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Identification of the HSPB4/TLR2/NF-κB axis in macrophage as a therapeutic target for sterile inflammation of the cornea

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Sterile inflammation underlies many diseases of the cornea including serious chemical burns and the common dry eye syndrome. In search for therapeutic targets for corneal inflammation, we defined the kinetics of neutrophil infiltration in a model of sterile injury to the cornea and identified molecular and cellular mechanisms triggering inflammatory responses. Neutrophil infiltration occurred in two phases: a small initial phase (Phase I) that began within 15 min after injury, and a larger second phase (Phase II) that peaked at 24–48 h. Temporal analysis suggested that the neuropeptide secretoneurin initiated Phase I without involvement of resident macrophages. Phase II was initiated by the small heat shock protein HSPB4 that was released from injured keratocytes and that activated resident macrophages via the TLR2/NF-κB pathway. The Phase II inflammation was responsible for vision-threatening opacity and was markedly suppressed by different means of inhibition of the HSPB4/TLR2/NF-κB axis: in mice lacking HSPB4 or TLR2, by antibodies to HSPB4 or by TNF-α stimulated gene/protein 6 that CD44-dependently inhibits the TLR2/NF-κB pathway. Therefore, our data identified the HSPB4/TLR2/NF-κB axis in macrophages as an effective target for therapy of corneal inflammation.

INTRODUCTION

Sterile inflammation is now recognized to play a key role in many diseases that include myocardial infarction, stroke, Alzheimer's disease and atherosclerosis (Chen & Nuñez, 2010; Rock et al, 2010; Spite & Serhan, 2010). The molecular and cellular responses of sterile inflammation include over 20 nonmicrobial endogenous stimuli referred to as damage-associated molecular patterns (DAMPs). DAMPs signal through pattern recognition receptors (PRRs) on resident macrophages to activate at least three intracellular pathways to up-regulate the expression of pro-inflammatory cytokines (Chen & Nuñez, 2010). In spite of the intense interest in the field, a series of important questions has remained unanswered to date, including whether some DAMPs identified from experiments *in vitro* play important roles *in vivo*, whether some DAMPs play redundant roles, and whether inflammatory responses in different tissue involve different DAMPs that are specific for the injured tissues (Matzinger, 2007). Therefore, to identify therapeutic targets, it is probably essential to identify the major DAMP or DAMPs that play essential roles in initiating sterile inflammation in a specific disease and tissue.

The cornea is an attractive system to investigate sterile inflammation since it is readily accessible to experimental manipulations *in vivo* and *in vitro*. Moreover, sterile inflammation underlies many diseases of the cornea that include vision-threatening conditions such as chemical burns and quality-of-life-deteriorating diseases such as dry eye syndrome (Moss et al, 2008; Wagoner, 1997). To search for novel therapeutic targets for sterile inflammation, we adopted a model of chemical and mechanical injury in which the cornea was injured by exposure to ethanol and mechanical scraping to remove both the epithelium

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and limbal stem cells. We first defined the kinetics of neutrophil infiltration and then examined the temporal sequence of genes expressed in the cornea to identify factors that triggered the infiltration of neutrophils into the cornea. We observed that the injury provoked two distinct phases of neutrophil infiltration: a small initial phase (Phase I) that began within 15 min and reached a plateau between 4 and 8 h and a much larger second phase (Phase II) that peaked at 24–48 h. Analysis of the two phases demonstrated that Phase I was stimulated by the neuropeptide secretoneurin (SN). The second, more massive infiltration of neutrophils in Phase II was stimulated by the small heat shock protein HSPB4 that was synthesized and released by injured keratocytes of the corneal stroma and that acted as a DAMP to activate resident macrophages through the TLR2/NF-κB signalling pathway. The inflammatory response in Phase II was largely responsible for vision-threatening opacity. Phase II was dramatically suppressed by inhibition of the HSPB4/TLR2/NF-κB axis in resident macrophages: (i) by depletion of resident macrophages, (ii) by administration of antibodies to HSPB4, (iii) in mice lacking HSPB4, (iv) in mice lacking TLR2 or (v) by administration of TNF-α stimulated gene/protein 6 (TSG-6) that inhibits TLR2/NF-κB signalling in macrophages in a CD44-dependent manner.

RESULTS

Two phases of neutrophil infiltration after sterile injury to the cornea

We injured the corneas of Lewis rats by exposing them to 100% ethanol for 30 s and scraping of the cornea and limbus to remove both the epithelium and limbal stem cells. Most of the infiltrating cells in the cornea were neutrophils early after ethanol/scraping injury as shown in our previous experiments (Oh et al, 2010). Thus, we followed neutrophil infiltration by assays for myeloperoxidase (MPO) that is stored within neutrophil granules (Borregaard & Cowland, 1997). The neutrophil infiltration occurred in two phases. There was a small initial phase that began within 15 min and reached a plateau level at 4–8 h (Phase I in Fig 1A). After the plateau, a much larger infiltration of neutrophils followed and reached a maximum at 24–48 h (Phase II in Fig 1A). The neutrophils then gradually disappeared in a recovery phase over 48 h to 7 days.

Search for candidate stimuli for Phase I and Phase II

As a strategy to identify candidate stimuli that initiated the two phases, we used microarrays to survey the response of the cornea to injury. We focused our attention on the genes up-regulated early after an injury under the assumption that the molecules up-regulated in the tissues in response to an injury might act as the major stimuli to initiate the sterile inflammation. Based on the temporal pattern of gene expression, we classified the up-regulated genes into the following three groups (Fig 1B, Supporting Information Fig S1): Group A, genes that were up-regulated at 4 h and returned towards normal at 24 h; Group B, genes that were up-regulated at 4 h and remained steady at 24 h; and Group C, genes that were gradually up-regulated over 24 h in parallel with neutrophil infiltration. A total of 842 genes were up-regulated in Group A, 108 genes were up-regulated in Group B and 307 genes were up-regulated in Group C (Supporting Information Fig S1). We focused on the

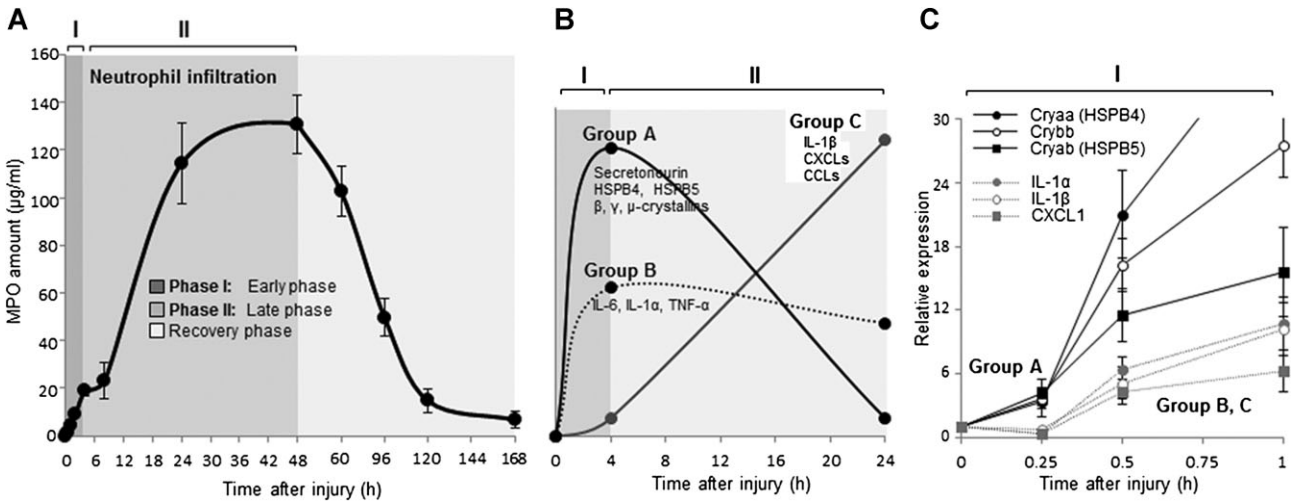

Figure 1. Time course of cellular and molecular changes in the rat cornea after sterile injury.

A. Time course of neutrophil infiltration in the cornea as measured by MPO. Neutrophils infiltrated the cornea in two phases: Phase I, a small initial phase that began within 15 min and reached a plateau level at 4–8 h; Phase II, a larger infiltration of neutrophils that followed and peaked at 24–48 h. Then neutrophils gradually decreased over 7 days that reflected the recovery phase. $n = 5$ at each time-point.

B. Microarray data of the cornea at 0, 4 and 24 h after sterile injury. The up-regulated genes were classified into three groups: Group A, genes that were up-regulated at 4 h and returned towards normal at 24 h; Group B, genes that were up-regulated at 4 h and remained steady at 24 h; Group C, genes that were gradually up-regulated over 24 h in parallel with neutrophil infiltration. $n = 4$ at each time-point.

C. Real-time RT PCR assays of representative genes in Groups A, B and C. $n = 5$ per group at each time-point. Error bars represent means ± s.e.m.

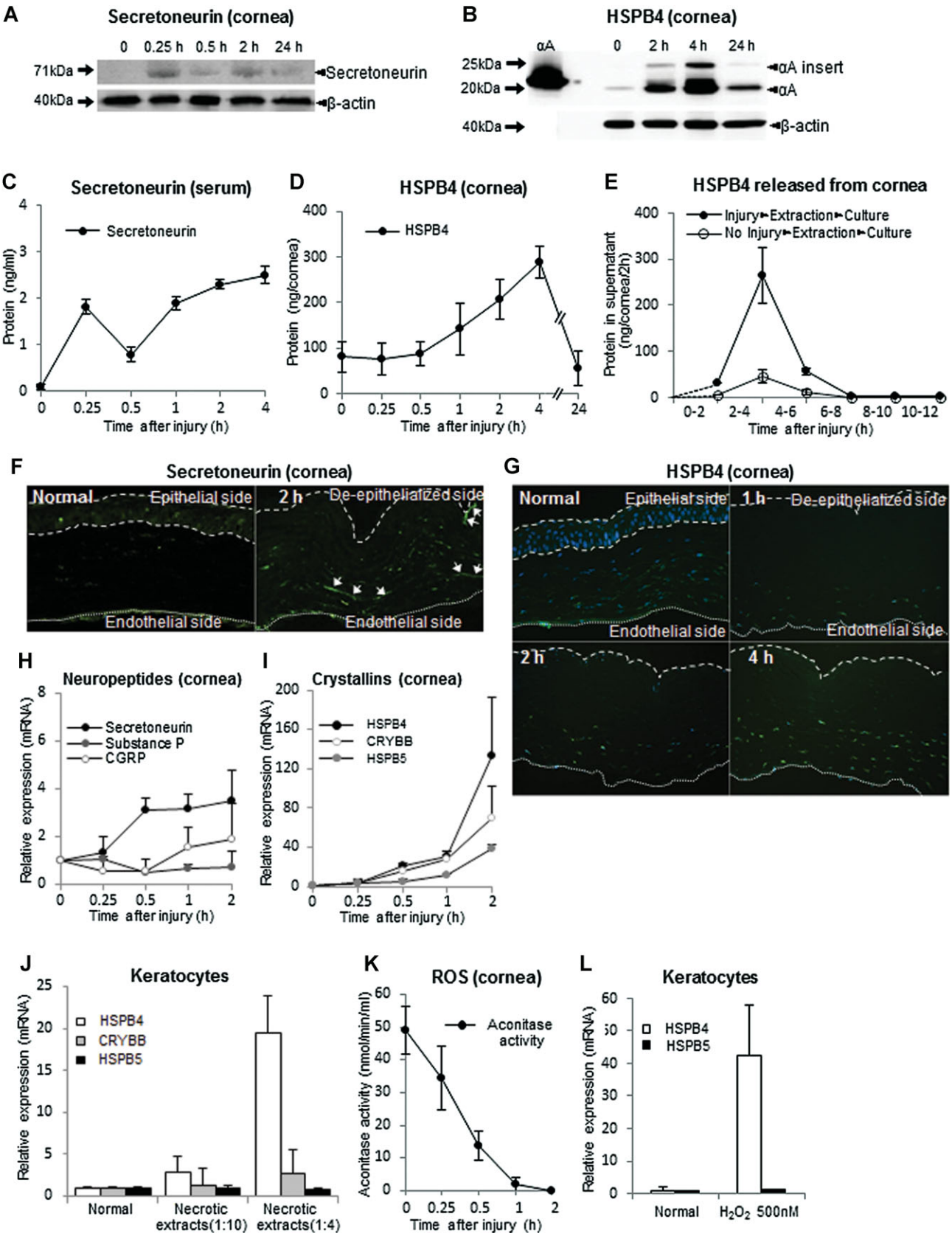

Figure 2.

Group A genes because they were expressed earlier and thus more likely to include stimuli for Phase I and II (Fig 1C). Most of the Group A genes were transcripts for nerve/neurotransmission-related proteins and structural proteins of the eye (Supporting Information Table S1 and Fig S1). Most of the Group B genes were the molecules related to apoptosis/death and defense response (Supporting Information Table S1 and Fig S1). Most of Group C molecules were pro-inflammatory chemokines and cytokines (Supporting Information Table S1 and Fig S1) that peaked late in the inflammatory responses (Supporting Information Fig S2). From the category of Group A genes, we selected the following as attractive candidates for inflammatory signals: (i) the neuropeptide SN because it is stored in nerve endings (Troger et al, 2005) and was previously shown to activate the chemotactic migration and transendothelial extravasation of blood cells (Helle, 2010; Taupenot et al, 2003) and (ii) two small heat shock proteins, HSPB4 and HSPB5, because some heat shock proteins (HSPs) were previously shown to act as DAMPs (Henderson & Pockley, 2010; Joly et al, 2010; Quintana & Cohen, 2005; van Wijk & Prakken, 2010).

SN as a candidate stimulus for Phase I and HSPB4 for Phase II

Data on the time course of expression were consistent with SN serving as an initiating stimulus for Phase I. SN was not detected in extracts of uninjured cornea or the serum, but it appeared both in corneal extracts and the serum of the rats within 0.25 h of the injury (Fig 2A and C). The levels of SN in extracts of injured cornea and the serum decreased temporarily at 0.5 h after the initial release and then increased again at 2 h apparently as a result of increased expression of the gene (Fig 2A, C, F and H). We measured the levels of SN in the serum in order to evaluate the levels of SN released outside of corneal nerve endings in response to an injury as a distinct form, not SN stored inside sensory nerve endings in the cornea (Troger et al, 2005). Since the expression pattern of SN in the serum after injury corresponded with that of SN in the cornea, the results suggested that the source of SN in the serum after corneal injury was the sensory nerve endings in the injured cornea. In fact, immunohistochemistry of injured cornea showed an increased expression of SN along the nerves in the posterior corneal stroma at 2 h following injury (Fig 2F).

On the other hand, there were little changes in the expression of genes for substance P and calcitonin gene-related peptide (CGRP), two other neuropeptides known to be expressed abundantly in the cornea (Fig 2H; Troger et al, 2007).

Similar data on the time course of expression were consistent with HSPB4 serving as a stimulus for Phase II. Uninjured cornea contained low levels of HSPB4 protein, but the amount increased beginning after 0.5 h of the injury and reached a peak at 4 h (Fig 2B, D, and G). A similar time course was observed in the release of HSPB4 into the medium in experiments in which cornea was injured *in vivo* and then incubated *ex vivo* (Fig 2E). As expected from the microarray data, real-time RT PCR assays demonstrated increased expression of mRNAs for HSPB4 and related genes from Group A (Fig 2I). However, the increases in expression of HSPB4 were delayed compared to the increase in the mRNA for SN (compare Fig 2H and I). Immunohistochemistry of injured cornea was consistent with these results. The expression of HSPB4 in the corneal stroma was increased at 4 h following injury (Fig 2G).

Keratocytes in the corneal stroma released HSBP4 in response to injury

To define the cellular origin of HSPB4 in the injured cornea, keratocytes, that are fibroblast-like cells from the corneal stroma, were incubated with extracts of the cornea in which necrosis was induced by homogenization and repeated cycles of freezing–thawing. The necrotic extracts induced the expression of HSPB4 in keratocytes (Fig 2J). The necrotic extracts, however, did not significantly increase the expression of a second small HSP, HSPB5 or a third Group A gene, βB-crystallin, in keratocytes. Most of sterile inflammation accompanies the generation of reactive oxygen species (ROS) (Martinon, 2007; Naik & Dixit, 2011). As expected, assays of injured cornea demonstrated a rapid decrease in aconitase activity, a reflection of an increase in ROS (Fig 2K). The incubation of keratocytes with H_2O_2 to increase ROS resulted in increased expression of HSPB4 in keratocytes (Fig 2L). Therefore, the results suggested that injury to the cornea generated ROS, and an increase in ROS stimulated keratocytes to synthesize HSPB4.

SN stimulated neutrophil infiltration of Phase I, and HSB4 stimulated Phase II

Injection of recombinant SN (0.2 ng corresponding to the amount of SN detected by ELISA in the rat cornea at 15 min after

Figure 2. Increased expression of SN and HSPB4 in the rat cornea and keratocytes after sterile injury.

A,B. Western blots of SN and HSPB4 in the cornea. SN was immediately released into the cornea after injury. HSPB4 rapidly increased after injury and reached a peak at 4 h.

C. SN was increased in the blood of injured rats in the same temporal sequence as its expression in the cornea. $n = 5$ at each time-point. Error bars represent means ± s.e.m.

D. HSPB4 increased rapidly in the cornea after injury with a peak at 4 h. $n = 5$ at each time-point. Error bars represent means ± s.e.m.

E. HSPB4 was released from injured cornea to the media during incubation in culture. $n = 5$ per group at each time-point. Error bars represent means ± s.e.m.

F,G. Immunohistochemistry of the cornea after injury demonstrated increases of SN within 2 h and increases of HSPB4 within 4 h.

H,I. Real-time RT PCR assays of selected Group A genes (neuropeptides, small HSPs and crystallins) in the cornea. $n = 5$ per group at each time-point. Error bars represent means + s.e.m.

J. Real-time RT PCR assays of selected Group A genes (small HSPs and crystallins) in keratocytes incubated with extracts of necrotic cornea. $n = 5$ per group. Error bars represent means + s.e.m.

K. Assays for aconitase activity in the injured cornea as a measure for ROS. $n = 5$ at each time-point. Error bars represent means ± s.e.m.

L. Keratocytes expressed HSPB4 in response to incubation with H_2O_2 to generate ROS. $n = 5$ per group. Error bars represent means + s.e.m. HSPB.

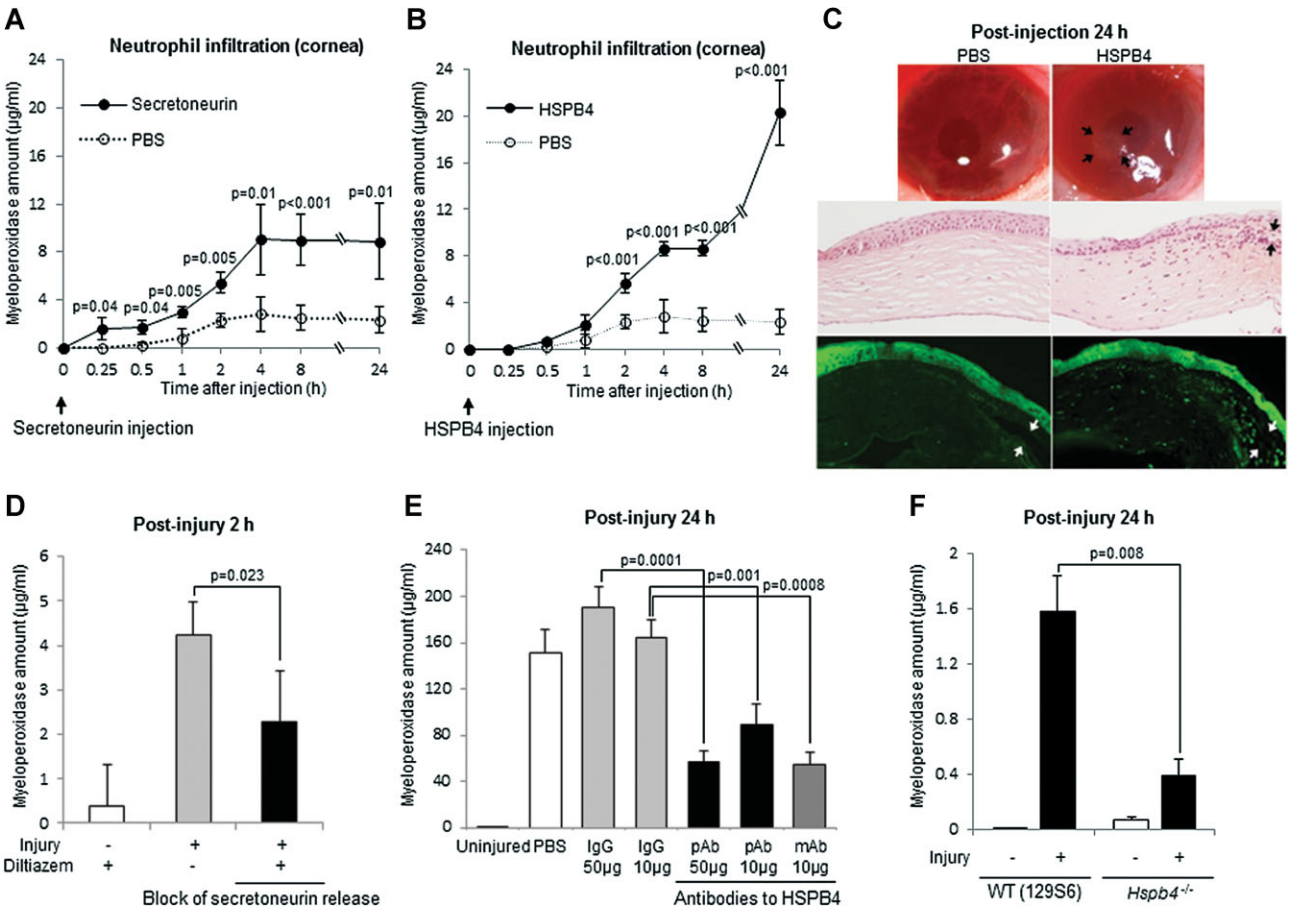

Figure 3. SN reproduced the Phase I inflammatory response and HSPB4 reproduced Phase II.

A. The injection of the recombinant SN (0.2 ng) into the corneal stroma of rats reproduced Phase I, but not Phase II. $n = 5$ per group at each time-point. *p* values, *versus* control (PBS-treated). Error bars represent means ± s.e.m.

B. Injection of the recombinant HSPB4 (100 ng) into the corneal stroma reproduced Phase I and Phase II. $n = 5$ per group at each time point. *p* values, *versus* control (PBS-treated). Error bars represent means ± s.e.m.

C. The corneal opacity developed around the injection site (arrows) of HSPB4 (100 ng) 24 h after injection (**top panels**). Sections were stained with H&E or immunostained for neutrophil elastase. Increased neutrophil infiltration was observed around the site of HSPB4 injection (arrows) (**middle and bottom panels**).

D. Topical administration of a calcium channel blocker (Diltiazem) 15 min prior to injury significantly decreased Phase I neutrophil infiltration in the cornea after sterile injury. $n = 5$ per group. Error bars represent means + s.e.m.

E. Subconjunctival injection of either polyclonal (pAb) or monoclonal (mAb) antibodies to HSPB4 significantly decreased Phase II neutrophil infiltration in the cornea after sterile injury. $n = 5$ per group. Error bars represent means + s.e.m.

F. HSPB4-knockout mice (*Hspb4*$^{-/-}$) developed significantly less Phase II inflammatory response in the cornea after sterile injury than wild-type control mice (129S6/SvEvTac). $n = 8$ per group. Error bars represent means + s.e.m.

injury) into the stroma of the cornea stimulated the neutrophil infiltration of Phase I, but not Phase II (Fig 3A). The effect of SN injection was concentration-dependent. The injection of 1 ng SN significantly increased the amount of MPO in the cornea (Supporting Information Fig S3). The Phase I response was partially negated in the cornea after chemical/mechanical injury by topical application of a calcium channel blocker Diltiazem (Fig 3D) that inhibits the release of neuropeptides (Gonzalez et al, 1993). The results, therefore, indicated that SN served as a major stimulus for Phase I, although they did not exclude the possibility that SN acted in concert with other signals released by the injured cornea.

An intrastromal injection of recombinant HSPB4 (100 ng, which corresponded to the amount of HSPB4 detected by ELISA in the rat cornea at 15 min after injury) that was pyrogen-free (see Materials and Methods section) stimulated the neutrophil infiltration of both Phase I and Phase II (Fig 3B and C). Reproduction of Phase I by HSPB4 injection was partly explained by the protein being injected earlier than it appeared in the tissue by the injury to the cornea (Fig 2B, D, E and G). Injection of recombinant HSPB4 also reproduced the corneal opacity and neutrophil infiltrates that were localized around the site of injection (Fig 3C). To confirm that the effect of HSPB4 injection was specific to HSPB4, we also injected HSPB5 having

a similar molecular weight (23 kDa) to HSPB4. HSPB5 did not induce a significant change in MPO compared to PBS injection (Supporting Information Fig S4). The role of HSPB4 was confirmed by experiments in which antibodies to the protein were injected into the subconjunctival space immediately after chemical/mechanical injury was made to the cornea. The antibodies to HSPB4 significantly inhibited the Phase II inflammatory response in the cornea after the injury (Fig 3E). In addition, the neutrophil infiltration of Phase II in the cornea after the injury was significantly decreased in the corneas of HSPB4 knockout mice compared to their wild-type controls (Fig 3F).

HSPB4 induced Phase II by activating resident macrophages through TLR2/NF-κB signalling

To identify the cells that responded to HSPB4 in the cornea, we depleted resident macrophages from the cornea of rats by subconjunctivally injecting clodronate liposome and then injected recombinant HSPB4 into the corneal stroma. Macrophage depletion was confirmed with immunostaining for the macrophage-specific markers CD68 and CD11b (Supporting Information Fig S5). The inflammation was markedly decreased in the cornea after chemical/mechanical injury when resident macrophages were depleted prior to an injury (Fig 4), indicating a crucial role of resident macrophages in sterile injury-induced inflammation of the cornea. Similarly, HSPB4 did not reproduce Phase II in rats in which macrophages were depleted (Fig 5A), suggesting that the effects of HSPB4 were dependent on the presence of resident macrophages. In parallel experiments, we observed that Phase II, but not Phase I, was significantly suppressed in the cornea after sterile injury by an intraocular injection of the anti-inflammatory protein TSG-6 (Fig 5B) that inhibits TLR2/NF-κB signalling in macrophages (Choi et al, 2011; Lesley et al, 2004). Also, TSG-6 significantly suppressed the Phase II inflammatory response caused by an intrastromal injection of HSPB4 (Fig 5C). Furthermore, the HSPB4-induced inflammatory response in Phase II was significantly decreased in the corneas of TLR2 knockout mice, compared to wild-type control mice (Fig 5D). The results, therefore, suggested that HSPB4 induced Phase II by activating TLR2/NF-κB signalling in resident macrophages.

To test the effect of HSPB4 on macrophages, macrophages in culture were incubated with extracts of necrotic corneas. The necrotic extracts increased the expression of the pro-inflammatory cytokines IL-1α, IL-1β and IL-6 in macrophages (Fig 5E). However, heat inactivation of the extracts completely abrogated the effect of necrotic extracts on macrophages, suggesting that the macrophage-activating factor(s) in the necrotic cornea was protein(s). Addition of either polyclonal or monoclonal antibodies to HSPB4 significantly suppressed the effect of the necrotic extracts on macrophage activation, indicating that one of the active factor(s) in the necrotic cornea was HSPB4 (Fig 5E). Also, recombinant HSPB4 increased the expression of the pro-inflammatory cytokines by macrophages in a concentration-dependent manner (Fig 5F). In addition, HSPB4 caused translocation to the nucleus of the NF-κB complex in macrophages (Fig 5G). To test whether HSPB4 signalled through the TLR2/NF-κB pathway, we used a reporter cell line transfected to assay the TLR2-mediated activation of the NF-κB signalling (HEK-TLR2). Experiments in a HEK-TLR2 cell line demonstrated that the necrotic extracts of the cornea increased TLR2/NF-κB signalling and that antibodies to HSPB4 inhibited

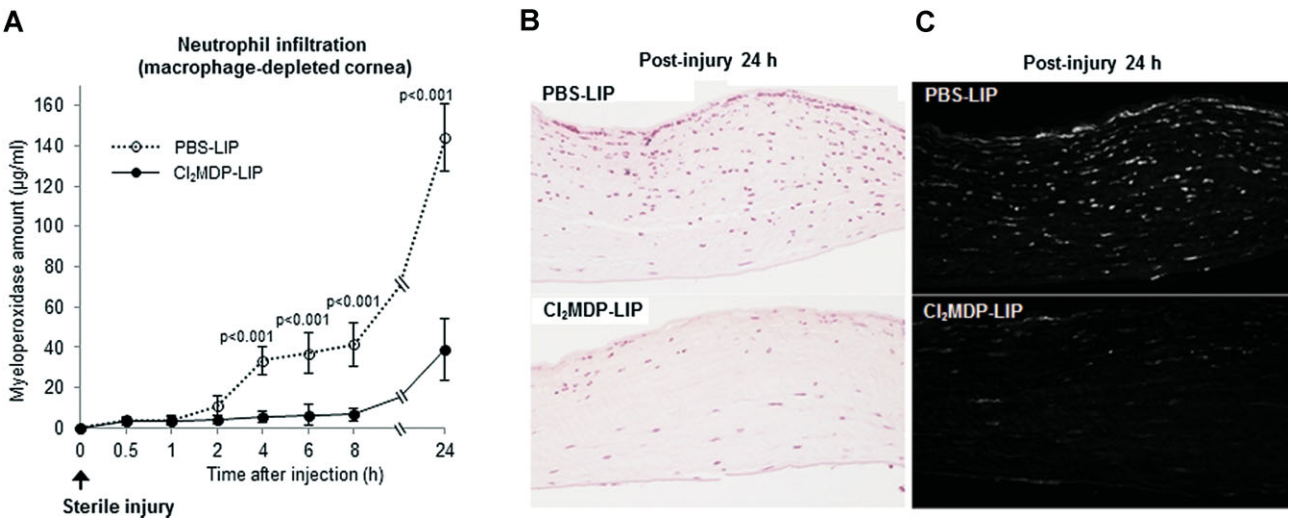

Figure 4. Sterile injury-induced inflammation was markedly decreased in the cornea after depletion of resident macrophages.

A-C. Sterile injury was made to the rat cornea after resident macrophages were depleted by subconjunctival injections of clodronate-encapsulated liposome (Cl_2MDP-LIP) on day −2 (2 days before injury) and day 0 (immediately after injury). The cornea was evaluated for neutrophil infiltration by assays for MPO (**A**), H&E staining (**B**) and immunostaining for neutrophil elastase to identify neutrophils (**C**). (**A**) Neutrophil infiltration measured by MPO was markedly decreased in the cornea 24 h after injury by injection with clodronate-encapsulated liposome, compared to PBS-encapsulated liposome-injected controls (PBS-LIP). $n = 5$ per group at each time-point. *p* values, *versus* control (PBS-LIP-treated). Infiltration of inflammatory cells (**B**) and neutrophils (**C**) was also markedly decreased in the macrophage-depleted cornea. Error bars represent means + s.e.m.

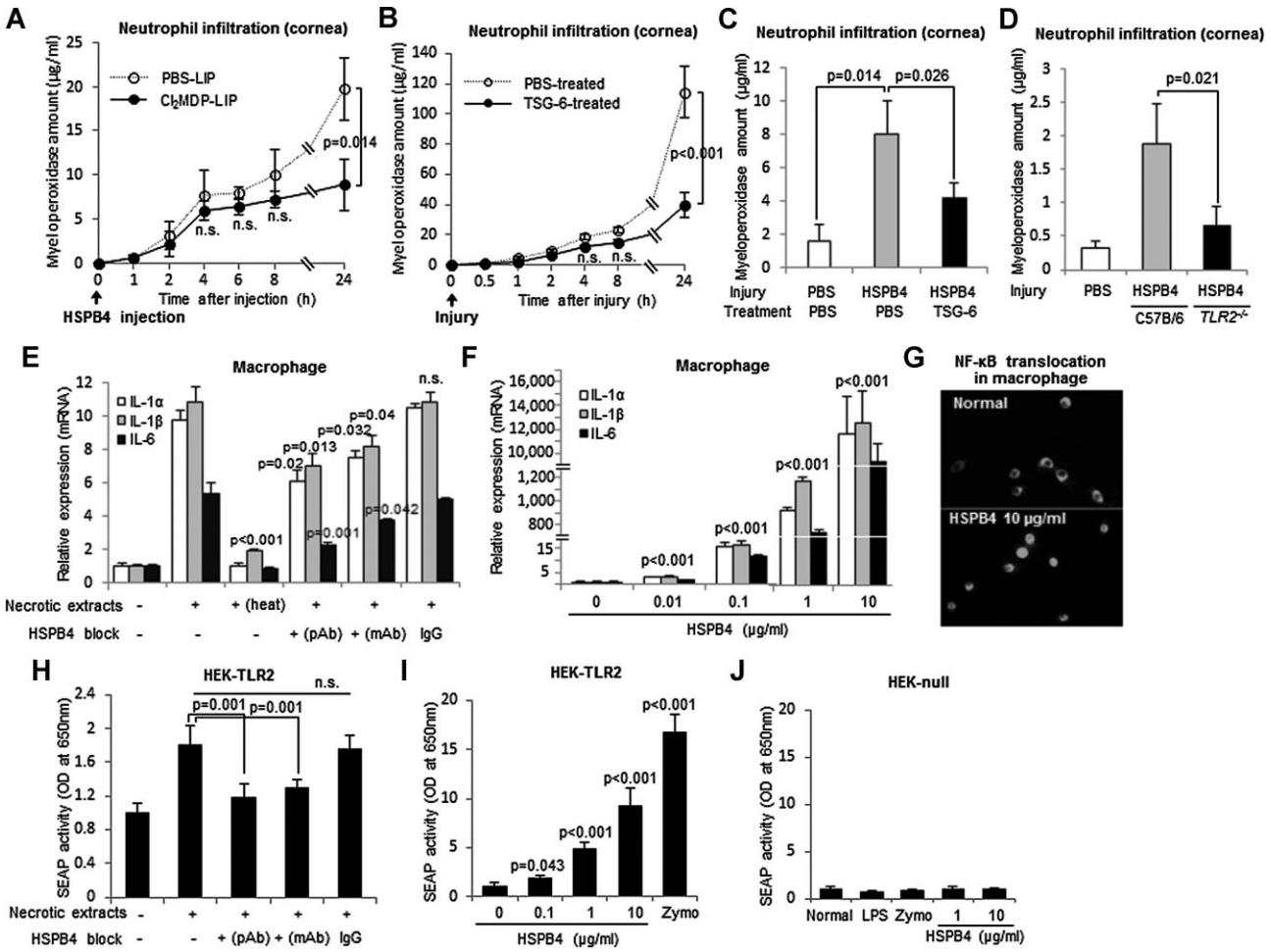

Figure 5. HSPB4 induced the Phase II inflammatory response by activating resident macrophages through TLR2/NF-κB signalling.

A. Intrastromal injection of HSPB4 (100 ng) did not induce the Phase II response when corneal macrophages were depleted by subconjunctival injection of clodronate-encapsulated liposome (Cl_2MDP-LIP). $n = 5$ per group at each time point. p values, *versus* control (PBS-LIP-treated). Error bars represent means ± s.e.m.

B. Sterile injury to the cornea did not induce Phase II after intraocular injection of TSG-6 (2 μg), an inhibitor of TLR2/NF-κB signalling in resident macrophages. $n = 5$ per group at each time point. p values, *versus* control (PBS-treated). Error bars represent means ± s.e.m.

C. Intraocular injection of TSG-6 (2 μg) significantly suppressed the HSPB4-induced neutrophil infiltration in the cornea at 24 h after injury, suggesting the importance of TLR2/NF-κB signalling in the action of HSPB4. $n = 5$ per group. Error bars represent means + s.e.m.

D. Also, HSPB4 induced significantly lower infiltration of neutrophils in the cornea of TLR2 knockout mice ($TLR2^{-/-}$) at 24 h after injury ($n = 4$ per group), which demonstrated that HSPB4 primarily acted through TLR2. Error bars represent means + s.e.m.

E. Necrotic corneal extracts, but not heat-treated extracts, activated macrophages in culture to express pro-inflammatory cytokines. Treatment with polyclonal (pAb) or monoclonal (mAb) antibodies to HSPB4 significantly reduced macrophage activation by necrotic extracts, suggesting that HSPB4 in the extracts was responsible for effects on macrophage activation. $n = 6$ per group. p values, versus necrotic extracts-treated and antibody-untreated group. Error bars represent means + s.e.m.

F. Activation of macrophages by recombinant HSPB4 was concentration-dependent. $n = 6$ per group. p values, *versus* no HSPB4 treatment. Error bars represent means + s.e.m.

G. Recombinant HSPB4 caused nuclear translocation of the NF-κB complex in macrophages.

H. Necrotic corneal extracts stimulated the TLR2/NF-κB pathway in cells expressing TLR2 (HEK-TLR2). Antibodies to HSPB4 significantly inhibited the effects. $n = 6$ per group. Error bars represent means + s.e.m.

I,J. HSPB4 concentration-dependently stimulated NF-κB signalling in cells expressing TLR2, but had no effect in cells without either receptor (HEK-null). $n = 6$ per group. p values, *versus* no HSPB4 treatment. Error bars represent means + s.e.m.

the effect (Fig 5H). Recombinant HSPB4 stimulated the NF-κB signalling in the same cell line in a concentration-dependent manner (Fig 5I). HSPB4 had no effect on the reporter cell line without either receptor (Fig 5J). Of note, other Group A molecules such as HSPB5 and βB-crystallin had no effect on pro-inflammatory cytokine production in macrophages or on NF-κB activation in HEK-TLR2 cells (Supporting Information Fig S6). Also, SN did not induce the expression of pro-inflammatory

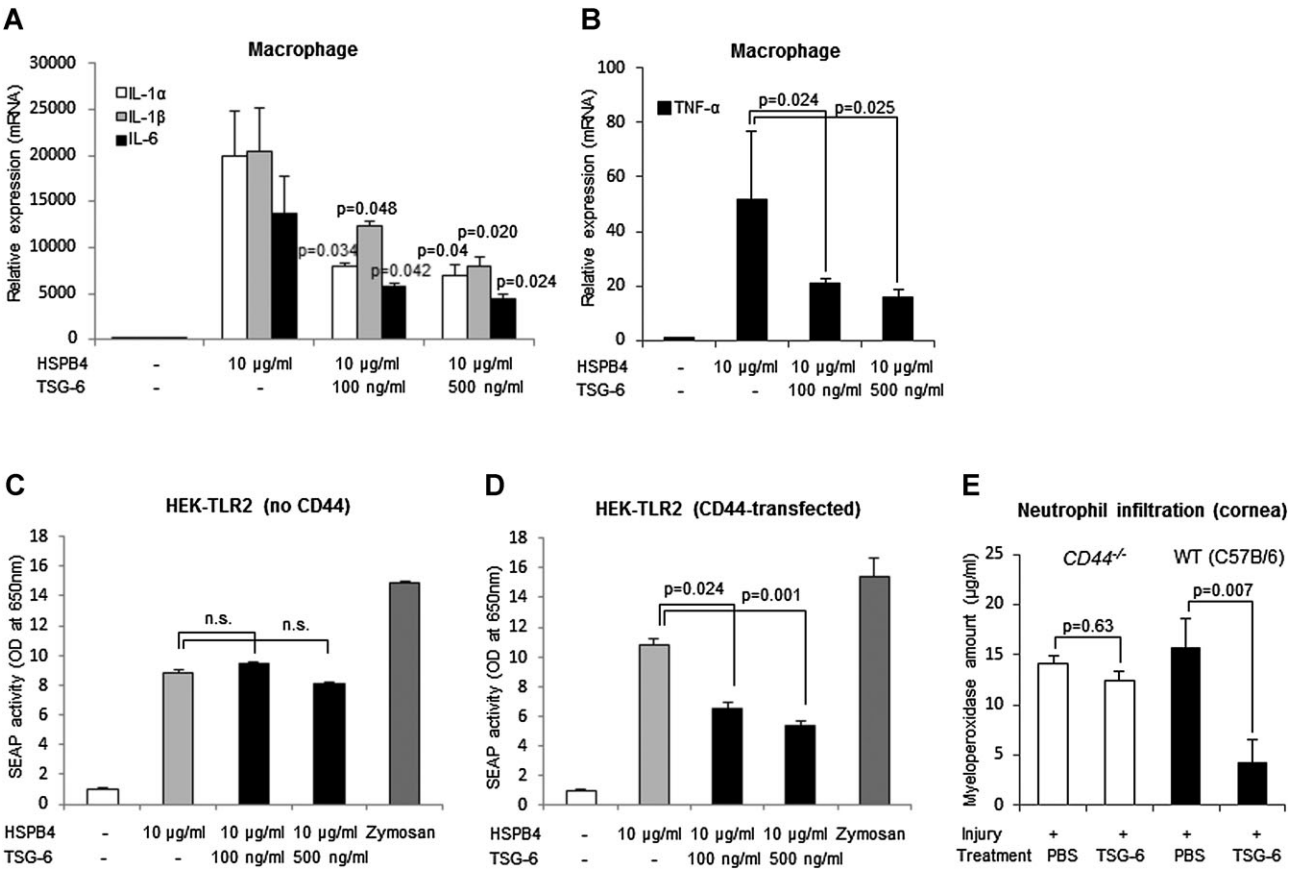

Figure 6. TSG-6 inhibition of HSPB4-activated macrophages and the requirement for CD44.

A,B. Dose-dependent suppression by TSG-6 of macrophages activated by HSPB4. $n = 6$ per group. p values, *versus* PBS treatment. Error bars represent means + s.e.m.

C. TSG-6 had no effect on NF-κB signalling in HEK-TLR2 cells that did not express CD44. $n = 8$ per group. Error bars represent means + s.e.m.

D. Inhibition by TSG-6 of NF-κB signalling in HEK-TLR2 cells expressing CD44. $n = 8$ per group. Error bars represent means + s.e.m.

E. TSG-6 did not suppress the inflammatory response at 24 h after injury in transgenic mice with null alleles for CD44 ($CD44^{-/-}$), whereas TSG-6 significantly inhibited inflammation in the cornea of wild-type mice expressing CD44 (C57B/6). $n = 10$ per group. Error bars represent means + s.e.m.

cytokines in either macrophages or keratocytes in culture (Supporting Information Fig S7). Similarly, HSPB4 did not stimulate keratocytes in culture to express pro-inflammatory cytokines (Supporting Information Fig S7).

Together, the results indicated that HSPB4 was a principal DAMP for Phase II inflammation and acted through the activation of resident macrophages in the cornea.

TSG-6 suppressed HSPB4-induced activation of macrophages in a CD44-dependent manner

Based on the observation that TSG-6 suppressed Phase II, not Phase I (Fig 5B and C), we tested the hypothesis that TSG-6 might suppress the HSPB4-induced activation of macrophages. We found that an addition of recombinant TSG-6 decreased the expression of pro-inflammatory cytokines in macrophages that were stimulated by HSPB4 (Fig 6A and B). Since the protein was previously shown to inhibit TLR2/NF-κB signalling in macrophages by interaction with CD44 on macrophages (Choi et al, 2011; Lesley et al, 2004), we examined whether the effects of TSG-6 was CD44-dependent. As a result, recombinant TSG-6 decreased NF-κB signalling in the HEK-TLR2 reporter cells stably expressing CD44 (Fig 6C and D). However, TSG-6 had no effect on the parent cell line that did not express CD44. Also, TSG-6 had no effect on the Phase II inflammatory response after chemical/mechanical injury to the cornea of CD44 knockout mice (Fig 6E), indicating that the action of TSG-6 was CD44-dependent. Together, TSG-6 interacted with CD44 on macrophages and thereby decreased the activation of TLR2/NF-κB signalling that had been induced by HSPB4 released from injured cornea.

DISCUSSION

As summarized in Fig 7, the results demonstrated that there were two distinct phases of neutrophil infiltration in the cornea after sterile injury: (i) a small initial Phase I that began within 15 min and reached a plateau level in 4–8 h and (ii) a much

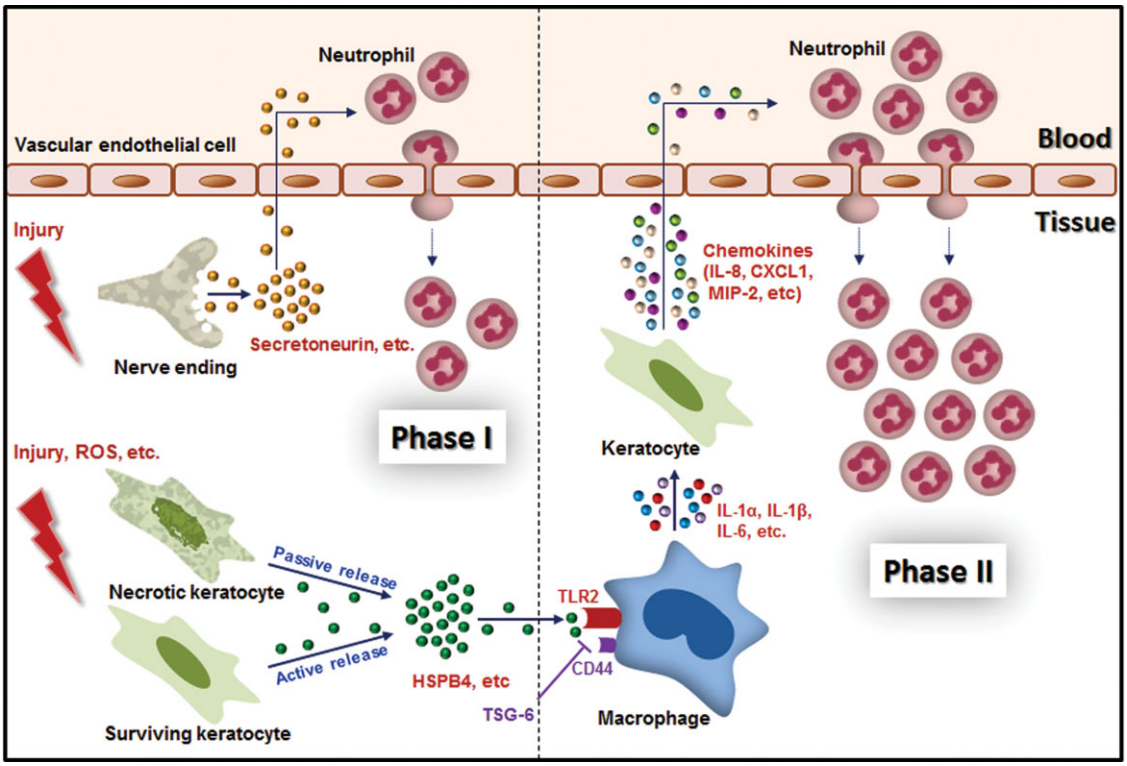

Figure 7. Graphic summary of sterile inflammation in the cornea. Immediately after injury, SN is released from nerve endings in the cornea to recruit circulating neutrophils and induce an initial inflammatory response (**Phase I**). Then necrotic or injured keratocytes release HSPB4 in response to injury and oxidative stress. The HSPB4 activates resident macrophages in the cornea via TLR2/NF-κB signalling pathway to produce pro-inflammatory cytokines including IL-1 and IL-6. The pro-inflammatory signals released by resident macrophages are amplified by keratocytes that produce chemokines to further recruit large amount of neutrophils (**Phase II**). TSG-6 inhibits the initial activation of resident macrophages by modulating TLR2/CD44/NF-κB signalling and thereby decreases the Phase II inflammatory response.

larger Phase II with a peak at 24–48 h. In search for the stimuli for Phase I and II responses, we screened for the molecules up-regulated in the cornea in each phase after an injury on the assumption that the molecules produced actively by tissues in response to an injury might act as the major stimuli to initiate the sterile inflammation.

The major stimulus for the Phase I response was the neuropeptide SN. Intrastromal injection of recombinant SN reproduced the Phase I response, and an inhibitor of the release of neuropeptides blocked the effect. As indicated by experiments with macrophage depletion, Phase I did not require participation of resident macrophages. SN did not elicit the further, intense infiltration of neutrophils in Phase II that was mediated by activation of resident macrophages. Therefore, the Phase I response was independent of the Phase II response and was not severe enough to cause corneal opacity occluding the visual axis. Nevertheless, neuropeptides are attractive candidates for the stimuli for the initial rapid inflammatory responses of the tissues because they are preformed and rapidly released from nerve endings by injury. Neuropeptides may be particularly important in the response of the cornea to an injury since the cornea is one of the most heavily innervated tissues in the body (Nishida, 2005). SN is a member of the granin family of neuropeptides/neurotransmitters that contributes to innate immunity by activating chemotactic migration and transendothelial extravasation of immune cells (Troger et al, 2005; Helle, 2010). Importantly, SN was shown to be as effective as TNF-α in stimulating transmigration of neutrophils through an endothelial cell barrier (Kähler et al, 2001, 2002). Also, recent *in vivo* studies suggested that SN has an important role in common diseases such as myocardial infarction, rheumatoid diseases, or acute inflammatory bowel syndromes (Helle, 2010; Taupenot et al, 2003; Wiedermann, 2000).

The data demonstrated that the small heat shock protein HSPB4 was the principal stimulus for the Phase II response that is largely responsible for vision-threatening opacity of the cornea. Injury to the cornea actively released HSPB4. Addition of necrotic extracts from corneal tissues increased the synthesis of HSPB4 by stromal keratocytes, probably as a result of increased ROS and other stimuli in the necrotic cornea. HSPB4 free of pyrogens reproduced the Phase II response by activating resident macrophages via the TLR2/NF-κB signalling pathway. HSPB4 did not induce Phase II in the corneas depleted of resident macrophages. In culture, HSPB4 stimulated the TLR2/NF-κB signalling in macrophages to release a cascade of pro-inflammatory cytokines such as IL-1α and IL-1β. The critical

role of the HSPB4/TLR2/NF-κB axis in macrophages was confirmed by the observation that the intense inflammatory response in Phase II in injured cornea was greatly diminished by depletion of resident macrophages, by blocking HSPB4 with antibodies to HSPB4, in mice lacking HSPB4 or TLR2, or by administration of TSG-6 that inhibits TLR2/NF-κB signalling in macrophages in a CD44-dependent manner (Choi et al, 2011; Lesley et al, 2004).

The role of HSPB4 as a DAMP is consistent with previous observations with other HSPs such as HSP60, 70, 72 or 96 (Asea et al, 2000; Chen & Cao, 2010; Huang et al, 2009; Lehnardt et al, 2008; Wheeler et al, 2009). HSPB4 is a member of small molecular weight HSPs (small HSPs) that range in size from 15 to 30 kDa. Like other HSPs, small HSPs are induced by heat and other stimuli such as oxidative and inflammatory stresses, and they act as a chaperone to protect cells from damage produced by misfolded proteins. However, HSPs have paradoxical effects (Chen et al, 2007a; DeMeester et al, 2001; Kobba et al, 2011). Heat shock pretreatment induces intracellular HSPs to protect against inflammation, whereas after inflammatory injury has been primed or cell integrity has been compromised, heat shock induces the release of HSPs from damaged or stressed cells and exacerbates the cell injury. Furthermore, HSPs are known to facilitate the binding of other danger signals to immune cells and thus to enhance the immunogenicity (Chen & Cao, 2010). Consistent with these reports, we for the first time report here that a small HSP, HSPB4, stimulated macrophages *in vitro* through the TLR2/NF-κB pathway and acted as the principal DAMP *in vivo* for the Phase II inflammatory response in the injured cornea. In contrast, another small HSP, HSPB5, did not induce this response.

The identification of the HSPB4/TLR2/NF-κB axis in resident macrophages as the principal stimuli for the Phase II inflammatory response has several implications for potential therapies. One is that the results provide novel therapeutic targets for diseases of the corneal surface that are characterized by sterile inflammation and for which there currently are no effective therapies. The results suggest that modulating the HSPB4/TLR2/NF-κB axis with antibodies/antagonists to HSPB4 or with recombinant TSG-6 may be beneficial for over 500,000 Americans who sustain chemical injuries of the cornea each year. The results also provide a basis for developing therapies for other diseases of the eye or of other tissues. HSPB4 was up-regulated in the retina in models for uveitis (Rao et al, 2008; Saraswathy et al, 2010), glaucoma (Munemasa et al, 2009), age-related macular degeneration (Kapphahn et al, 2003) and hypoxic retinopathy (Miyara et al, 2008). Also, HSPB4 is expressed in nonocular tissues such as spleen, thymus, pancreas and kidney (Deng et al, 2010; Srinivasan et al, 1992). Therefore, the strategy of using TSG-6 or antibodies/antagonists to extracellular HSPs may present useful to treat a variety of inflammatory diseases where HSPs are the primary signals for inflammation such as ischemia-reperfusion injury (Gill et al, 2010). Still another implication of the results is that the strategy of using microarrays to follow the response of the tissue to injury may be useful to analyse the inflammatory responses and to identify therapeutic targets for inflammatory diseases of other tissues or organs.

MATERIALS AND METHODS

Animals

Lewis rats (LEW/Crl) were purchased from Charles River Laboratory (Wilmington, MA). HSPB4 knockout mice (*Cryaa*−/− or *Hspb4*−/−) were generated at the National Eye Institute by targeted gene disruption and were maintained in the 129S6/SvEvTac background (Brady et al, 1997). 129S6/SvEvTac mice were purchased from Taconic Farms, Inc. (Germantown, NY). TLR-2 knockout mice (B6.129-Tlr2tm1Kir/J), CD44 knockout mice (*CD44*$^{-/-}$; B6.Cg-Cd44tm1Hbg/J) and C57BL/6J were obtained from Jackson Laboratory (Bar Harbor, ME). All animals were used under a protocol approved by the Institutional Animal Care and Use Committee of Texas A&M Health Science Center College of Medicine.

Animal models of injury and treatment

Injury was created by applying 100% ethanol to the whole cornea including the limbus for 30 s followed by rinsing with 1 ml of balanced salt solution. Then the whole corneal and limbal epithelium was mechanically scraped using a surgical blade.

For injection of the recombinant human (rh) SN (PolyPeptide Laboratories, Hillerød, Denmark) or HSPB4 (Enzo Life Sciences, Plymouth Meeting, PA), proteins in 2 μl PBS (0.2 ng SN or 100 ng HSPB4 which corresponded to the amounts of SN or HSPB4 detected by ELISA in the rat cornea at 15 min after injury) were injected using a 32 gauge needle into the corneal stroma near the temporal limbus of the rat eyes. To confirm that the effect of HSPB4 injection was HSPB4-specific rather than an effect of recombinant protein, we also injected HSPB5 (100 ng/2 μl PBS, Enzo Life Sciences) having a similar molecular weight (23 kDa) to HSPB4 into the corneal stroma. For experiment in TLR2 knockout mice, 10 ng HSPB4 in 1 μl PBS or the same volume of PBS was injected into the corneal stroma of the mouse eyes. The proteins were sterilized prior to use. HSPB4 was purified by endotoxin binding columns according to the manufacturer's instructions (EndoClear, blue, Hycult Biotech Inc., Plymouth Meeting, PA) and tested to be free of detectable levels of Gram-negative bacterial endotoxins (<0.01 EU/ml) and proteins (<1 ng/ml) using the Limulus amoebocyte lysate kit (Hycult Biotech Inc.) and *Escherichia coli* HCP ELISA kit (Cygnus Technologies, Southport, NC).

For blocking the release of SN from nerve endings in the cornea, 20 μl of 2 mM Diltiazem solution in isotonic saline (Sigma–Aldrich, St. Louis, MO) was topically applied to the rat cornea 15 min prior to injury (Gonzalez et al, 1993). The blockage of SN release by Diltiazem instillation was confirmed by evaluating the serum level of SN after injury. For blocking HSPB4 in the cornea, either mouse monoclonal or rabbit polyclonal antibodies to rat HSPB4 (10 or 50 μg in 100 μl PBS) (ab14821 and ab5595, Abcam, Cambridge, MA) were subconjunctivally injected near the limbus immediately before the injury. The same concentration of isotype IgG was injected as control. The antibodies were sterilized prior to injection.

For macrophage depletion, either 100 μl of clodronate-encapsulated liposome (5 mg clodronate/ml, Encapsula Nano Sciences, Nashville, TN) or the same volume of PBS-encapsulated liposome (Encapsula Nano Sciences) was injected into the subconjunctival space near the limbus on day −2 (2 days before injury) and on day 0 (day of injury). The injection was evenly dispensed over four quadrants (25 μl each) so that a circular bleb around the cornea was formed.

To evaluate the effect of TLR2 inhibition on the injured cornea, rhTSG-6 (2 μg in 5 μL of PBS) (2104-TS, R&D Systems, Minneapolis, MN) or the same volume of PBS was injected into the anterior chamber of the rat eye immediately after injury. We used 2 μg TSG-6 because this was the maximal and the most effective dose as we previously demonstrated (Oh et al, 2010).

Cells and cell lines

Murine macrophages (RAW 264.7) were obtained from ATCC (Rockville, MD). Human embryonic kidney (HEK) 293 cells that expressed TLR2 or TLR4 with secreted alkaline phosphatase driven by an NF-κB promoter were purchased from InvivoGen (HEK-Blue™ -hTLR2 and HEK-Blue™ -hTLR4, San Diego, CA). Control cell line not expressing either TLR2 or TLR4 was also obtained (HEK-Blue™ -Null1, InvivoGen). Primary human keratocytes were obtained from ScienCell (Carlsbad, CA) and used at passage 5.

The stable cell line expressing human CD44 was generated in HEK-Blue™ -hTLR2 cells. The CD44 coding sequence (Origene, Rockville, MD) was inserted into the plasmid pcDNA 3.1 (Invitrogen, Carlsbad, CA) using Not1/Xba1 sites and the plasmid (pcDNA 3.1-CD44) transformed into *E. coli* (Subcloning Efficiency™ DH5a™ Competent Cells; Invitrogen) for cloning. The transfected cells were lifted by pipetting and plated in four 10 cm culture dishes in 10 ml of growth medium supplemented with 1× HEK-Blue™ Selection media (InvivoGen). The cells were kept in the selection media for 2 weeks, individual clones designated as HEK-hTLR2-CD44, and HEK-hTLR2-pcDNA were selected for expansion.

Cell injury induction

To investigate the effects of injured corneal cells *in vitro*, necrotic corneal tissue extracts were prepared. Rat corneas were homogenized in PBS (100 μl per one cornea) using a motor-driven homogenizer followed by five freeze-thaw cycles and incubated at 37°C for 5 h (Chen et al, 2007b). After centrifugation at 12,000 rpm for 5 min, the supernatants were obtained as necrotic extracts. Some of necrotic extracts were heat-treated (100°C, 20 min). Necrotic extracts were incubated with the cells in culture at 1:10 dilution for 2 h.

To evaluate the effect of HSPB4, either antibodies to HSPB4 (10 or 50 μg) (ab14821 and ab5595, Abcam) or isotype IgG antibodies were added to the cultures.

To study the effects of small HSPs or SN, HSPB4, HSPB5, βB-crystallin (0.001–10 μg/ml, Enzo Life Sciences), or SN (0.1–10 ng/ml) was added to cultures of the cells, and the cultures were incubated for 2 h. Prior to use, small HSPs were tested to be free of pyrogens after being purified by endotoxin binding columns (EndoClear, blue) using the Limulus amoebocyte lysate kit (Hycult Biotech Inc.) and *E. coli* HCP ELISA kit (Cygnus Technologies). To additionally rule out the possibility of bacterial or LPS contamination of small HSP preparations, all experiments were performed in the presence of polymyxin B (10 μg/ml) (Sigma–Aldrich) for neutralization of LPS. Moreover, for additional control experiments, small HSPs denatured by heat (100°C, 20 min) were used in parallel sets of experiments.

To examine the effect of TSG-6, 100 or 500 ng/ml of rhTSG-6 (R&D Systems) was added to the cultures of the cells.

To determine whether keratocytes express small HSPs in response to injury, either necrotic corneal extracts or H_2O_2 (100–500 nM) were added to the cultures of keratocytes.

Myeloperoxidase (MPO) assay

For a quantitative measure of neutrophil infiltration, the cornea was assayed for the MPO concentration (Rat MPO ELISA kit, HyCult biotech) as we previously reported (Oh et al, 2010). For protein extraction, the cornea was minced into small pieces and lysed in 150 μl of tissue extraction reagent (Invitrogen) containing protease inhibitor cocktail (Roche, Indianapolis, IN). The samples were sonicated on ice using an ultrasound sonicator. After centrifugation at 12,000 rpm at 4°C for 20 min, the supernatant was collected and assayed for levels of MPO.

Microarrays

For RNA extraction, the cornea was minced into small pieces, lysed in 600 μl of RNA isolation reagent (RNA Bee, Tel-Test Inc., Friendswood, TX) and homogenized using a motor-driven homogenizer. Total RNA was then extracted using RNeasy Mini kit (Qiagen, Valencia, CA). RNA target for microarrays was prepared using the 3′ IVT Express Kit (Affymetrix, Santa Clara, CA) according to manufacturer's instructions starting with 200 ng of total RNA. Total of 12.5 μg of cRNA was hybridized onto rat arrays (RG-230 2.0), and the arrays were stained, washed and scanned for fluorescence. Microarray data were normalized and analysed using the Partek Genomics Suite 6.4 (Partek, St. Louis, MO) and dChip software. For comparative analysis, data were filtered based on fold changes of two or more (either up- or down-regulated). For the hierarchical clustering analysis, data were filtered using coefficient of variation higher than 0.6 and presence call of at least 33%. Filtered genes were hierarchically clustered, and six clusters were selected on the similar level of hierarchy and studied for enriched Gene Ontology tags based on hypergeometric distribution. Data have been submitted to the ArrayExpress database.

Real-time RT PCR

Total RNA from the cornea or the cells was extracted as described above and used to synthesize double-stranded cDNA by reverse transcription (SuperScript III, Invitrogen). Real-time amplification was performed (Taqman Universal PCR Master Mix, Applied Biosystems, Carlsbad, CA) and analysed on an automated instrument (7900HT Fast Real-Time PCR System, Applied Biosystems). PCR probe sets were commercially purchased (Taqman Gene Expression Assay Kits, Applied Biosystems). For assays, reactions were incubated at 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s followed by 60°C for 1 min. For normalization of gene expression, 18S rRNA probe was used as an internal control.

Western blot

Clear lysates of protein from the cornea were prepared as described above and measured for the concentration. A total of 10 μg protein was fractionated by SDS-PAGE on 10% *bis–tris* gel (Invitrogen), transferred to nitrocellulose membrane (Invitrogen), and then blotted with antibodies against SN (Phoenix Pharmaceuticals, Burlingame, CA) or HSPB4 (ab14821, Abcam).

ELISAs

Protein was extracted from the cornea as described above and assayed for levels of pro-inflammatory cytokines and chemokines with commercial ELISA kits for IL-6, IL-1β and CXCL1/CINC-1 (Quantikine kit, R&D Systems), and for CCL2/MCP-1 (Immunoassay Kit, Invitrogen). For HSPB4 measurement, mouse monoclonal anti-rat antibody to HSPB4 (ab14821, Abcam) was used as a capture antibody (4 μg/ml),

The paper explained

PROBLEM:

Sterile inflammation underlies many diseases. Specifically, diseases of the cornea resulting from sterile inflammation range from vision-threatening disorders (*e.g.* chemical burn) to quality-of-life deteriorating diseases such as the dry eye syndrome that affects nearly 10% of the U.S. population. For all these conditions, the anti-inflammatory agents currently available are not effective and often produce adverse effects. Hence, searching for an effective therapeutic target for inflammation is critical. Here, we have taken advantage of the unique features of the cornea to analyse the process of sterile inflammation in detail and search for the stimuli that initiate the inflammatory response.

RESULTS:

First, we found that neutrophil infiltration in sterile inflammation occurred in two distinct phases: early Phase I and late Phase II. Next, we demonstrated that the neuropeptide SN was a major signal for the initial Phase I inflammatory response in the cornea. Also, the small heat shock protein HSPB4 was a principal signal for a late Phase II. HSPB4 stimulated neutrophil infiltration in Phase II by activating TLR2/NF-κB pathway in resident macrophages. We further showed that inhibition of the HSPB4/TLR2/NF-κB axis in resident macrophages suppressed Phase II inflammation in the cornea and rescued the cornea from vision-threatening corneal opacity.

IMPACT:

The identification of the HSPB4/TLR2/NF-κB axis in resident macrophages as the principal stimuli for the Phase II inflammatory response has important implications as a potential therapy for inflammatory diseases of the cornea. Also, the experimental approach we successfully used here can be applied to identify novel therapeutic targets for inflammatory diseases in other tissues.

and rabbit polyclonal anti-rat antibody to HSPB4 (ab5595, Abcam) was used as a secondary antibody (400 ng/ml).

Release of HSPB4 in injured cornea

To measure the amount of HSPB4 released from the injured cornea, the cornea was harvested immediately after injury and cultured in an incubator at 37°C with 5% CO_2 for 12 h. Every 2 h, the culture medium was changed, and the concentration of HSPB4 in the medium during each time frame was measured by ELISA.

Histopathology

The cornea was excised after the animal was sacrificed and fixed in 10% paraformaldehyde. The cornea was cut into 4 μm sections and stained with the hematoxylin–eosin (H&E) or subjected to immunohistochemistry. The formalin-fixed corneal section was deparaffinized with ethanol. The antigen was retrieved using a steamer in epitope retrieval solution (IHC WORLD, Woodstock, MD). The rabbit polyclonal anti-rat antibody to neutrophil elastase (1:200) (ab21595, Abcam), the mouse monoclonal anti-rat antibody to secretogranin II (1:200) (ab20245, Abcam), or the mouse monoclonal anti-rat antibody to HSPB4 (1:200) (ab14821, Abcam) were used as primary antibodies, and the anti-rabbit IgG (1:5000) (Abcam) or the anti-mouse IgG (1:5000) (Abcam) used as secondary antibodies. The DAPI solution (VECTASHIELD Mounting Medium; Burlingame, CA) was used for counterstaining.

Aconitase activity assay

To evaluate the oxidative damage in the cornea by injury (Ma et al, 2009), loss of aconitase activity in corneal lysates was measured using an Aconitase Assay Kit according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI).

NF-κB translocation assays

Macrophages were plated in eight well chamber slides (Lab-Tek II Chamber Slide; Nalge Nunc, Rochester, NY) and incubated for 1 h in 0.2 ml of 2% FBS in α-MEM with or without 10 μg/ml HSPB4. The cells were washed twice with PBS, fixed with 100% methanol for 5 min, and blocked with Image-iT™ FX Signal Enhancer (Invitrogen). The cells were then incubated with 1 μg/ml of anti-NF-κB p65 antibody (ab16502, Abcam) in blocking buffer (5% BSA in PBS) overnight at 4°C. The samples were incubated for 1 h with the anti-rabbit IgG (1:2000) (Alexa Fluor® 488, Invitrogen). The slides were visualized with a fluorescent microscopy (Eclipse 80i, Nikon, Melville, NY).

Statistical analysis

Comparisons of parameters among the groups were made by one-way ANOVA using SPSS software (SPSS 12.0, Chicago, IL). Differences were considered significant at $p < 0.05$.

Author contributions

JYO initiated and designed the project, performed the experiments, and wrote the manuscript; HC performed the western blotting, generated the stable cell line expressing CD44 and contributed conceptually to the project; RHL performed the NF-κB translocation study and helped design some of the experiments; GWR assisted in the animal and cell culture experiments and contributed to the writing of the manuscript; JHY performed the microarray assays; EW provided the *Hspb4*$^{-/-}$ mice; DJP supervised the project, interpreted data and wrote the manuscript.

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Supporting Information is available at EMBO Molecular Medicine online.

Conflict of interest statement: DJP is a member of the scientific advisory board of Temple Therapeutics LLC. The other authors have no conflict of interest.

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