Osteopontin Impairs Host Defense During Pneumococcal Pneumonia

Gerritje J. W. van der Windt,1,2 Arie J. Hoogendijk,1,2 Marcel Schouten,1,2 Tijmen J. Hommes,1,2 Alex F. de Vos,1,2 Sandrine Florquin,3 and Tom van der Poll1,2

1Center for Infection and Immunity Amsterdam (CINIMA), 2Center for Experimental and Molecular Medicine, 3Department of Pathology, Academic Medical Center, University of Amsterdam, the Netherlands

Background. Streptococcus pneumoniae is the most frequently isolated pathogen responsible for community-acquired pneumonia. Osteopontin is involved in inflammation during both innate and adaptive immunity.

Methods. To determine the role of osteopontin in the host response during pneumococcal pneumonia, osteopontin knockout (KO) and normal wild-type (WT) mice were intranasally infected with viable S. pneumoniae.

Results. Pneumonia was associated with a rapid increase in pulmonary osteopontin concentrations in WT mice from 6 h onward. Osteopontin KO mice showed a prolonged survival relative to WT mice, which was accompanied by diminished pulmonary bacterial growth and reduced dissemination to distant body sites. In addition, at 48 h after infection pulmonary inflammation was decreased in osteopontin KO mice as reflected by lower inflammation scores and reduced chemokine concentrations. In contrast to pneumococcal pneumonia, osteopontin deficiency did not influence bacterial growth in primary pneumococcal sepsis induced by direct intravenous infection, suggesting that the detrimental effect of osteopontin on antibacterial defense during pneumonia primarily is exerted in the pulmonary compartment. Moreover, recombinant osteopontin stabilized S. pneumoniae viability in vitro.

Conclusions. These results suggest that the pneumococcus misuses osteopontin in the airways for optimal growth and to cause invasive disease after entering the lower airways.

Bacterial pneumonia is a common and serious illness that is a leading cause of morbidity and mortality. Streptococcus pneumoniae (the pneumococcus) is the most frequently isolated pathogen responsible for community-acquired pneumonia [1, 2], and in recent sepsis trials this gram-positive bacterium was an important causative organism, especially in the context of pneumonia [3]. This, together with the increasing incidence of antibiotic resistance in this pathogen, stresses the importance of expanding our knowledge of the host defense mechanisms that influence the outcome in pneumococcal pneumonia [1, 4].

Osteopontin is a phosphorylated glycoprotein, expressed by a broad range of tissues and cells, that has been implicated as an important regulator of inflammation [5–7]. Osteopontin has an important role in innate and adaptive immunity by mediating inflammatory cell differentiation, maturation and migration, and cytokine production [5, 7–11]. Osteopontin especially seems to be involved in lung inflammation, as patients suffering from diverse pulmonary diseases, including interstitial pneumonia, tuberculosis, silicosis, and sarcoidosis, have displayed enhanced osteopontin expression in their lungs [12–16], whereas patients with idiopathic pulmonary fibrosis have shown increased osteopontin levels in bronchoalveolar lavage fluid (BALF) [17]. Furthermore, plasma osteopontin levels were dramatically elevated in patients with interstitial pneumonia [18] and in patients with sepsis, of whom almost half suffered from pneumonia as the primary site of infection [19]. Moreover, a functional role for
Osteopontin has been described in several experimental models of lung disease, including allergy and asthma [20–22], acute respiratory distress syndrome [16], and fibrosis [23, 24].

Considering the association between lung disease and sepsis on the one hand and pulmonary and systemic expression of osteopontin on the other hand, we here sought to determine the potential role of osteopontin in the host response during pneumococcal pneumonia.

MATERIALS AND METHODS

Mice
Nine- to twelve-week-old C57BL/6 wild-type (WT) mice were purchased from Harlan Sprague Dawley Inc. Osteopontin knockout (KO) mice (Jackson Laboratories) of a C57BL/6 genetic background were bred in the animal facility of the Academic Medical Center.

Study Design
The Animal Care and Use Committee of the University of Amsterdam approved all experiments. Pneumonia was induced by means of intranasal inoculation of 10^4 colony-forming units (CFU) of *S. pneumoniae* serotype 3 (ATCC 6303; American Type Culture Collection), as described elsewhere [25, 26]. Sepsis was induced by intravenous injection in the tail vein of 5 × 10^6 CFU of the same *S. pneumoniae* strain. Sample harvesting and processing, and determinations of bacterial loads and cell counts were performed as described elsewhere [25, 26]. For survival studies, mice were monitored for 4 d after infection.

Cell Cultures
In vitro stimulation of alveolar macrophages and respiratory epithelial cells was performed as described previously [27], using 5 × 10^4 MLE-15 cells and heat-killed *S. pneumoniae* (multiplicity of infection [MOI], 1:2, 1:20, and 1:200), 5 × 10^4 MH-S cells and growth-arrested *S. pneumoniae* (by mitomycin C, 50 μg/mL; Sigma; MOI, 1:6 and 1:60), or 1 × 10^6 primary alveolar macrophages and growth-arrested *S. pneumoniae* (by mitomycin C, 50 μg/mL; Sigma; MOI, 1:20 and 1:200). Supernatants were taken after 4 h of stimulation for MH-S cells and after 20 h of stimulation for primary alveolar macrophages and MLE-15 cells and stored at –20°C until assayed for osteopontin.

Assays
Osteopontin, myeloperoxidase, interleukin 1β, interleukin 4, interleukin 5, interleukin 13, interleukin 17, interleukin 33, keratinocyte-derived cytokine (KC), and macrophage inflammatory protein 2 (MIP-2) were measured by means of enzyme-linked immunosorbent assay (ELISA) (myeloperoxidase, Hycult; all others, R&D Systems). Monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor α (TNF-α), interleukin 6, interferon γ (IFN-γ), interleukin 12, and interleukin 10 were measured by means of cytometric bead array multiplex assay (BD Biosciences).

Histopathology
Paraffin lung sections were stained with hematoxin and eosin and semiquantitatively scored as described elsewhere [25].

Effect of Osteopontin on *S. pneumoniae* Viability, Phagocytosis, and Phagolysosomal Fusion
*S. pneumoniae* or *Staphylococcus* (S.) aureus (Newman strain) (1 × 10^6 bacteria/mL) was incubated in sterile normal saline in the presence of 0.8–800 ng/mL recombinant mouse osteopontin (rOPN; <1.0 endotoxin units per 1 μg as determined by the LAL assay; R&D Systems), 800 ng/mL boiled rOPN (30 min at 100°C), 800 ng/mL bovine serum albumin (BSA), or normal saline only at 37°C for 6 h. At indicated time points the number of bacteria was determined. Neutrophil phagocytosis and phagolysosomal fusion were determined as described elsewhere [28–30]. To determine the impact of alveolar macrophages on the growth of pneumococci, viable *S. pneumoniae* bacteria (5 × 10^6 CFU) were incubated with alveolar macrophages (1 × 10^5 cells), harvested from osteopontin KO and WT mice, for 2 h. Thereafter, the number of remaining live *S. pneumoniae* bacteria was determined.

Binding Assay
The capacity of osteopontin to bind *S. pneumoniae* was assessed in an ELISA-based assay. Microlon plates (Greiner Bio–one) were coated overnight at 4°C with growth-arrested 1 × 10^8 CFU/mL *S. pneumoniae* serotype 3 (ATCC 6303) or D39 (serotype 2) in phosphate-buffered saline (PBS), with PBS only (negative control), or with goat anti–mouse osteopontin (0.8 μg/mL in PBS; R&D Systems; positive control). Plates were blocked with 3% BSA/Tris-buffered medium (20 mM Tris, 150 mM NaCl, 1 mM CaCl_2, and 2 mM MgCl_2) for 1 h at room temperature. Biotin-labeled rOPN (R&D systems; 2 μg/mL) was added and incubated for 1 h at room temperature. Binding was detected with streptavidin conjugated with horseradish-peroxidase.

Statistical Analysis
All data are expressed as mean ± standard error of the mean except for bacterial counts, which are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. Comparisons for 2 groups were performed with the Mann-Whitney U test; comparisons for more than 2 groups, with the Kruskall Wallis followed by the Dunn test for multiple comparisons; survival, by means of Kaplan-Meier analysis followed by log-rank test; and comparisons of the numbers of positive and negative culture results, by means of the χ^2 test. A value of *P* < .05 was considered to reveal a statistically significant difference.

RESULTS

Osteopontin Levels Increase During *S. pneumoniae* Pneumonia
To obtain a first insight into a potential role for osteopontin during pneumococcal pneumonia, we measured osteopontin...
concentrations in lungs and plasma from WT mice at indicated time points after infection. Osteopontin concentrations in lungs were significantly elevated at 6 h, compared with baseline, and further increased at 48 h after infection (Figure 1A). Plasma levels increased more slowly and were significantly elevated only at 48 h after infection, compared with baseline (Figure 1B). To determine which pulmonary cells could release osteopontin upon stimulation with *S. pneumoniae*, we incubated alveolar macrophages and lung epithelial cells with *S. pneumoniae*. Osteopontin release by the alveolar macrophage cell line MH-S (Figure 1C), as well as by primary alveolar macrophages (Figure 1D), was enhanced upon *S. pneumoniae* stimulation, as compared with medium (both \( P < .05 \)). Lung epithelial cells constitutively released osteopontin, as described earlier [27, 29], which, however, was not further increased upon *S. pneumoniae* stimulation (data not shown).

Osteopontin Negatively Affects Mortality and Pulmonary Bacterial Growth During Pneumococcal Pneumonia

To investigate a potential role for osteopontin during the host response against pneumococci, we infected WT and osteopontin KO mice with a lethal dose of *S. pneumoniae* and followed them for 4 days. Osteopontin deficiency significantly prolonged survival: the median survival time was 61.5 h for WT mice, compared with 70.5 h for osteopontin KO mice (Figure 2A; \( P < .01 \)). Next, we wondered whether osteopontin deficiency affects bacterial growth in lungs, and therefore we infected WT and osteopontin KO mice with *S. pneumoniae*. At 6 h, osteopontin KO mice displayed significantly decreased bacterial loads in their lungs, compared with WT mice (Figure 2B; \( P < .01 \)). Although at 24 h pulmonary bacterial loads were similar in both groups, beyond this time point the number of pneumococci in lung homogenates only grew in WT mice and at 48 h after infection bacterial burdens were approximately 200-fold higher in WT mice than in osteopontin KO mice (\( P < .001 \)). Remarkably, whereas none of the mice in either group displayed positive blood or spleen culture results 6 h after infection, osteopontin KO mice displayed a significantly diminished dissemination of the infection at 24 h as reflected by the fact that *S. pneumoniae* could be cultured from the blood of only 3 of 8 osteopontin KO mice, compared with 7 of 8 WT mice, and from the spleen of only 1 of 7 osteopontin KO mice, compared with 7 of 8 WT mice (\( P < .05 \) and \( P < .01 \), respectively). In concurrence with the data on pulmonary growth, bacterial loads in the blood and spleen were further enhanced at 48 h after infection, but to a significantly lesser extent in osteopontin KO mice as compared with WT mice (Figure 2C-D; \( P < .001 \) and < .05, respectively). Together, these results suggest that osteopontin facilitates bacterial growth and early dissemination during severe pneumococcal pneumonia, which might explain the delayed mortality of osteopontin KO mice.

Reduced Pulmonary Inflammation in Osteopontin KO Mice

The extent of lung inflammation was determined using the semiquantitative scoring system described above. Already at 6 h after infection, all mice displayed mild pulmonary inflammation.
that slightly increased toward 24 h; at these early time points, the extent of lung inflammation was similar in both groups (Figure 3A–F). At 48 h, pulmonary inflammation had strongly increased in both groups; however, at this late time point the extent of lung pathology was much more severe in WT mice than in osteopontin KO mice, which was especially due to more endothelialitis and bronchitis and an enhanced percentage of the lung that was affected (Figure 3G–I; \(P < .01\), compared with WT).

In line, osteopontin KO mice showed significantly lower lung weights at 48 h after infection (0.20 ± 0.01 g vs 0.26 ± 0.01 g in WT mice; \(P < .01\)), supporting our data on reduced pulmonary inflammation in the absence of osteopontin.

Osteopontin Deficiency Does Not Influence Neutrophil Recruitment

Osteopontin has been shown to be chemotactic for neutrophils in vitro and in vivo [11, 31–34]. Considering the role of neutrophils in an adequate host response to \(S. pneumoniae\) [35–37], we determined neutrophil influx into the lungs of osteopontin KO and WT mice after intranasal infection with \(S. pneumoniae\) by quantifying the number of neutrophils in BALF obtained 6 h after infection and by measuring myeloperoxidase concentrations in whole lung homogenates harvested at 6, 24, or 48 h. Neutrophil counts were similar in the BALF of osteopontin KO and WT mice at 6 h (0.48 ± 0.15 cells/mL vs 0.79 ± 0.32 \(\times 10^3\) cells/mL; \(P > .05\)); likewise, lung myeloperoxidase levels were not significantly different between mouse strains (data not shown).

Cytokine and Chemokine Levels During Pneumococcal Pneumonia

Cytokines and chemokines play an important role in host defense during bacterial pneumonia [1, 38]. Therefore, we measured the concentrations of T helper 1 cytokines (IFN-\(\gamma\) and interleukin 12), T helper 2 cytokines (interleukin 4, interleukin 5, interleukin 13, and interleukin 33), proinflammatory cytokines (TNF-\(\alpha\), interleukin 1\(\beta\), interleukin 6, and interleukin 17), the anti-inflammatory cytokine interleukin 10, and chemokines (KC, MIP-2, and MCP-1) in lungs. At 6 h after infection, all mediators were similar in WT and osteopontin KO mice or below detection limits (data not shown). Similarly, at 24 h no differences were found between the 2 groups in the pulmonary levels of any of these mediators (Table 1). Whereas lung cytokine levels remained similar in both mouse strains at 48 h, except for interleukin 33 levels that were increased in the lungs of osteopontin KO mice (\(P < .05\)), chemokine levels were decreased in lungs from osteopontin KO mice, compared with WT mice (Table 1; KC, \(P = .06\); MIP-2, \(P < .01\); and MCP-1, \(P < .05\)). In addition, we measured the concentrations of TNF-\(\alpha\), interleukin 6, IFN-\(\gamma\), and MCP-1 in plasma and spleen tissue. The concentrations of these mediators were similar in WT and osteopontin KO mice at 24 h; however, at 48 h after infection,
Osteopontin KO mice displayed strongly decreased concentrations of all 4 mediators in plasma (Table 1) and spleen tissue (data not shown).

Osteopontin Stabilizes *S. pneumoniae* Viability In Vitro

The reduced bacterial outgrowth in the absence of osteopontin might be explained by an effect of osteopontin on the phagocytosis capacity of or phagolysosomal fusion in osteopontin KO neutrophils or by a direct effect of osteopontin on *S. pneumoniae*. Phagocytosis capacity and phagolysosomal fusion were not altered in osteopontin KO neutrophils, compared with WT neutrophils (data not shown). Considering the reduced bacterial loads in the lungs of osteopontin KO mice early after infection, we determined the impact of alveolar macrophages on the growth of pneumococci. For this we incubated viable *S. pneumoniae* with osteopontin KO and WT alveolar macrophages for 2 h and determined the number of pneumococci thereafter. Alveolar macrophages did not kill the pneumococcal strain used as reflected by unchanged bacterial counts at 2 h relative to 0 h; bacterial counts detected at 2 h of culture did not differ between osteopontin KO and WT macrophages (1.2 ± 0.07 × 10^5 bacteria/mL vs 1.1 ± 0.04 × 10^5 bacteria/mL, respectively).

To establish a possible direct effect of osteopontin on pneumococcal growth, we incubated *S. pneumoniae* in sterile normal saline at a concentration similar to the inoculum that we used for infection experiments for 1–6 h in the presence or absence of increasing concentrations of rOPN (0.8–800 ng/mL) and determined the viability of the bacteria. The number of viable pneumococci decreased over time when incubated in normal saline only. We found that 800 ng/mL of rOPN stabilized the viability of *S. pneumoniae*, whereas lower osteopontin concentrations had no effect (Figure 4A). In a separate experiment, we confirmed the effect of rOPN on the viability of *S. pneumoniae* and further showed that boiled osteopontin or BSA did not exert
such an effect (Figure 4B). These data suggest that osteopontin serves as a growth factor for S. pneumoniae in vitro. Recently, osteopontin was demonstrated to bind to Staphylococcus aureus and Streptococcus agalactiae [39]. Therefore, we were interested to determine the capacity of osteopontin to bind S. pneumoniae. For this we coated ELISA plates with either S. pneumoniae ATCC 6303 (the serotype 3 strain used in the in vivo studies) or S. pneumoniae D39 (serotype 2) and assessed their capacity to bind biotinylated rOPN. Both pneumococcal strains were found capable of binding osteopontin (both P < .01 vs buffer; Figure 4C). To establish whether osteopontin binding to bacteria could be a generalized process for stabilization, we determined S. aureus viability in the presence of osteopontin. In this setting, however, these bacteria remained viable in the presence of saline only, and osteopontin did not affect this (data not shown).

**Osteopontin Does Not Impair the Immune Response Upon Intravenously Administered S. pneumoniae**

We wondered whether the detrimental effect of osteopontin on host defense against S. pneumoniae was primarily present in the lungs (with, as a consequence thereof, enhanced bacterial dissemination to distant organs) or also was demonstrable in the systemic compartment. To study this, we injected S. pneumoniae directly intravenously into osteopontin KO and WT mice, thereby bypassing the role of osteopontin in the lungs upon primary pulmonary infection. Osteopontin was detectable at relatively high concentrations in the circulation of uninfected WT mice, confirming the data presented in Figure 1B; intravenous S. pneumoniae injection resulted in a modest increase in the plasma concentrations of osteopontin, which did not reach statistical significance (at 0 h, 69 ± 6 ng/mL; at 48 h, 95 ± 11 ng/mL; P ≥ .05). Unlike the clear phenotype of osteopontin KO mice in pneumococcal pneumonia, osteopontin KO and WT mice displayed similar bacterial loads in the blood, lung, spleen, and liver at 24 and 48 h after intravenous administration of S. pneumoniae (Figure 5). In addition, TNF-α, interleukin 6, IFN-γ, and MCP-1 concentrations in plasma and spleen were indistinguishable between both mouse strains at both time points (data not shown). Taken together, these data suggest that the negative effect of osteopontin on the bacterial response during pneumococcal pneumonia is due to a pulmonary rather than a systemic effect of osteopontin.

**DISCUSSION**

To the best of our knowledge, the present study is the first to investigate the functional role of osteopontin during...
gram-positive bacterial pneumonia and sepsis. In pneumococcal pneumonia, osteopontin KO mice displayed a survival benefit and an improved antibacterial defense, as reflected by lower bacterial loads in their lungs, reduced dissemination to distant body sites, and less pulmonary inflammation. In contrast, during primary pneumococcal sepsis, osteopontin deficiency did not affect bacterial growth. We further showed that osteopontin stabilizes *S. pneumoniae* viability in vitro. Our data suggest that osteopontin facilitates the growth of pneumococci in the lungs after primary infection of the respiratory tract, subsequently resulting in enhanced dissemination of the infection.

Knowledge of the production of osteopontin during acute inflammatory diseases is limited. Recently, patients with bacterial pneumonia and sepsis. In pneumococcal pneumonia, osteopontin KO mice displayed a survival benefit and an improved antibacterial defense, as reflected by lower bacterial loads in their lungs, reduced dissemination to distant body sites, and less pulmonary inflammation. In contrast, during primary pneumococcal sepsis, osteopontin deficiency did not affect bacterial growth. We further showed that osteopontin stabilizes *S. pneumoniae* viability in vitro. Our data suggest that osteopontin facilitates the growth of pneumococci in the lungs after primary infection of the respiratory tract, subsequently resulting in enhanced dissemination of the infection.

Knowledge of the production of osteopontin during acute inflammatory diseases is limited. Recently, patients with bacterial
sepsis, who predominantly suffered from pneumonia, were reported to have elevated circulating levels of osteopontin [19]. Although several investigations revealed enhanced osteopontin expression during subacute and chronic pulmonary inflammation [12, 14–17, 21, 40], knowledge of osteopontin production during bacterial respiratory tract infection is more limited. Infection with Francisella novicida induced osteopontin mRNA in mouse lungs [41], whereas we observed elevated pulmonary levels of osteopontin during murine gram-negative pneumonia caused by either Klebsiella pneumoniae [29] or Burkholderia pseudomallei [27]. To date, studies on osteopontin levels during gram-positive bacterial infection have not been reported. We show here that airway infection with viable S. pneumoniae results in a rapid increase of osteopontin in lung tissue with a more gradual rise in plasma osteopontin levels. Moreover, in concurrence with previous studies reporting alveolar macrophages to secrete osteopontin during inflammation [5, 16, 42] and gram-negative bacterial or mycobacterial infection [27, 29, 43], we identified alveolar macrophages to be the most likely source of pulmonary osteopontin during pneumococcal pneumonia.

In this study we reveal that endogenous osteopontin impairs host defense during pneumococcal pneumonia. The experiments in which S. pneumoniae was injected directly into the circulation, revealing similar bacterial growth in osteopontin KO and WT mice in all body sites examined, suggest that the effect of osteopontin on pneumococcal infection primarily is exerted in the lungs. In accordance with this notion, bacterial loads in lung tissue of osteopontin KO mice with pneumococcal pneumonia were already reduced 6 h after infection, which at 24 h was associated with a clearly diminished spreading of pneumococci to the blood and spleen in these animals. Of note, at 24 h pulmonary bacterial burdens were similar in osteopontin KO and WT mice, whereas at 48 h bacterial loads were approximately 200-fold lower in the lungs of the former mouse strain. These data suggest that the innate immune system is unable to compensate for the apparently detrimental effect of osteopontin on the growth of S. pneumoniae in the lungs in the early phase of pneumococcal pneumonia (when the defense mechanism predominantly relies on resident cells such as alveolar macrophages and the respiratory epithelium), as well as in the late phase of the infection (when the growing bacterial load overwhelms innate defense mechanisms provided by infiltrating neutrophils).

Osteopontin did not have an effect on pulmonary inflammation during the early phase of pneumococcal pneumonia; however, at 48 h after infection less inflammation was present in the lungs of osteopontin KO mice. The attenuated inflammatory response in osteopontin KO mice at this late time point was also reflected in reduced chemokine levels in lung homogenates and lower plasma concentrations of cytokines and chemokines. Likely, the diminished local and systemic inflammatory response associated with osteopontin deficiency was the result of the much lower bacterial loads in the lungs and blood, providing a less potent proinflammatory stimulus. Indeed, earlier studies have demonstrated a clear positive correlation between the bacterial load and the extent of inflammation during pneumococcal pneumonia [44, 45]. Remarkably, osteopontin KO mice displayed higher interleukin 33 levels in lung homogenates at 48 h after infection. Additional studies are needed to investigate the effect of osteopontin on interleukin 33 release during severe infection.

Osteopontin has been shown to be chemotactic for neutrophils in vitro and in vivo [11, 31–34], and neutrophil recruitment to the lungs is essential for a protective host response against S. pneumoniae [35–37]. We found no evidence for a role of osteopontin in neutrophil influx into the lungs of mice with pneumococcal pneumonia, as reflected by similar myeloperoxidase levels in lung homogenates of WT and osteopontin KO mice throughout the infection and by similar neutrophil counts in BALF harvested from both mouse strains early (6 h) after infection [11]. Recently, it has been shown that osteopontin is able to bind to S. aureus and S. agalactiae, thereby mediating phagocytosis through α,β integrins on monocytes [39]. Although we here observed that osteopontin is also able to bind S. pneumoniae, our results reveal that, for phagocytosis of this pathogen by neutrophils, osteopontin is not crucial, as the assay was performed in whole blood in which in WT samples (but not in osteopontin KO samples) constitutive osteopontin was present. Interestingly, we found that osteopontin stabilizes the viability of S. pneumoniae in vitro. This effect was dose dependent and specific, as boiled osteopontin and BSA were not effective. Osteopontin concentrations used in vitro were higher than those measured in vivo; however, measurements of osteopontin in whole lung homogenates are unlikely to reflect osteopontin levels at sites where high numbers of pneumococci reside, especially since these bacteria themselves stimulate the release of osteopontin locally. Thus, although these data may not accurately reflect the in vivo situation and therefore should be interpreted with caution, these data suggest that osteopontin may be misused by the pneumococcus during infection of the lower airways.

In conclusion, we show here for the first time that osteopontin impairs the antibacterial response against pneumoccoci in the lungs, at least in part by stabilizing the viability of this gram-positive pathogen.

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