allergic airway inflammation.

To investigate the role of HA polymer length, we evaluated LMW-HA in our model. This material was generated by sonication of the same HMW-HA used to make XHA and was identical in chemical composition. Because this LMW-HA was thiolated and crosslinker was added, it was also expected to covalently bind to OVA. However, it did not reduce total BAL counts or eosinophilia (Figures 1F and IG).

As a control for HA composition, we evaluated alginate (ALG), a polymer with

**Figure 1.** (Continued). ladder of 400–100 kD hyaluronan (HA) is also shown. (*B*) Agarose gel electrophoresis of unmodified 1.56 × 106 445 Da HMW-HA thiolated, 500 kD HMW-HA ± polyethylene (glycol) diacrylate (PEGDA) crosslinker (XHA) and controls. XHA was crosslinked overnight. All reagents were purchased commercially, and the aforementioned HA size characteristics are per the manufacturers' information. Data in *A* and *B* are each representative of three experiments. (*C*) Schematic of the OVA-induced allergic airway inflammation protocol. (*D*) Total cell counts and (*E*) leukocyte subsets in bronchoalveolar lavage (BAL) fluid of mice rechallenged with OVA/XHA. Data in *D* and *E* are representative of eight independent experiments. Total cell counts (*F*) and eosinophils (*G*) in BAL fluid of mice treated with OVA, OVA/XHA, low-molecular-weight hyaluronan (LMW-HA), or alginate (ALG). $^*P < 0.05$ by Student's *t* test. SE is shown. Macs, macrophages.

charge and size properties similar to those of HMW-HA. It did not reduce total BAL counts or eosinophilia (Figures 1F and IG). The same held true for collagen (data not shown).

We then questioned whether chemical modification of HA is important. Chemically unmodified (nonthiolated) HMW-HA did not significant reduce BAL fluid counts (*see* Figure E1A in the online supplement). Conversely, thiolated HA without inclusion of the PEGDA crosslinker was sufficient to prevent allergic airway inflammation in our model (Figure E1B). However, thiolated HA can be expected to crosslink spontaneously on reaction with ambient oxygen, making the relative importance of thiolation versus crosslinking difficult to ascertain. Therefore, to standardize this crosslinking, we consistently added PEDGA to our XHA preparations.

Together, these data indicate that thiolation and perhaps crosslinking are necessary for the effectiveness of XHA.

### XHA Reduces Physiologic, Molecular, and Histologic Measurements of Allergic Airway Inflammation

Together with reduced cellular readouts of inflammation (decreased BAL fluid total counts and eosinophilia), OVA/XHA treatment reduced airway hyperresponsiveness as measured by change in lung resistance to increasing doses of methacholine challenge (Figures 2A–2C), pulmonary draining lymph node (PDLN) cell counts (Figure 2D), and OVA-specific IgE (Figure 2E). Histological analysis of lung sections likewise confirmed a decrease in cellular recruitment in mice treated with OVA/XHA (Figures 2F–2H). In addition, OVA/XHA-treated animals exhibited a decreased expression of Th2 cytokine mRNA (IL-4 and IL-13), but not the Th1 cytokine IFN-γ or the Th17 cytokine IL-17, in lung tissue (Figures 2I–2L).

Together, these physiologic, histologic, molecular, and immunologic data strongly support the conclusion that OVA/XHA inhibits allergic airway inflammation in response to airway allergens.

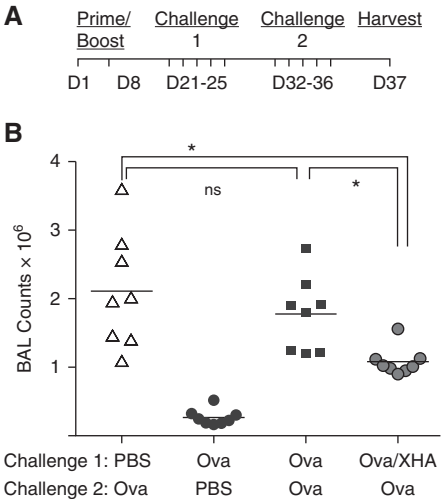


**Figure 3.** Antiinflammatory effects of XHA on allergic airway inflammation are durable for 2 weeks. (*A*) Schematic of the protocol used to evaluate the durability of OVA/XHA-mediated tolerization. (*B*) BALF cell counts in response to challenge with OVA alone, in mice that received OVA/XHA 12–16 days earlier. Data are representative of three independent experiments. $^*P < 0.05$ by Student's *t* test. SE is shown. ns, not significant.

### Antiinflammatory Effects of OVA/XHA on Allergic Airway Inflammation Are Durable for 2 Weeks

We also investigated if these OVA/XHA-mediated effects were durable over time. Animals were sensitized to OVA in albumin on Days 1 and 8 and were then challenged intranasally with OVA/XHA daily for challenge 1 (Days 21–25). These same animals were then rechallenged with OVA alone for challenge 2 (Days 32–36). BAL fluid cell counts were then assessed on Day 37. A schematic of this protocol is shown in Figure 3A. We found that OVA/XHA-exposed mice were protected from allergic airway inflammation in response to OVA, despite having last received OVA/XHA 12–16 days earlier (Figure 3B). This indicates that OVA/XHA-induced immune tolerance is durable in this model.

When we extended the time period between challenge 1 and challenge 2, so that the animals last received OVA/XHA 22–26 days earlier, the mice were no longer protected from OVA-induced allergic airway inflammation (Figure E2). This indicates that OVA/XHA-mediated suppression of allergic airway inflammation is durable but nonpermanent.

### OVA/XHA Treatment Inhibits Local Allergen-Specific Responses

We also asked whether XHA could prevent allergic airway inflammation in response to a different allergen, cockroach antigen (CRA). Using a protocol using IN sensitization with CRA (36), we found that CRA/XHA treatment reduced cellular recruitment significantly in the PDLN (Figure E3A), but this effect did not reach statistical significance in the BAL (Figure E3B). This distinction may reflect the fact that inflammation in the CRA model is less pronounced in the BAL, relative to the OVA model.

We also investigated whether treatment with XHA and one allergen could induce tolerance to another allergen using the protocol in Figure E3C. When mice sensitized to OVA (on Days 1 and 8) subsequently received CRA/XHA (challenge 1; Days 21–25), they nonetheless developed robust inflammation in response to subsequent exposure to OVA (challenge 2; Days 32–36) (Figure E3D). This suggests that XHA does not induce tolerance to allergens with which it is not coadministered.

We then asked whether OVA/XHA treatment results in diminished allergen-specific responses *ex vivo*. OVA-specific responses were diminished *ex vivo* in PDLN cells isolated from animals treated previously with OVA/XHA (Figures 4A and 4B), whereas polyclonal responses to anti-CD3/28 antibodies were not reduced (Figure 4C). In contrast, splenocyte counts were not diminished by OVA/XHA treatment (Figure 4D), and splenocytes isolated from mice that received either OVA or OVA/XHA had comparable proliferation in response to both OVA, as well as to anti-CD3/28 Ab *ex vivo* (Figures 4E and 4F).

These data indicate that local, antigen-specific immune responses are inhibited after OVA/XHA treatment of mice sensitized previously to OVA. Furthermore, it is notable that polyclonal, systemic responses were not reduced, indicating that OVA/XHA treatment does not lead to generalized immune suppression.

One possible explanation for our results is that formulation of antigen with XHA leads to sequestering of antigen and a lower effective delivery of the antigen. To evaluate this, we repeated our *in vivo* assay using Dye quenched Ovalbumin (DQ OVA) conjugated to the pH-insensitive BODIPY (4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene) dye, which fluoresces green on proteolytic degradation of DQ OVA. Using this tool, we observed that coupling OVA to XHA did not affect antigen uptake and processing by lung resident antigen-presenting cell subsets (including $CD11b^+$ $CD11c^+$ DC and

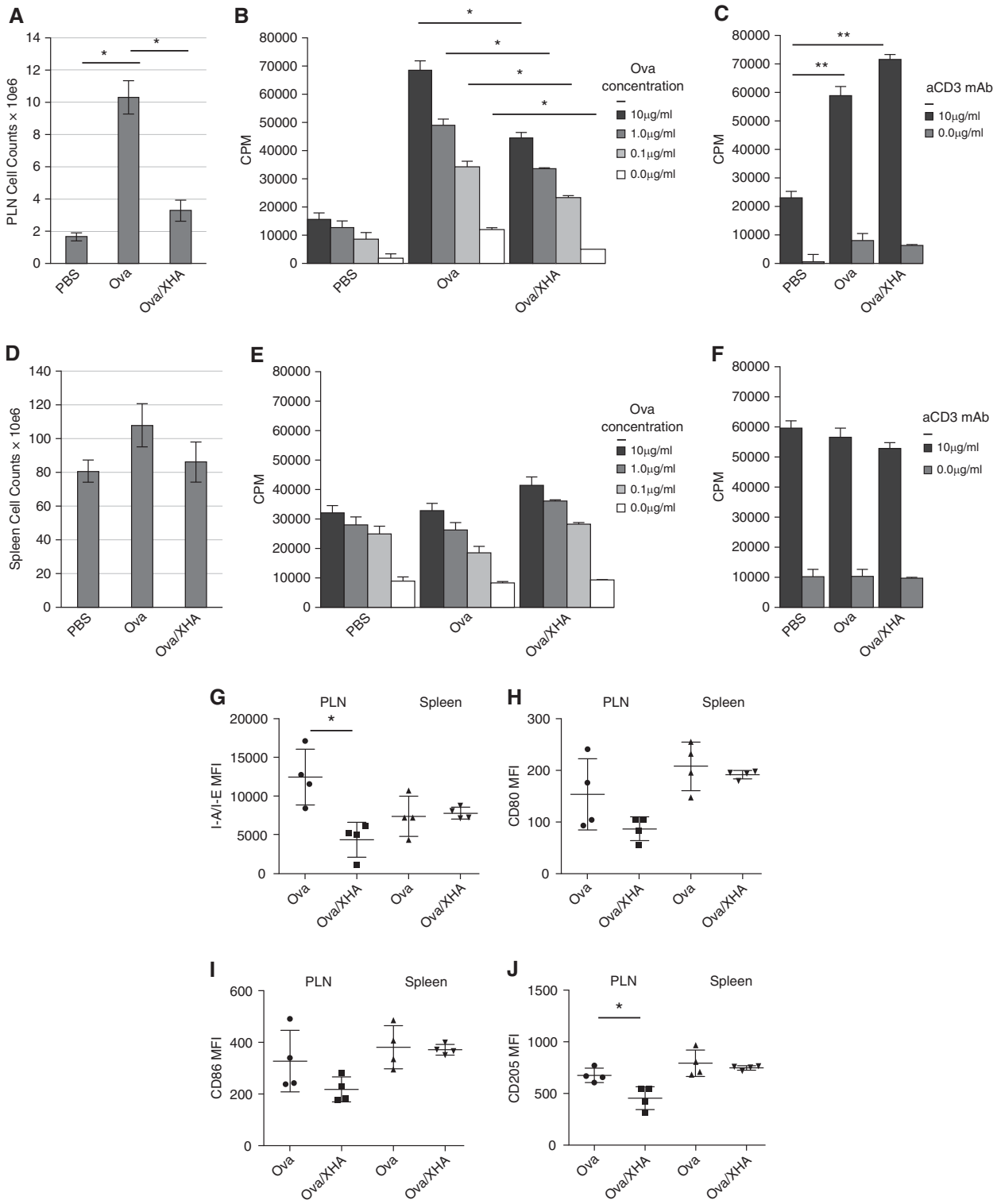


**Figure 4.** XHA treatment inhibits proliferative responses to allergen but not to polyclonal stimulus. (*A–C*) PDLN lymphocytes and (*D–F*) splenocytes were isolated from animals sensitized to OVA and then challenged with PBS, OVA, or OVA/XHA. Cell counts in (*A*) PDLN and (*D*) spleens from mice treated with PBS or OVA or OVA/XHA are shown. Lymphocytes and splenocytes were then stimulated *ex vivo* with either (*B* and *E*) OVA or (*C* and *F*) anti-CD3 at the concentrations indicated for 72 hours. Tritiated thymidine was added for the final 24 hours. Data are representative of two independent experiments. In a separate experiment, PDLN lymphocytes and splenocytes were stained directly *ex vivo* for (*G*) I-A/I-E, (*H*) CD80, (*I*) CD86, and (*J*) CD205. MFIs were calculated relative to antibody-appropriate isotype controls. $^{*}P < 0.05$; $^{**}P < 0.01$. Data are representative of two experiments. CPM, counts per minute; MFI, median fluorescence intensity; PLN, pulmonary lymph node.

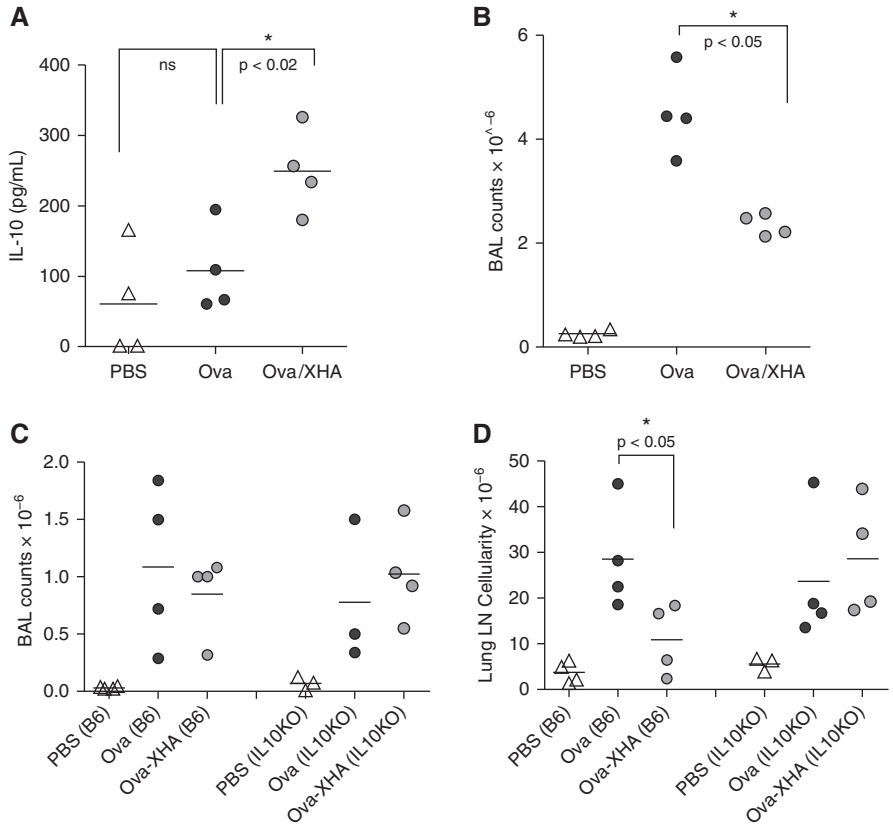


**Figure 5.** OVA/XHA effects are IL-10 dependent. Mice sensitized previously to OVA were administered intranasal OVA or OVA/XHA. Cells were isolated from total BAL fluid via centrifugation (4 × 1 ml flushes of the lung with PBS) and were activated *in vitro* with anti-CD3 for 48 hours. (*A*) IL-10 levels in cell culture supernatants as measured by ELISA. (*B*) BAL fluid counts for the same animals as in *A*. The same protocol was repeated in IL-10$^{-/-}$ mice, and total cell counts were measured in (*C*) BAL fluid and (*D*) PLN. *$P < 0.05$. LN, lymph node.

CD11c$^+$ CD103$^+$ DC) (Figure E4). The gating strategy for these cells is shown in Figure E5 and their absolute numbers are shown in Figure E6. Similarly, we observed that coupling OVA to XHA did not affect the fraction of DQ OVA$^+$ DC present in the PDLN (Figures E7 and E8). These data indicate that formulation of OVA/XHA does not lead to immune ignorance via a lower effective delivery of antigen.

### OVA/XHA Effects on Immune Tolerance Are IL-10 Dependent

To investigate how our findings are consistent with established mechanisms of tolerance, we evaluated whether OVA/XHA treatment promotes IL-10 production. We specifically examined IL-10 because it is known to contribute to tolerance to airway allergens (37) and because, as we reported previously, HMW-HA promotes IL-10 production by multiple cell types *in vitro* (10, 38). We indeed saw that OVA/XHA treatment promotes IL-10 production (Figures 5A and 5B). Moreover, the tolerizing effect of XHA was lost in IL-10–deficient mice (Figures 5C and 5D). From these data, we concluded that the mechanism of OVA/XHA-mediated tolerance is IL-10 dependent.

### XHA Prevents DC Maturation *In Vivo*

The data in Figure 4 suggest a role for antigen presentation in XHA-mediated inhibition of allergic airway inflammation responses. Consistent with this, CD11c$^+$ DC in PDLN from OVA/XHA-treated mice had reduced expression of the cell-surface markers involved in antigen presentation and costimulation, including I-A/I-E (MHC-II) and CD205, compared with mice treated with OVA alone. However, the effects on the costimulatory markers CD80 and CD86 were not statistically significant. These effects were observed in CD11c$^+$ DC from PDLN but not the spleen (Figures 4G–4J). These particular cell surface markers were examined because of their role in antigen presentation and costimulation.

Similar results were seen in CD11c$^+$ DC in BAL fluid. CD11c$^+$ DC isolated from mice treated with OVA/XHA had elevated expression of CD62L (Figures E9A andE9B) (a marker of immature DC) and reduced levels of I-A/I-E, CD80, and CD86 compared with similar cells from animals treated with OVA alone (Figures E9C to E9H). Together with the PDLN data above, these results are indicative of decreased local DC maturation *in vivo* on OVA/XHA treatment.

We considered whether OVA/XHA treatment might lead to altered recruitment of specific DC subsets known to be involved in allergic asthma. X OVA/HA treatment was associated with an increase in the fraction of CD11b$^+$CD11c$^+$ cells in the BAL fluid of previously sensitized animals (Figures E10A–E10D). These cells were both I-A/I-E$^+$ and LY6C$^+$ (Figures E10E and E10F), a profile consistent with recently recruited LY6C$^+$ monocytes, a cell subset capable of driving inflammation in a number of different settings (39–41). However, the absolute numbers of these CD11b$^+$CD11c$^+$ cells were not increased on OVA/XHA treatment (Figure E6), indicating that most of their increase reflects reduced numbers of other cell populations involved in allergic airway inflammation responses. We did not observe heightened numbers of CD11c$^+$ CD103$^+$ DC or CD3$^-$CD19$^-$CD11c$^+$mPDCA plasmacytoid DC (Figures E10G and E10H). These data suggest that OVA/XHA treatment does not meaningfully alter the representation of these DC populations.

### XHA Prevents DC Maturation *In Vitro*

We next evaluated whether XHA could influence the *in vitro* maturation of bone marrow–derived DC (BMDC). Immature BMDC were cultured on plates coated with 1% XHA and controls and were treated with LPS to promote their maturation. In this setting, XHA treatment of BMDC prevented up-regulation of I-A/I-E, CD80, and CD86 and down-regulation of CD14 and CD62L, resulting in retention of an immature DC phenotype, compared with those treated with LPS alone. Culturing cells on plates coated with HMW-HA had a more modest effect, whereas soluble LMW-HA actually promoted DC

maturation, as has been reported previously (17, 19) (Figures 6A–6F). These studies were performed with plate-bound 1% XHA to facilitate direct contact between this material and the cells. Analogous but less potent effects were seen with 1% soluble XHA (Figure E11). This inhibition of maturation was also evident at the level of cell morphology. LPS and LMW-HA promoted DC spreading and development of dendrites, whereas culturing cells in the setting of a coating of 1% XHA prevented this (Figures 6G–6J). ALG did not inhibit antigen presentation (Figure E12). Consistent with these phenotypic

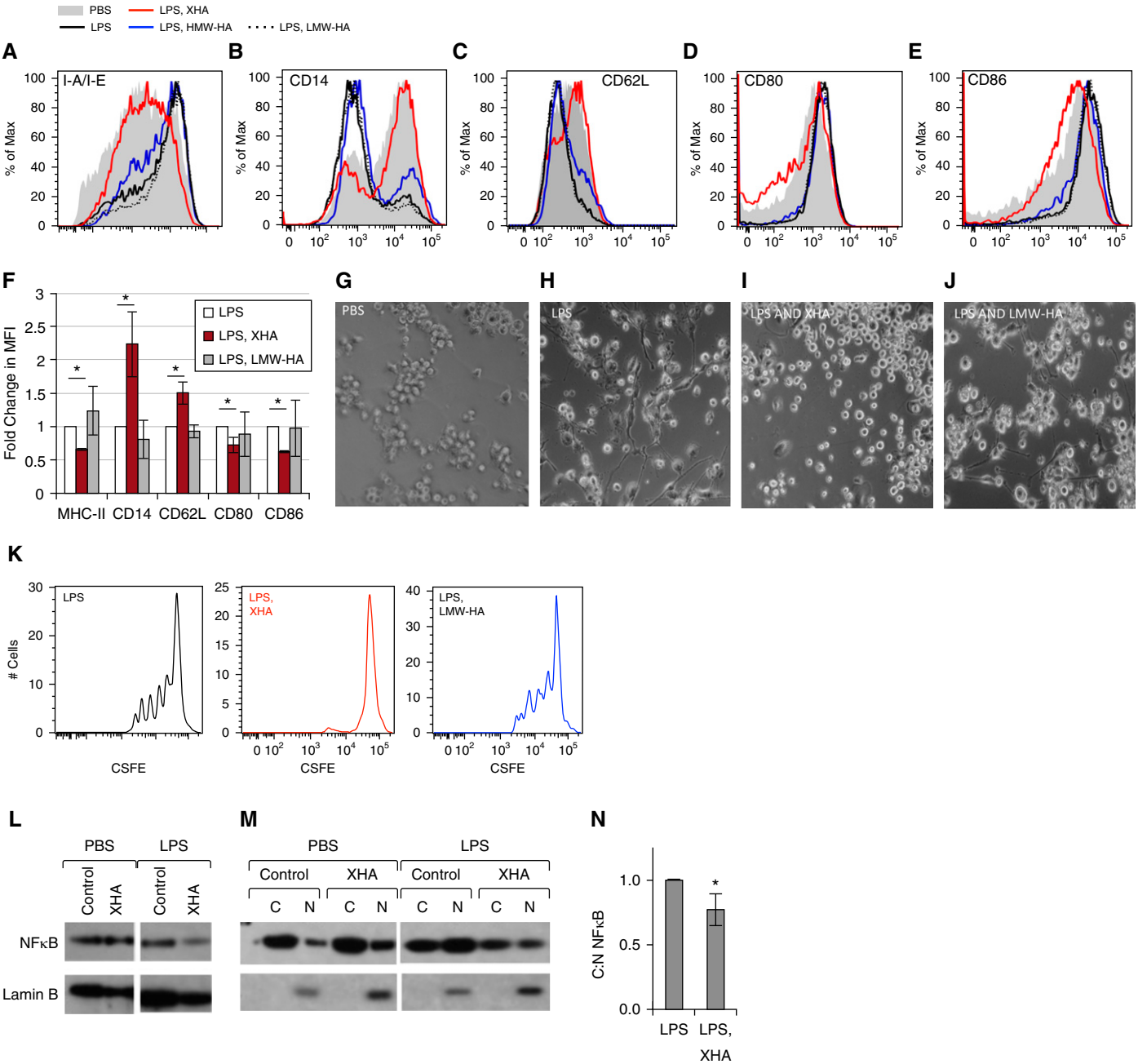


**Figure 6.** XHA prevents bone marrow–derived dendritic cell (BMDC) maturation in response to LPS. BMDC were treated with LPS for 24 hours in the setting of a 1% coating of XHA or soluble LMW-HA. Representative histograms showing the change in cell surface markers including (*A*) I-A/I-E, (*B*) CD14, (*C*) CD62L (*D*) CD80, and (*E*) CD86 after treatment with XHA and controls. (*F*) Fold change in MFI for the same markers as in *A–E*. This figure includes data from three independent experiments. Representative appearance of BMDC cultured in the setting of (*G*) PBS, (*H*) LPS, (*I*) LPS and XHA, or (*J*) LPS and LMW-HA. (*K*) Proliferation in response to OVA, as measured by carboxyfluorescein succinimidyl ester (CFSE) dye dilution, for $CD4^+$T491 cells isolated from animals immunized previously with OVA and cultured for 72 hours with BMDC cultured previously for 24 hours with LPS ± XHA or LMW-HA. Western blot for the p65 subunit of nuclear factor-κB (NF-κB) for (*L*) whole cell lysates or (*M*) the corresponding cytosolic (C) and nuclear (N) fractions of dendritic cells, treated with PBS or LPS ± XHA. (*N*) Ratios of C to N NF-κB in lysates for three independent experiments. $^*P < 0.05$ by Student's *t* test. SE is shown.

profiles, plate-bound 1% XHA-treated DC were less efficient at antigen presentation as assessed by proliferation of T cells isolated from animals immunized previously with OVA and labeled with carboxyfluorescein succinimidyl ester (CFSE) (Figure 6K).

### XHA Inhibits NF-κB Nuclear Translocation

Given that LPS is known to drive DC maturation through NF-κB–dependent mechanisms, we questioned whether plate-bound 1% XHA inhibits NF-κB signaling in BMDC treated with LPS. We assessed this by examining translocation of the p65 subunit of active NF-κB from the cytosol to the nucleus with or without LPS treatment for 24 hours. We observed a relatively constant level of total amount of NF-κB, as measured by the ratio between NF-κB and the nuclear protein lamin B1 (Figure 6L). However, plate-bound 1% XHA inhibited nuclear translocation of NF-κB instigated by LPS treatment (Figure 6M). This increased the C:N ratio, as shown for five independent experiments (Figure 6N). Together, these data suggest that plate-bound 1% XHA inhibits DC maturation and NF-κB signaling in response to proinflammatory signals.

### XHA Effects Are CD44 Dependent

To evaluate whether this OVA/XHA-mediated inhibition of allergic airway inflammation was dependent on CD44, the

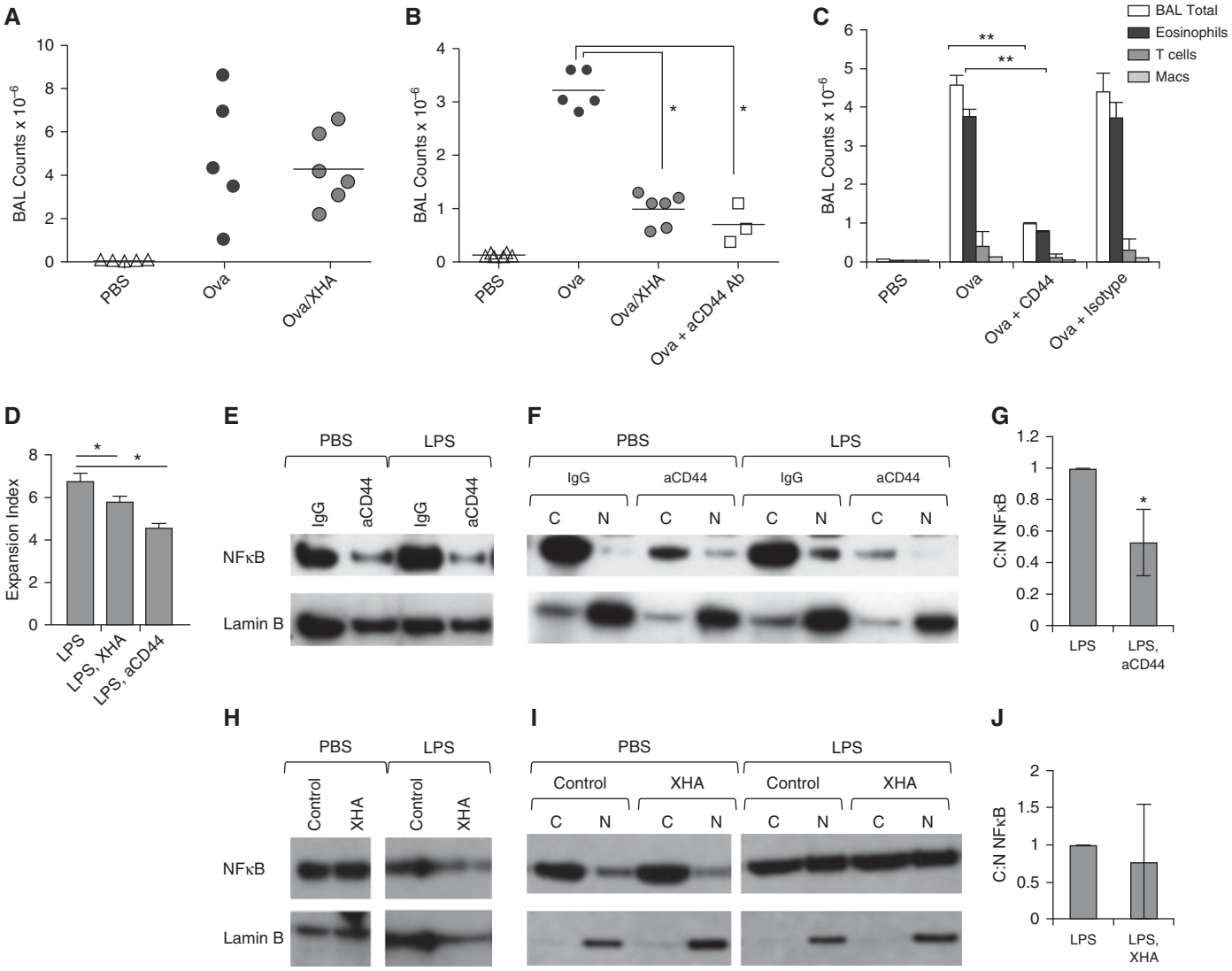


**Figure 7.** XHA-mediated inhibition of allergic airway inflammation is CD44 dependent. (*A*) BAL cell counts for $CD44^{-/-}$ mice after rechallenge with OVA or OVA/XHA. (*B*) BAL cell counts for wild-type ($CD44^{+/+}$) mice treated with OVA, OVA/XHA, or anti-CD44 antibody (Ab). (*C*) BAL cell counts for mice treated as in *B* but now for different leukocyte subsets. $^{*}P < 0.05$. Data in *A–C* are representative of three experiments. (*D*) Proliferation in response to OVA, as measured by CFSE dye dilution, for $CD4^{+}$T cells isolated from animals immunized with OVA and cultured for 72 hours with BMDC cultured previously for 24 hours with LPS ± XHA or anti-CD44 Ab. Western blots for the p65 subunit of NF-κB generated using (*E*) whole cell lysates or (*F*) the corresponding C and N fractions of dendritic cells treated with PBS or LPS ± aCD44 Ab or an isotype-matched control IgG. (*G*) Ratios of C to N NF-κB in lysates of dendritic cells treated as in *F*, now for three independent experiments. (*H–J*) Similar data as shown in Figures 6L–6N only now generated using cells from $CD44^{-/-}$ mice. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t* test. SE is shown.

HA receptor, we repeated these studies in $CD44^{-/-}$ mice. Strain-matched BalbC. $CD44^{-/-}$ mice had robust airway inflammation despite treatment with OVA/XHA (Figure 7A), indicating that CD44 is required for XHA-mediated inhibition of airway inflammation. Administration of anti-CD44 Ab IN to wild-type BalbC mice together with OVA during the rechallenge stage decreased total BAL cells (Figure 7B) and eosinophilia (Figure 7C), suggesting that this treatment had effects analogous to those of XHA. *In vitro* treatment with plate-bound CD44 antibodies (which might be expected to crosslink CD44 in a manner analogous to that of HMW-HA [42, 43]) likewise limited the LPS-mediated effects on DC antigen presentation, as assessed by proliferation of CFSE-labeled T cells (Figure 7D). Consistent with this, plate-bound CD44 antibodies also inhibited nuclear translocation of NF-κB instigated by LPS treatment (Figures 7E–7G). NF-κB nuclear translocation induced by stimuli other than LPS, including Pam3Csk4 and tumor necrosis factor-α, were also inhibited by aCD44 Ab (Figure E13). These data indicate that XHA inhibits NF-κB signaling via a CD44-dependent pathway, consistent with established roles for CD44 in the negative regulation of TLR signaling (44–46).

To evaluate whether XHA-mediated inhibition of NF-κB nuclear translocation was dependent on CD44, the HA receptor, we repeated the assay in Figures 6L–6N now using BMDC derived from $CD44^{-/-}$ mice. We observed that, in the absence of CD44, plate-bound 1% XHA had a nonsignificant impact on NF-κB signaling (Figures 7H–7J).

Together, these data support a model whereby OVA/XHA prevents DC maturation in a CD44-dependent manner, thereby limiting efficient presentation of allergens to previously activated, allergen-specific cells.

## Discussion

We have discovered that HMW-HA, a polymer characteristic of healthy lung tissue, promotes tolerance to airway allergens in an IL-10–dependent manner. This effect required chemical stabilization of HA to prevent its catabolism. This thiolated and crosslinked preparation, which we call XHA, improved the physiologic (responses to methacholine challenge), histologic (airway infiltrates), and immunologic (cytokine levels and BAL fluid counts) aspects of allergic airway inflammation. These effects included elements of both the acute response (e.g., OVA-specific IgE levels) and late-phase responses (e.g., eosinophil counts) in mice sensitized previously to allergens. These data suggest that the antiinflammatory cues that intact HMW-HA provides can be harnessed to treat hypersensitivity to airway allergens in this model.

Although our data are consistent with the generally antiinflammatory properties of HMW-HA, the chemical modifications that produced XHA were necessary for its effectiveness in our models. Thiolation enhances stability against HA'ases and, together with crosslinking, may prevent rapid catabolism of XHA. Crosslinking transforms HA into networks of interlinked polymers of increased effective size and may also contribute to the stability of XHA *in vivo*.

One possible explanation for our results is that, because of lower effective delivery of antigen to the tissue, the formulation of antigen with XHA leads to immune ignorance, rather than immune tolerance. Several lines of evidence argue against this interpretation. First, we demonstrated that coupling OVA to XHA does not affect antigen uptake and processing by lung resident antigen-presenting cells, nor their migration into the draining lymph nodes. Second, we observed effects using allergens (OVA and CRA) with different physical properties, making a specific masking effect unlikely. Third, tolerizing effects were not seen using LMW-HA, a material with chemical composition identical to that of XHA. Fourth, tolerance was not seen in the setting of ALG, a polymer with properties similar to those of HA that does not interact with CD44, the major cell-surface HA-binding transmembrane glycoprotein (47). Fifth and finally, XHA was not efficacious in $CD44^{-/-}$ mice, and this would not be expected to be the case in the setting of immune ignorance. Together, these lines of evidence refute the argument that immune ignorance is responsible for the effects observed here.

Our data indicate that XHA promotes an immature DC phenotype and thereby prevents efficient presentation of allergens to previously activated, allergen-specific cells. Indeed, XHA treatment trumps the proinflammatory effects of LPS on DC maturation and NF-κB signaling. These data are perhaps consistent with roles for immature DC in the induction of IL-10–based immune tolerance (48). Our data do not, however, rule out XHA effects on other cell types present in the inflamed lung.

The effects of OVA/XHA are CD44 dependent. This is consistent with reports that CD44 signaling inhibits NF-κB DNA activation (44, 45, 49) and also with reports that both MyD88 expression levels and nuclear translocation of NF-κB are increased in $CD44^{-/-}$ mice (44, 45). We propose that XHA may be a biomimetic of the naturally occurring complexes of HMW-HA and hyaladherins that provide what we have called "tissue integrity signals" that dampen inflammation in healing or uninjured tissues (15). To our knowledge, XHA is the first technology to use an endogenous tolerogen.

XHA may have therapeutic potential in allergic asthma. The current standard of care, desensitization, prevents allergic responses by the administration of daily treatments of allergens (50). The durability of XHA over time (∼2 wk) potentially offers a substantial advantage over these daily regimens. Because HMW-HA is a natural component of human tissues, it is highly biocompatible and would not be expected to promote toxicities (51) such as those associated with inhaled particulate matter (52). Our vision is that antigen/XHA solutions could be administered as an intranasal spray or rinse to treat allergic asthma and sinusitis or be adapted to enhance current desensitization protocols.

### Conclusions

The finding that a simple, biocompatible modification (thiolation) protects HMW-HA from catabolism also has broad applicability. HMW-HA is Food and Drug Administration approved for many indications (53–55). The use of thiolated HA as a starting material may radically improve the efficacy of these products by dramatically increasing their longevity. ■

**Author disclosures** are available with the text of this article at www.atsjournals.org.

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