Mycobacterium tuberculosis Chaperonin 60.1 Inhibits Leukocyte Diapedesis in a Murine Model of Allergic Lung Inflammation

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Chaperonin 60.1 from *Mycobacterium tuberculosis* suppressed allergic lung inflammation and bronchial hyperresponsiveness in mice by a mechanism that is yet to be clarified. To investigate the possible antiinflammatory mechanism(s) of action of Cpn60.1 in a model of allergic lung inflammation, ovalbumin (OVA)-allergic mice were pretreated with Cpn60.1 intranasally 20 minutes before each OVA aerosol challenge in a total of three treatments. Readouts were performed 24 hours after last challenge. Pretreatment with Cpn60.1 (1.0–0.001 μg) significantly inhibited the number of eosinophils in bronchoalveolar lavage fluid (OVA, 49.2 ± 12.3 versus Cpn60.1 [1 μg dose], 90.4 ± 2.3 × 10^4 cells/ml) and IL-5 release (OVA, 43 ± 8.5 versus Cpn60.1 [1 μg dose], 3 ± 11 pg/ml) but increased IL-12 levels (OVA, 50 ± 24 versus Cpn60.1 [1 μg dose], 103 ± 13 pg/ml). The effect of Cpn60.1 on cell recruitment and IL-5, but not IL-12, release was abolished in TLR-4 knockout mice. Intravital microscopy demonstrated that Cpn60.1 reduced chemokine-mediated leukocyte rolling and transmigration across the vessel wall (rolling cells: eotaxin, 11.7 ± 1.1 versus Cpn60.1 [1 μg dose], 2.8 ± 1 cells in 30 s). Similarly, Cpn60.1 reduced eotaxin-induced leukocyte migration *in vitro* (eotaxin, 17.3 ± 3.3 versus Cpn60.1 [0.1 μg dose], 3.3 ± 0.4 cells × 10^4/ml). Immunostaining demonstrated that Cpn60.1 inhibits VCAM-1 and increases vascular endothelial–cadherin expression in lung vascular tissue, suggesting that the antiinflammatory effect of Cpn60.1 is partly mediated by altering the expression of adhesion molecules. This study shows that Cpn60.1 inhibits leukocyte diapedesis by a TLR-4 and an adhesion molecule–dependent mechanism in allergic inflammation in mice.

Keywords: chaperonin; asthma; inflammation; eosinophils; Toll-like receptors

A number of epidemiological studies suggest that the prevalence of asthma correlates inversely with immunity toward a number of infectious diseases, including *Mycobacterium tuberculosis* (1), *Trypanosoma gondii*, and *Helicobacter pylori* (2). In addition, an inverse correlation between vaccination with bacillus Calmette-Guérin (BCG) and atopic diseases has been demonstrated (1, 3). BCG is a vaccine against tuberculosis that is prepared from a strain of the attenuated live bovine tuberculosis bacillus *Mycobacterium bovis*, suggesting that bacterial components could be responsible for this effect. However, there are reports showing that an increase in the production of Th1-like cytokines can worsen the severity of asthma (4) and exacerbate the intensity of the inflammatory response in the lung (5), suggesting that the increase in prevalence of allergic diseases could be due to mechanisms other than an imbalance between Th1 and Th2 immune pathways.

Chaperonins are proteins that assist the noncovalent folding or unfolding and the assembly or disassembly of proteins. Chaperonins in all bacteria are encoded by the essential groEL and groES genes, also called cpn60 and cpn10, respectively. *M. tuberculosis* has two genes encoding Cpn60 proteins, termed Cpn60.1 and Cpn60.2, also known as heat shock proteins 60 and heat shock protein 65, respectively (6). Cpn60.1 is released *in vitro* when the bacterium is in a quiescent state and is not a requirement for the survival of the bacteria but appears to be an important agent in the regulation of the cytokine-dependent granulomatous response in *M. tuberculosis* infection (7, 8). It has also been demonstrated that Cpn60.1 is a potent inducer of inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12, TNF-α, and GM-CSF and the monocyte-deactivating cytokine IL-10 from human peripheral monocytes *in vitro* (9) and IL-1 and IL-6 from murine macrophages (7).

We have previously demonstrated in a murine model of allergic inflammation that exposure to chaperonins isolated from *M. tuberculosis* significantly inhibited the recruitment of eosinophils to the airways and bronchial hyperresponsiveness (BHR) in OVA-immunized mice (10). Moreover, pretreatment with the chaperonin Cpn60.1 resulted in decreased levels of IL-5 and IL-4 without altering the levels of Th1 cytokines, supporting the hypothesis that the effect of this protein on the inflammatory response is not simply due to an imbalance in the Th1/Th2 response.

The aim of this study was to investigate the possible mechanism(s) underlying the antiinflammatory effect of *M. tuberculosis* chaperonin 60.1 (Cpn60.1) in a murine model of allergic lung inflammation.

CLINICAL RELEVANCE

There is an inverse correlation between immunization against tuberculosis and the incidence of allergic diseases in humans. Experimentally, chaperonins isolated from *Mycobacterium tuberculosis* significantly inhibit the recruitment of eosinophils to the airways and bronchial hyperresponsiveness in ovalbumin-immunized mice by mechanisms that are yet to be established. We demonstrate for the first time that treatment with mycobacterial chaperonins significantly suppresses leukocyte chemotaxis *in vitro* and diapedesis *in vivo* by a TLR-4 and adhesion molecule dependent mechanism, suggesting that these agents may be of use in the treatment of allergic diseases.

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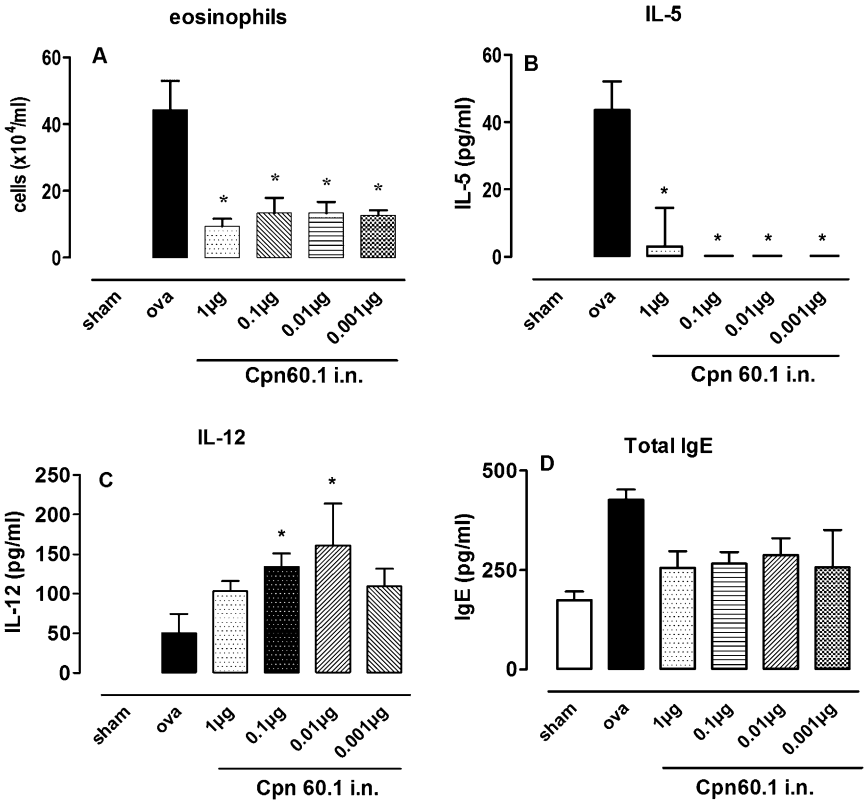

Figure 1. Effect of Cpn60.1 on allergic inflammation in the lung. (*A*) Number of eosinophils. (*B*) IL-5 in BAL fluid. (*C*) IL-12 in BAL fluid. (*D*) Total IgE in serum collected 24 hours after OVA challenge in sham-immunized mice (sham), OVA-immunized mice (ova), and OVA-immunized treated with Cpn60.1 (Cpn60.1 intranasally). *Vertical lines* represent SEM of 6 to 18 animals per group. $^*P < 0.05$ for the ova group.

MATERIALS AND METHODS

Additional information about the methods can be found in the online supplement.

Antigen-Induced Allergic Inflammation

Female BALB/c mice (Charles River Laboratories, Margate, UK), male and female C57Bl/6 mice (Charles River), male and female TLR-2 KO and TLR-4 KO (11, 12), and male and female MKK3 knockout (KO) mice (13) between 6 and 10 weeks old were immunized intraperitoneally with 10 μg of OVA (type V; Sigma Chemical Co., Gillingham, UK) adsorbed to a saturated solution of aluminum hydroxide as described previously (10). Experiments were approved by the Home Office under The Animals (Scientific Procedures) Act (1986) and by the Ethics Committee of King's College London.

Treatment with Cpn60.1

Animals received Cpn60.1 (0.001–1.0 μg) or saline by nasal instillation 20 minutes before each challenge (Days 14–16), receiving a total of three instillations. For intravital microscopy experiments, Cpn60.1 (1 μg) or saline was injected in the scrotal sac of allergic mice 15 minutes before eotaxin injection (100 ng).

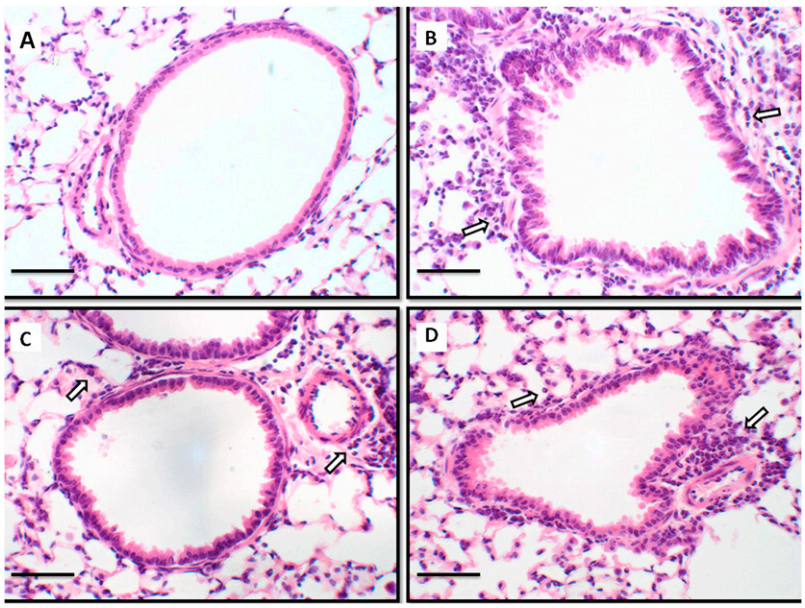

Figure 2. Photomicrographs of lung tissue stained with standard hematoxylin and eosin method collected 24 hours after OVA challenge. (*A*) Sham-immunized mice. (*B*) OVA-immunized mice. (*C* and *D*) OVA-immunized mice treated with 1.0 and 0.001 μg Cpn60.1, respectively. *Bars* represent 50 μm length; *white arrows* denote areas of eosinophil accumulation.

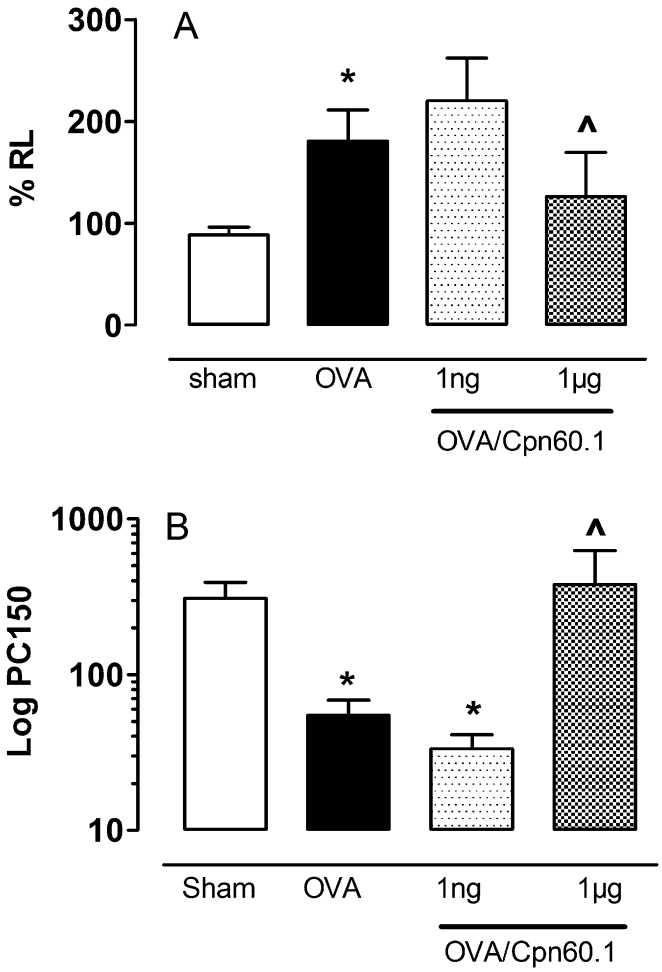

Figure 3. Bar graph representing (*A*) peak response of total lung resistance (% increase above postsaline value) and (*B*) log of PC 150 in response to aerosolised methacholine in sham-immunized (*open column*; $n = 8$); OVA-immunized (*closed column*; $n = 8$); OVA-immunized, treated with 1 ng/dose of Cpn60.1 (*light-shaded column*; $n = 8$); and OVA-immunized, treated with 1 μg of Cpn60.1 (*dark-shaded column*; $n = 8$). *Vertical lines* represent SEM. *$P < 0.05$ compared with sham control mice. ^$P < 0.05$ for OVA-sensitized mice.

Bronchoalveolar Lavage

Twenty-four hours after the last antigen challenge, mice were anesthetized with urethane (2 g/kg intraperitoneally) (Sigma Chemical Co.). A cannula was inserted into the exposed trachea, and three 0.5-ml aliquots of sterile saline were injected into the lungs as described previously (10).

Bronchoalveolar Lavage Cytokine Levels

Levels of IL-5, IL-12, and total IgE were measured in bronchoalveolar lavage (BAL) fluid by ELISA (eBioscience, Hatfield, UK, and BD Pharmingen, Oxford, UK) following instructions from the manufacturer.

Histological Analysis

Formalin-fixed tissues were embedded in paraffin, and 4-μm sections were cut and stained with H&E (Sigma-Aldrich, Gillingham, UK) for examining leukocyte infiltration microscopically (Zeiss Axioscope). A Nikon Digital Camera (DXM1200; Nikon, Kingston upon Thames, UK) and Lucia image analysis software (version 4.61; Nikon) were used to obtain images of the lungs.

Respiratory Lung Mechanics

Respiratory lung mechanics and airways responsiveness to methacholine were measured according to methods previously described in our laboratory (10).

Chemotaxis Assay

Bone marrow (BM) cell migration was examined using 96-well chemotaxis plates (ChemoTx; NeuroProbe, Gaithersburg, MD) with 3 μm pore size polycarbonate filters. Cells were obtained from OVA-immunized mice. The bottom wells of the plate were filled with 30 μl of Dulbecco's modified Eagle medium containing 5 μg/ml of polymixin B with or without 1 ng/ml of eotaxin. After 3-hour incubation at 37°C, migrated cells were removed and counted using a modified Neubauer chamber.

Intravital Microscopy

To examine the recruitment of cells from the microvasculature of the cremaster muscle, Cpn60.1 (1 μg per dose) was administered subcutaneously into the shaved scrotal sac of OVA-sensitized C57/Bl6, TLR-2, or TLR-4 mice. Ten minutes later, the animals received eotaxin (100 ng per dose, subcutaneously). Four hours later, the animals were surgically prepared for intravital microscopy.

Immunohistochemistry

Primary antibodies used were rat anti-mouse CD31 (PECAM-1, clone 390), rat antimouse CD144 (vascular endothelial [VE]-cadherin, clone aBioBV13), and rat anti- mouse CD106 (VCAM-1, clone 429) (eBioscience UK) and polyclonal rabbit IgG as isotype control (Sigma-Aldrich UK).

Statistical Analysis

Data were analyzed using ANOVA (1 or 2 fixed effect design) followed where appropriate by a *post hoc* test (Sidak; SPSS version 18). Differences between mean values considered significant if $P < 0.05$.

RESULTS

The Effect of Cpn60.1 on Eosinophil Migration to the Lung Lumen

The total number of cells quantified in BAL fluid obtained from OVA-immunized mice (OVA) 24 hours after OVA challenge was significantly higher compared with nonsensitized sham mice (OVA, $143.3 \pm 13.3 \times 10^4$ cells/ml versus Sham, $28.6 \pm 2.55 \times 10^4$ cells/ml; $n = 10$–18; $P < 0.05$). This was reflected by a significantly greater number of eosinophils recruited to the airways in OVA mice ($44.3 \pm 8.6 \times 10^4$ cells/ml) versus Sham mice ($0.03 \pm 0.03 \times 10^4$ cells/ml; $P < 0.05$) (Figure 1A). The number of neutrophils was less than 3% of the total cell counts and not significantly different between groups (data not shown). The intensity of the inflammatory response was significantly reduced in animals treated with Cpn60.1 (1, 0.1, 0.01, and 0.001 μg). This was reflected by a reduced total number of eosinophils in BAL fluid ($P < 0.05$) (Figure 1A).

The Effect of Cpn60.1 on Cytokine Release

The levels of IL-5 quantified in the BAL fluid obtained from OVA-immunized mice 24 hours after OVA challenge was significantly higher compared with nonsensitized Sham mice (OVA, 43.6 ± 8.6 pg/ml versus Sham, below detection limits; $n = 10$–18; $P < 0.05$) (Figure 1B). IL-5 release was significantly reduced in animals treated with Cpn60.1 (1, 0.1, 0.01, and 0.001 μg) where levels of IL-5 were reduced below the detection limit of the assay (Figure 1B). Analysis of IL-12 levels in BAL fluid revealed an increase in the levels of this cytokine obtained from OVA-immunized and challenged mice compared with nonsensitized sham mice (OVA, 50.1 ± 24.1 pg/ml versus Sham, below detection limits; $n = 6$–10; $P < 0.05$) (Figure 1C). The levels of IL-12 were further

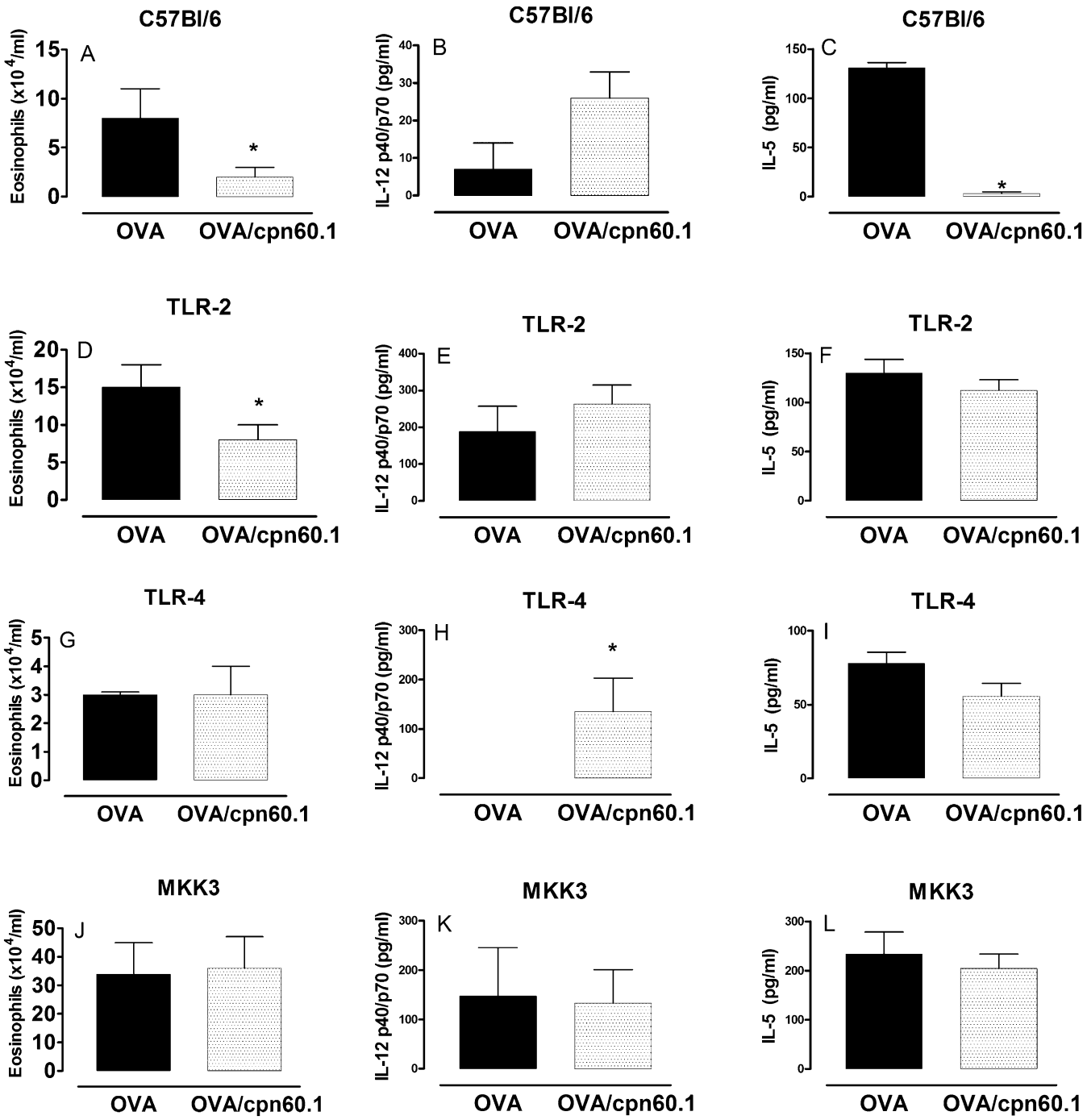

Figure 4. Effect of Cpn60.1 on the number of eosinophils (*A*, *D*, *G*, and *J*), IL-12 levels (*B*, *E*, *H*, and *K*), and IL-5 levels (*C*, *F*, *I*, and *L*) in BAL fluid collected 24 hours after OVA challenge in OVA-immunized mice (OVA) and OVA-immunized treated with Cpn60.1 (1 μg/ml intranasally; OVA/cpn60.1) in C57Bl/6, TLR-2, TLR-4, and MKK3 KO mice. *Vertical lines* represent SEM of 5 to 8 animals per group. *$P < 0.05$ for the ova group.

increased in animals treated with Cpn60.1 in comparison with OVA-immunized mice, reaching statistical significance with the 0.1 and 0.01 μg doses of Cpn60.1 ($P < 0.05$) (Figure 1C). Levels of total IgE present in the serum collected from OVA-immunized mice 24 hours after OVA challenge were significantly higher compared with nonsensitized sham mice (OVA, 326.2 ± 25.8 pg/ml versus Sham, 174.5 ± 21.7 pg/ml; $n = 6$–9; $P < 0.05$). The levels of IgE were diminished in animals treated with Cpn60.1 in comparison with OVA-immunized mice, although this failed to achieve statistical significance ($P > 0.05$) (Figure 1D).

The Effect of Cpn60.1 on Eosinophil Migration into the Lung Tissue

Histological analysis of lung tissue collected from OVA-immunized mice showed an intense accumulation of eosinophils in the peribronchial area in comparison with sham-immunized mice (Figures 2A and 2B). Lung tissue collected from animals treated with a 1-μg dose of Cpn60.1 showed a mild accumulation of eosinophils in the perivascular area but not around the airways (Figure 2C). Animals treated with the 0.001-μg dose of Cpn60.1 showed an accumulation of eosinophils in the peribronchial area similar to

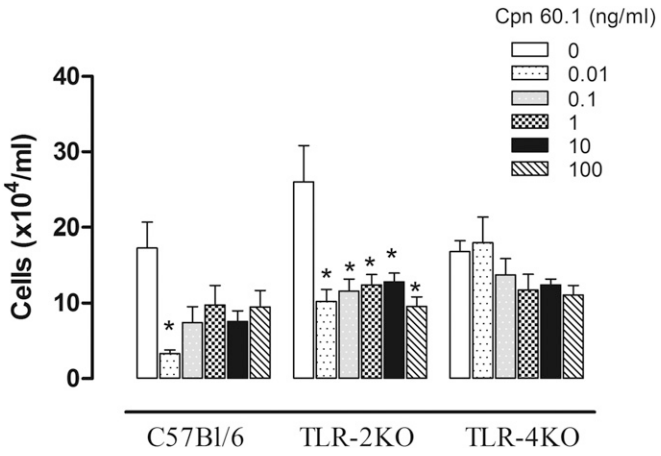

Figure 5. Effect of Cpn60.1 on leukocyte chemotaxis *in vitro*. Bone marrow cells were collected from C57Bl/6, TLR-2, and TLR-4 KO mice previously immunized with OVA. Cells were pretreated with Cpn60.1 (0.01–100 ng/ml) in log increments for 15 minutes in the presence of IL-5 (5 ng/ml). Migration in response to eotaxin (1 ng/ml) was measured after 3 hours of incubation at 37°C. Results were tested in duplicate and each column represents the mean of three experiments. *$P < 0.05$ for untreated cells.

nontreated OVA-immunized mice (Figure 2D), although cell numbers were significantly lower in BAL fluid (Figure 1A).

The Effect of Cpn60.1 on Bronchial Hyperresponsiveness

Twenty-four hours after the last OVA challenge, there was a significant increase in BHR in response to methacholine in OVA-sensitized mice (Figure 3), which was reflected by an increase in airway reactivity (peak response; Figure 3A) and sensitivity (PC150; Figure 3B) compared with nonsensitized sham mice. Treatment with 1 μg of Cpn60.1 significantly inhibited the increase in sensitivity and reactivity to methacholine (Figures 3A and 3B) compared with untreated OVA-sensitized and OVA-challenged mice. In contrast, treatment with 1 ng of Cpn 60.1 did not significantly alter the bronchial responsiveness to methacholine (Figures 3A and 3B).

The Effect of Cpn60.1 on Eosinophil Migration to the Lung Lumen in C57Bl/6, TLR-2, TLR-4, and MKK3 KO mice

The total number of cells quantified in BAL fluid obtained from C57Bl/6 OVA-immunized mice 24 hours after OVA challenge was increased compared with nonsensitized sham mice (OVA, $61.1 \pm 11.8 \times 10^4$ cells/ml versus Sham, $26.1 \pm 2.6 \times 10^4$ cells/ml; $n = 5$). This was reflected by a significantly higher number of eosinophils recruited to the airways in OVA mice ($7.5 \pm 2.1 \times 10^4$ cells/ml) versus Sham mice ($0.01 \pm 0.03 \times 10^4$ cells/ml; $P < 0.05$). Treatment with Cpn60.1 did not significantly alter the total number of cells in the lung of OVA-immunized C57Bl/6 mice ($72.2 \pm 11 \times 10^4$/ml) but significantly reduced the number of eosinophils in BAL fluid ($P = 0.018$) (Figure 4A). The total number of cells in TLR-2, TLR-4, and MKK3 KO mice was not significantly altered by Cpn60.1 compared with OVA-sensitized mice (data not shown) but significantly reduced the number of eosinophils in the lungs of TLR-2 KO mice ($P = 0.001$) (Figure 4D). However, the inhibitory effect of Cpn60.1 was absent in TLR-4 KO and MKK3 KO mice where there was no significant difference in the numbers of eosinophils recruited to the lung between treated and untreated groups ($P > 0.05$) (Figures 4G and 4J).

The Effect of Cpn60.1 on Cytokine Release in C57Bl/6, TLR-2, TLR-4, and MKK3 KO Mice

The levels of IL-5 quantified in the BAL fluid obtained from C57Bl/6 OVA-immunized mice 24 hours after OVA challenge was significantly higher compared with nonsensitized Sham mice. IL-5 release was significantly reduced in animals treated with 1 μg of Cpn60.1 (Figure 4C). Similarly, the levels of IL-5 in OVA-treated TLR-2 KO and MKK3 KO mice were not significantly different between Cpn60.1-treated mice (Figures 4F and 4L, respectively). In TLR-4 KO mice, there was no significant difference in the levels of IL-5 between treated and untreated groups (Figure 4I).

Analysis of IL-12 levels in BAL fluid revealed an increased level in C57Bl/6 OVA-immunized mice pretreated with 1 μg of Cpn60.1 compared with untreated OVA animals (Figure 4B). Analysis of IL-12 release in TLR-2 KO and MKK3 KO mice showed no significant difference between OVA-sensitized mice and OVA-sensitized mice pretreated with Cpn60.1 (Figures 4E and 4K, respectively). However, a significant difference in levels of IL-12 was detected between TLR-4 KO OVA-sensitized mice and OVA-sensitized mice pretreated with Cpn60.1 ($P = 0.015$) (Figure 4H).

The Effect of Cpn60.1 in Cell Chemotaxis *In Vitro*

To investigate whether Cpn60.1 directly affected cell migration, BM cells from OVA-immunized mice were tested for their ability to migrate in response to eotaxin *in vitro*. Cells obtained from OVA-sensitized C57/Bl6 mice did not migrate in response to medium alone. However, cells migrated positively in response to eotaxin (1 ng/ml) (Figure 5). In contrast, pretreatment of BM cells with 0.01 ng/ml of Cpn60.1 significantly inhibited this positive migration (Figure 5). A similar response was observed with BM cells collected from TLR-2 KO mice where all concentrations of Cpn60.1 significantly reduced cell chemotaxis *in vitro* (100–0.01 ng/ml) (Figure 5). Although BM cells collected from TLR-4 KO mice were capable of migrating in response to eotaxin, pretreatment with Cpn60.1 did not significantly alter this response (Figure 5).

The Effect of Cpn60.1 in Cell Migration *In Vivo*

We used intravital microscopy of the vasculature of the cremaster muscle to investigate whether Cpn60.1 altered the initial steps of the transmigration process (Figure 6). There were no significant signs of leukocyte rolling or arrest within the cremaster muscle microvasculature of OVA-sensitized mice treated with saline subcutaneously (Figure 7A and Video E1 in the online supplement). Four hours after subcutaneous administration of 100 ng/ml of eotaxin, a pronounced arrest and accumulation of cells outside the vessels was observed (Figures 6A–6H and 7B and Video E2). The accumulation of cells in the extravascular tissue of C57Bl/6 and TLR-2 KO mice pretreated with 1 μg of Cpn60.1 injected into the muscle was significantly reduced (Figures 6A, 6B, and 7C and Video E3). Similarly, C57Bl/6 and TLR-2 KO mice showed a significantly increased adhesion of cells to the vessel wall when compared with untreated mice (Figures 6D and 6E). In contrast, treatment with Cpn60.1 did not significantly alter cell accumulation in the extravascular tissue or rolling or adhesion of the cells in TLR-4 KO mice (Figures 6C, 6F, 6I and 7D and Video E4).

Effect of Cpn60.1 on VCAM-1 and VE-Cadherin Expression in Lung Tissue

To investigate whether the effect of Cpn60.1 on cell migration observed *in vivo* is mediated by changes to adhesion protein expression in the lung vascular tissue, we stained frozen lung

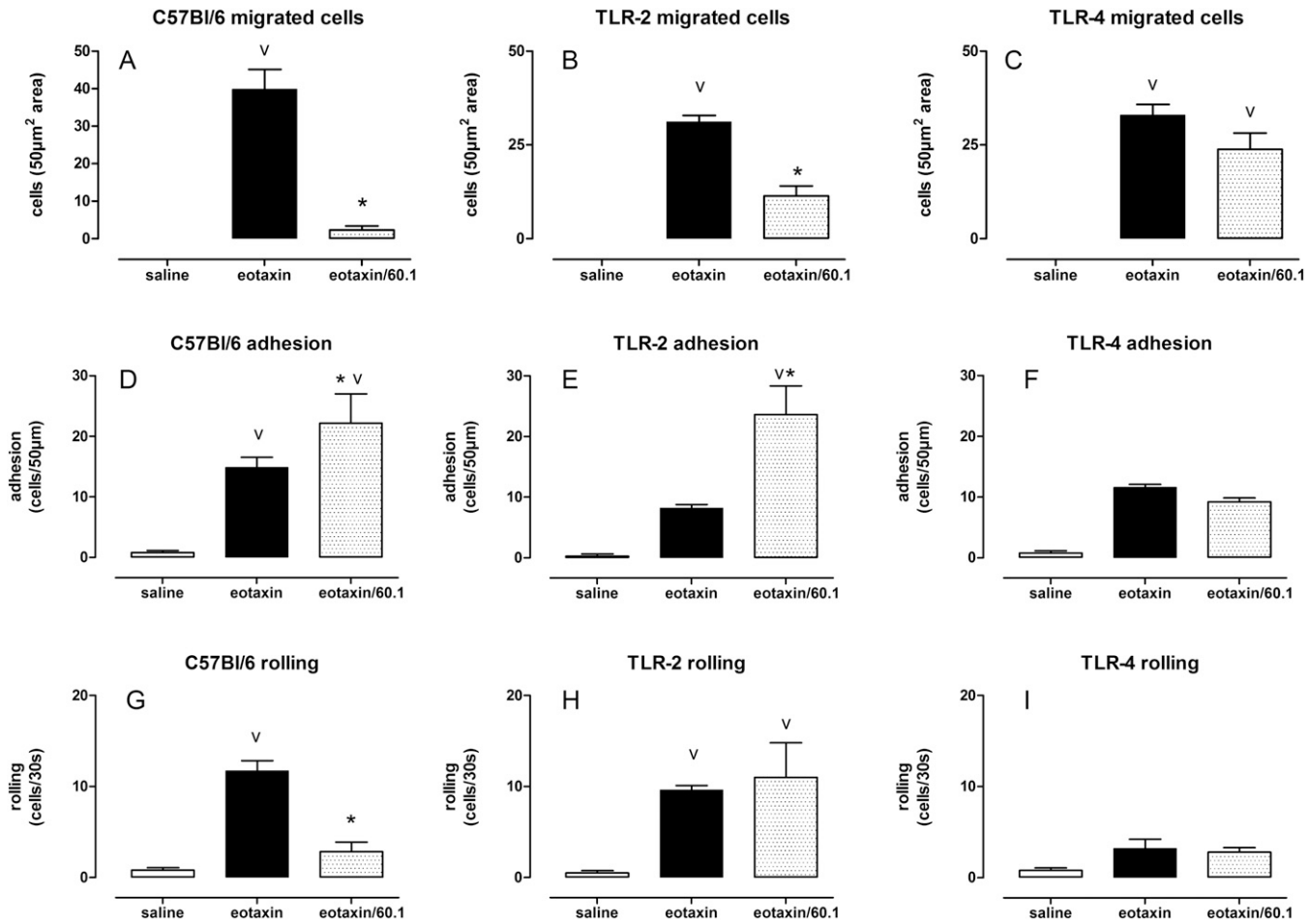

Figure 6. Effect of Cpn60.1 on cell migration *in vivo*. Intravital examination of recruitment of cells in response to eotaxin injected on the cremaster muscle of C57Bl/6 (*A*, *D*, and *G*), TLR-2 KO mice (*B*, *E*, and *H*), and TLR-4 KO mice (*C*, *F*, and *I*). *Bars* represent the average of rolling, adhesion, and cell counting of three animals per group, three recording per animal. $^{*}P < 0.05$ for the untreated mice. $^{V}P < 0.05$ for the saline-treated mice.

sections collected from Sham-sensitized and OVA-sensitized C57Bl/6 mice with antibodies against PECAM-1, VCAM-1, and VE-cadherin. Sensitization and challenge with OVA increased the expression of VCAM-1 in the lung vascular tissue in comparison to Sham-sensitized animals (Figure 8A). In contrast, the expression of VCAM-1 was significantly reduced in allergic mice pretreated with 1 μg of Cpn60.1 in comparison to untreated allergic mice (Figure 8A). VE-cadherin expression was reduced in lung tissue collected from OVA-sensitized in comparison to Sham-sensitized mice (Figure 8B). However, pretreatment with Cpn60.1 significantly enhanced the expression of VE-cadherin (Figure 8B). PECAM-1 expression was not detected in any of the samples analyzed.

DISCUSSION

We have previously demonstrated that Cpn60.1 from *M. tuberculosis* inhibited the migration of eosinophils into the lung, Th2 cytokine release, and BHR in a murine model of allergic inflammation (10). The mechanisms responsible for these effects remained to be established. In the present study, we show that Cpn60.1 inhibited eosinophil migration and IL-5 release in a murine model of allergic inflammation in the lung at lower concentrations than previously described. In fact, Cpn60.1 inhibited eosinophil migration and cytokine release in the lung at concentrations as low as 1 ng per dose. The histological analysis of the tissue shows for the first time that Cpn60.1 treatment of mice not only inhibited the migration of eosinophils into the lung lumen but also inhibited the migration of these cells from the perivascular area to the peribronchial area. However, histological analysis of the tissue also shows that, although 1 ng of Cpn60.1 significantly inhibits the migration of eosinophils from the airways to the lung lumen, it does not inhibit the migration of the cells from the vessels to the peribronchial area in the tissue, suggesting that the concentration of Cpn60.1 *in situ* is an important factor contributing to the antiinflammatory effect of this chaperonin and that Cpn60.1 may also influence the migration of leukocytes through tissues. It also suggests dissociation between the effect of this chaperonin on transendothelial and transepithelial cell migration. The effect of this chaperonin on cell migration observed in the tissue correlates with the effect observed on BHR. Treatment with 1 μg, but not 1 ng, of Cpn60.1 was effective in inhibiting sensitivity and reactivity of the airways to methacholine, suggesting dissociation at least between eosinophil numbers in BAL and BHR. Furthermore, although we have lowered the concentration of Cpn60.1 1,000-fold between 1 μg and 1 ng, we did not observe a dose–response effect of this chaperonin in our *in vivo* model. A possible explanation for this phenomenon is that we have achieved a maximal effect, even at these low concentrations. We could have used picogram amounts of this protein to establish submaximal effects on eosinophil recruitment. However, we did show that 1 ng of Cpn60.1 failed to protect against bronchial hyperresponsiveness, whereas a higher dose (1 μg) was effective (Figure 3).

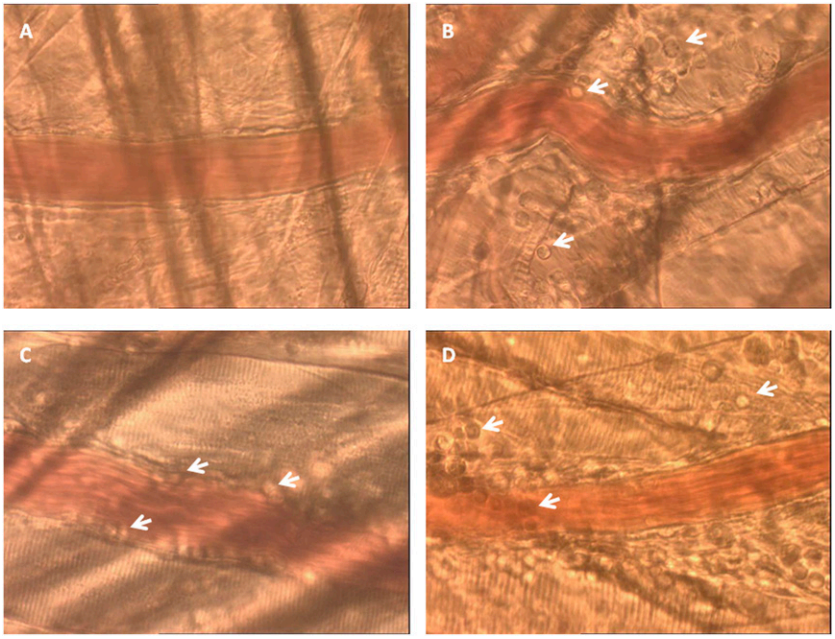

Figure 7. Photographic stills of cremaster muscle microvessels, corresponding to Videos E1 through E4. (*A*–*C*) Images were recorded using C57Bl/6 mice treated with saline (*A*) or 100 ng of eotaxin (subcutaneously) (*B*) or pretreated with 1 μg of Cpn60.1 (*C*) and TLR-4 KO mice pretreated with Cpn60.1 (subcutaneously) (*D*). *White arrows* highlight leukocytes outside and inside the vascular tissue.

Cpn60s are powerful inducers of a number of cytokines *in vitro*, including IL-12, IL-1, IL-4, IL-6, IL-8, IFN-γ, and TNF-α (9). Our study shows for the first time that Cpn60.1 can induce IL-12 release *in vivo*. Moreover, in this study we have demonstrated that the effect of Cpn60.1 on cell accumulation in the lung *in vivo* is dependent on TLR-4 and partially dependent on TLR-2, confirming previous observations made *in vitro* (7). More importantly, we have demonstrated that TLR-4 KO mice pretreated with Cpn60.1 are able to produce significant levels of IL-12 when challenged with OVA, whereas the effect on eosinophil migration to the lung is abolished. These observations strongly suggest that, although Cpn60.1 induces IL-12 release *in vivo*, this is not the mechanism by which this chaperonin inhibits cell migration. Moreover, the inability of TLR-2 KO mice to produce significantly higher levels of IL-12 when pretreated with Cpn60.1 suggests that the effect of this chaperonin on IL-12 release *in vivo* is dependent on TLR-2 but not on TLR-4. The inability of MKK3 KO mice to respond to Cpn60.1 also suggests that these effects are dependent on p38 phosphorylation. MKK3 is upstream of p38MAPK and is activated by mitogenic and environmental stress and participates in the MAPK-mediated signaling cascade. MKK3 phosphorylates and thus activates

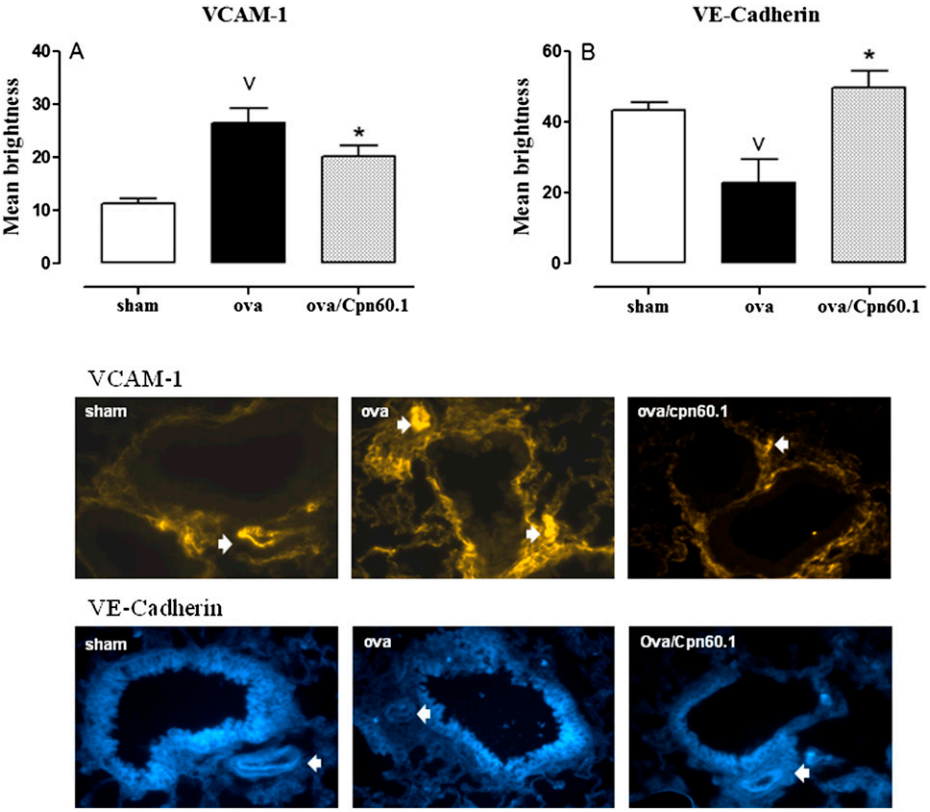

Figure 8. Analysis of VCAM-1 (*A*) and VE-cadherin (*B*) expression by immunohistochemistry staining of frozen lung tissue collected from C57Bl/6 sensitized and challenged with OVA. Photographs show expression of VCAM-1 and VE-cadherin in Sham-sensitized animals, OVA-sensitized animals, and OVA-sensitized animals pretreated with 1 μg of Cpn60.1. Values express mean ± SEM of three mice per group. $^{*}P = 0.003$ ova for ova/Cpn60.1 in *graph A*; $^{*}P = 0.0006$ ova for ova/Cpn60.1 in *graph B*. $^{V}P < 0.05$ for the sham group.

MAPK14/p38-MAPK (13). Our findings support previous observations made *in vitro* where exposure of human monocytes to Cpn60.1 caused rapid activation of p38 with kinetics of phosphorylation comparable to those observed in response to LPS (14).

The histological analysis of lung tissue obtained from OVA-challenged mice pretreated with 1 μg of Cpn60.1 shows a decreased accumulation of cells around the peribronchial tissue, suggesting an effect of this chaperonin on transendothelial cell migration. This idea was supported by our observations using intravital microscopy of the microvasculature of the cremaster muscle from OVA-sensitized mice. Our results demonstrated the ability of Cpn60.1 to alter cell migration *in vivo* by a TLR-4–dependent, but not a TLR-2–dependent, mechanism. These results were confirmed *in vitro* where Cpn60.1 inhibited cell migration in a chemotaxis assay by a TLR-4–dependent mechanism, suggesting that this chaperonin has the ability to directly affect leukocyte movement.

To investigate whether Cpn60.1 inhibits cell migration by a mechanism involving the expression of adhesion molecules, we stained frozen lung sections with fluorescent markers specific for VCAM-1, PECAM-1, and VE-cadherin. Our results show that pretreatment with Cpn60.1 inhibits expression of VCAM-1, but not PECAM-1, in the lung microvasculature of OVA-sensitized mice. This result supports previous findings showing that pretreatment of mice with BCG inhibited VCAM-1 expression in the lung microvasculature in a murine model of allergic lung inflammation that was associated with a decrease in eosinophil number and Th2 cytokine release (15). In contrast, we did not observe significant differences in the expression of PECAM-1 between groups. However, it has been shown that C57Bl/6 mice have the unique ability to compensate for the loss or blockade of this adhesion molecule expression, suggesting that our results might be strain specific (16). Our results also show that pretreatment with Cpn60.1 increases expression of VE-cadherin in the lung of OVA-sensitized compared with untreated mice. VE-cadherin is a strictly endothelial specific adhesion molecule located at junctions between cells and has been implicated in the preservation of the integrity of these junctions. Mechanisms that regulate VE-cadherin–mediated adhesion are important for the control of vascular permeability and leukocyte diapedesis (17). It has been recently shown that VE-cadherin can regulate eosinophils migration *in vitro* (18), but to our knowledge this is the first time that VE-cadherin expression has been implicated in the anti-inflammatory effect of chaperonins *in vivo*.

In conclusion, our findings show that Cpn60.1 can exert an anti-inflammatory action in the lung by inhibiting cell diapedesis by a TLR-4– and cell adhesion molecule–dependent mechanism.

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

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