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Accumulation of Ym1 and formation of intracellular crystalline bodies in alveolar macrophages lacking heparanase

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ABSTRACT

Heparanase is a heparan sulfate (HS) degrading endoglucuronidase that has been implicated in cell migration and inflammatory conditions. Here we used mice deficient of heparanase (*Hpse*^{-/-}) to study the impact of heparanase on airway leukocytes. Normal numbers of macrophages and lymphocytes were present in bronchoalveolar lavage fluid of *Hpse*^{-/-} mice, indicating that heparanase is not essential for proper homing of leukocytes to airways. While lymphocytes from *Hpse*^{-/-} mice displayed normal morphology, *Hpse*^{-/-} alveolar macrophages showed a striking, age-dependent appearance of granule-like structures in the cytoplasm. Transmission electron microscopy revealed that these structures corresponded to membrane-enclosed crystalline bodies that closely resembled the intracellular crystals known to be formed by the HS-binding protein Ym1, suggesting that heparanase deficiency is associated with intracellular Ym1 deposition. Indeed, applying immunocytochemistry, we found markedly higher levels of intracellular Ym1 protein in *Hpse*^{-/-} versus *WT* alveolar macrophages, and there was a significant correlation between levels of Ym1 protein detected by immunoblotting and amounts of crystalline material in BAL cells. Biosynthetic radio-labeling of the macrophages revealed accumulation of non-degraded HS chains in *Hpse*^{-/-} alveolar macrophages. Together, these findings implicate heparanase in normal processing of HS in macrophages, and indicate that heparanase regulates intracellular Ym1 accumulation and crystal formation in airways.

Keywords: heparanase knockout mice; heparan sulfate; chitinase-like protein; alveolar macrophages; airway inflammation

Abbreviations: BAL, bronchoalveolar lavage; BMMØ, bone marrow-derived macrophage; DTH, delayed type hypersensitivity; ECM, extracellular matrix; GAG, glycosaminoglycan; *Hpse*, heparanase; HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; TEM, transmission electron microscopy.

1. Introduction

Heparan sulfate proteoglycans (HSPGs) consist of protein cores to which sulfated and thereby negatively charged glycosaminoglycans (GAGs) of heparan sulfate (HS) type are attached. HSPGs are ubiquitously expressed, being present on the cell surface of most cell types as well as being one of the main components of the extracellular matrix (ECM). Cell surface HSPGs, e.g. of the syndecan or glypican families (Alexopoulou et al., 2007; Filmus et al., 2008), mediate cell-cell and cell-matrix contacts and function as co-receptors for various growth factors (Mythreya and Blobel, 2009). In addition, cell surface HSPGs lining the vascular endothelium act as sites for leukocyte attachment, thereby facilitating migration of blood-borne cells into tissues (Wang et al., 2005).

Heparanase is an endoglucuronidase that cleaves HS chains (Pikas et al., 1998). Heparanase may thus modify the structure of preformed HSPGs and hence regulate the plethora of processes in which HSPGs are implicated. Indeed, previous studies have suggested a role for heparanase in various pathophysiological conditions, including tumor metastasis (Escobar Galvis et al., 2007; Vlodaysky and Friedmann, 2001), experimental autoimmune disease (Lider et al., 1989), amyloidosis (Li et al., 2005) and delayed type hypersensitivity (DTH) reactions (Edovitsky et al., 2006). A role for heparanase in tumour metastasis is supported by the strong correlation between levels of heparanase expression and metastatic potential of B16 melanoma (Vlodaysky et al., 1999) and T lymphoma (Goldshmidt et al., 2002b) cells. Further, it has been shown by RNA interference that suppression of heparanase expression ameliorates DTH reactions (Edovitsky et al.,

2006) as well as reduces lung colonization of B16 melanoma cells (Edovitsky et al., 2004). The role of heparanase in normal cell and tissue physiology is however not well characterized, although a role in tissue remodeling, vascularization and hair growth has been suggested applying transgenic mice overexpressing heparanase (Zcharia et al., 2004).

It is important to point out that most of the functional implications for heparanase have been derived from correlative or *in vitro* observations, and that detailed investigations of the *in vivo* function of heparanase have been hampered by the lack of relevant tools. The recent generation of a mouse strain lacking heparanase expression has opened new possibilities for determining its *in vivo* function (Zcharia et al., 2009). Heparanase deficient (*Hpse*^{-/-}) mice do not demonstrate any major abnormalities under normal conditions, possibly due to an increased expression of matrix metalloproteases, compensating for the loss of heparanase-mediated ECM degradation (Zcharia et al., 2009). Moreover, heparanase deficiency results in accumulation of non-degraded HS chains in different tissues.

Heparanase is highly expressed by numerous types of tumor cells, and by various cells of the immune system (Vlodavsky et al., 1992), including mast cells (Gong et al., 2003), eosinophils (Temkin et al., 2004), dendritic cells (Benhamron et al., 2006) and monocytes/macrophages (Sasaki et al., 2004). However, the role of heparanase in the context of immune cell function is only partially understood. Here we addressed this issue by studying the impact of heparanase deficiency on various leukocyte populations present in airways. We show that the absence of heparanase does not affect homing of lymphocytes or macrophages into the

airways, nor does it affect lymphocyte morphology. However, lack of heparanase caused an accumulation of Ym1, a chitinase-like protein, accompanied by a striking appearance of intracellular crystalline bodies in alveolar macrophages. Moreover, the HS chains in heparanase mutant macrophages were longer than in *WT* macrophages. Together, these findings implicate heparanase in the regulation of macrophage homeostasis.

2. Materials and Methods

2.1. Mice and collection of bronchoalveolar lavage

Heparanase-deficient (*Hpse*^{-/-}) mice were generated as described (Zcharia et al., 2009) and were bred and maintained in the animal facility at Uppsala Biomedical Centre. Experimental groups were age and sex matched and *Hpse*^{+/+} littermates (*WT* mice) were used as controls. All animal experiments were approved by the local ethical committee. Bronchoalveolar lavage (BAL) fluid was collected by rinsing the lungs twice with 1 ml HBSS. Total numbers of cells were counted and cytospin slides were prepared. In separate experiments the remaining cells were centrifuged and prepared for TEM or immunoblot analysis, and the cell-free BAL-supernatants were stored in -20° until used.

2.2. Transmission electron microscopy (TEM)

BAL cells from 7 or 17 months old *Hpse*^{-/-} and *WT* mice were pooled from 2 mice per group and prepared for TEM as described (Braga et al., 2007). Electron micrographs were generated using a Hitachi electron microscope (Hitachi Ltd., Tokyo, Japan) (Lukinius et al., 1995).

2.3. Differential cell count and morphology

Cytospin slides were stained with May-Grünwald/Giemsa (Merck, Darmstadt, Germany) and the numbers of different airway cells were determined by differential counting of at least 200 cells per slide. The altered morphology of alveolar macrophages, i.e. the presence of granules in the cytoplasm, was quantified by blinded scoring of at least 200 cells per slide. Cells with visual

granules were given scores (1-3) corresponding to the degree of granulation and no score (0) was given for cells lacking granules. An average score per counted cell was determined. To evaluate the presence of highly sulfated GAGs, cells were stained with Toluidine blue or Alcian blue as described (Braga et al., 2007; Reimer et al., 2006).

2.4. Immunocytochemistry

For immunocytochemistry, cytospin slides were fixed in ice-cold acetone for 5 minutes. Cells were permeabilized and blocked by incubation for 30 minutes with PBS containing 0.1% saponin, 0.5% BSA, and 10% goat serum. Endogenous peroxidase was blocked with PBS containing 0.3% H₂O₂ and 0.3% goat serum for 5 minutes. Cells were stained for 1 hr with anti-Ym1 antibodies (StemCell Technologies, Vancouver, Canada) or anti-mouse IgG (H+L) antibodies (Chemicon international, Temecula, CA, USA) diluted 1:200 in a blocking buffer (PBS with 0.1% saponin, 0.5% BSA, 2% goat serum, and 0.42mM NaCl). Rabbit IgG (Vector Laboratories, Burlingame, CA, USA) was used as a negative control. Cells were then incubated for 1 hr with biotinylated goat anti-rabbit IgG diluted 1:200 in blocking buffer. The Vectastain® Elite ABC reagent and DAB substrate kit for peroxidase were used for development (Vector Laboratories). Slides were counterstained for 2 minutes with Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA) and mounted with VectaMount (Vector Laboratories).

2.5. Generation of bone marrow derived macrophages

To generate conditioned medium for macrophage differentiation, we cultured the colony stimulating factor (CSF)-1 secreting cell line L929 (provided by Dr Ulrich

Blank, Inserm U699, Paris, France) in DMEM supplemented with 5% FCS, 50 µg/ml penicillin, 60 µg/ml streptomycin and 2mM L-glutamine (SVA, Uppsala, Sweden). Culture densities were initially 3×10^5 cells/ml, and after 8 days of culture, supernatants were collected, filtered and stored at -20°C until used. Bone marrow cells from eight months old *WT* or *Hpsc*^{-/-} mice were collected from femura and tibia by flushing the bones with 2.5 ml PBS. Red blood cells were removed by incubating cells for 2 minutes in 200 µl lysis buffer (0.15M NH₄Cl, 0.1mM EDTA, pH7.4). Cells were cultured overnight in complete medium (DMEM supplemented with 15% FCS, 10% L929-conditioned medium, 50 µg/ml penicillin, 60 µg/ml streptomycin and 2mM L-glutamine). Non-adherent cells, including monocyte precursors, were collected and 3×10^5 cells/ml were added to Petri dishes and cultured for 10 days in complete medium before used in further analyses.

2.6. FACS analysis

To confirm the purity of bone marrow derived macrophages, flow cytometry was used to identify presence of cells expressing the macrophage marker CD11b. Cells were washed twice in PBS and diluted in flow cytometry buffer (0.5%FCS/PBS). Samples of 0.2×10^6 cells were stained for 30 minutes on ice with 0.5µg PE-labeled anti-CD11b antibodies or with 0.5µg PE-labeled isotype controls (ImmunoTools, Friesoythe, Germany). After washing two times, cells were analyzed using a FACScan® flow cytometer and the CELLQuest™ 3.3 software (Becton Dickinson, San Jose, CA, USA).

2.7. Biosynthetic labeling and analysis of HS

Bone marrow derived macrophages from *WT* or *Hpsc^{-/-}* mice were cultured at 0.56×10^6 cells/ml in sulfate free medium supplemented with 2 mM L-glutamine, 1% pest, 10% L929-conditioned medium, 10% FCS (dialyzed against PBS), and 100 μ Ci/ml Na³⁵SO₄ (specific activity 1,500 Ci/mmol; PerkinElmer, Waltham, MA, USA). After 24 hours, HS was isolated from medium or cell lysates as described (Escobar Galvis et al., 2007). The purified ³⁵S-labeled HS was analyzed on a superose-6 column (GE Healthcare Biosciences, Uppsala, Sweden) in a buffer of 0.5M NH₄HCO₃.

2.8. Immunoblot analysis

Ten μ l of BAL fluid or BAL cell pellets from 13-17 months old *WT* or *Hpsc^{-/-}* mice were separated under reducing conditions on 12% SDS-PAGE gels. Proteins were subsequently blotted onto polyvinylidene difluoride membranes, followed by incubation with LI-COR blocking buffer (LI-COR Biosciences, Cambridge, UK) for 1h at room temperature. Membranes were stained o.n. at 4°C with anti-Ym1 (StemCell Technologies) antibodies diluted 1:1000 in blocking buffer. After washing with 0.1% Tween 20 in TBS, the membrane was incubated with IR Dye 800-conjugated donkey anti-rabbit Ig (LI-COR Biosciences, dilution 1:1000) in blocking buffer. Visualization and densitometry analyses were performed using Odyssey IR imaging system (LI-COR Biosciences). Data were measured as counts/mm² and normalized to the loading control, i.e. results are expressed as the ratio between sample value for Ym1 and the corresponding value for β -actin.

2.9. Statistical analyses

Statistical differences between *WT* and *Hpsc^{-/-}* mice were determined by Mann-Whitney U test, and correlations were analyzed by Spearman test, using the GraphPad Prism 4.0 (GraphPad Software, Inc, San Diego, CA, USA).

3. Results

3.1. Normal homing of leukocytes to airways in the absence of heparanase

Heparanase has previously been implicated in inflammatory processes and cell migration (Benhamron et al., 2006; Edovitsky et al., 2006; Sasaki et al., 2004), although the precise impact of heparanase on leukocyte populations *in vivo* has not been evaluated. To address this issue, we examined the effect of heparanase deficiency on leukocytes present in bronchoalveolar lavage (BAL) fluid. As shown in Fig 1A, the BAL cell populations from both *WT* and *Hpse*^{-/-} mice were mainly composed of macrophages. There were very few lymphocytes (1.3% in average) and almost no neutrophils or eosinophils in BAL, indicating that there was no spontaneous ongoing inflammatory reaction in the airways. There was a tendency for higher numbers of macrophages and lymphocytes in the *Hpse*^{-/-} mice, but the difference between *WT* and *Hpse*^{-/-} mice did not reach statistical significance (Fig. 1A). These results demonstrate that, under normal conditions, heparanase has no major impact on migration of macrophages and lymphocytes to the airway lumen.

3.2. Granule-like structures in *Hpse*^{-/-} alveolar macrophages

Examination of the morphology of May-Grünwald/Giemsa-stained BAL leukocyte populations showed that *Hpse*^{-/-} lymphocytes did not differ morphologically from the corresponding *WT* cells (not shown). In contrast, *Hpse*^{-/-} macrophages showed a striking presence of purple-stained intracellular, granule-like structures, which was not seen in *WT* macrophages (Fig. 1B). A systematic scoring for the presence of granule-like structures in alveolar macrophages from 3-17 months old *WT* and *Hpse*^{-/-} mice showed that the extent of granulation correlated strongly with the age

of animals, with a more pronounced granulation seen in old (i.e. 7 and 13-17 months) vs. young (i.e. 3 months) mice (Fig. 1B and C). Although some signs of granulation were also seen in alveolar macrophages from old *WT* mice, the extent and intensity of granulation was markedly higher in corresponding macrophages derived from *Hpse*^{-/-} mice (Fig. 1B and C). Notably, granule-like structures were not seen in peritoneal macrophages from *WT* or *Hpse*^{-/-} mice (not shown), suggesting that this phenotype was restricted to the airways.

Potentially, the increased granulation of alveolar macrophages might be associated with accumulation of cellular HS due to the absence of heparanase. To address this possibility, we stained for sulfated GAGs using Alcian blue and Toluidine blue (Fig. 2). Compared with peritoneal macrophages, alveolar macrophages stained more intensely with Alcian blue (Fig. 2A), suggesting a higher level of cell-associated GAGs in airways as compared to peritoneal macrophages. However, there was no significant difference in the staining intensity between *WT* and *Hpse*^{-/-} macrophages. Moreover, alveolar macrophages did not demonstrate metachromatic staining with Toluidine blue (Fig. 2B), indicating that there was no substantial accumulation of highly sulfated GAGs in these cells.

3.3. Needle-shaped crystalline bodies in *Hpse*^{-/-} alveolar macrophages

To further examine the nature of the granule-like structures present in *Hpse*^{-/-} macrophages, alveolar macrophages were examined by transmission electron microscopy (TEM). The TEM analysis revealed a striking morphology of electron dense and needle-shaped crystalline bodies in alveolar macrophages derived from *Hpse*^{-/-} animals, structures that were totally absent in corresponding *WT* cells (Fig.

3). The crystalline bodies were surrounded by membranes, indicating that they had developed within membrane-enclosed organelles such as lysosomes, ER or Golgi.

3.4. Accumulation of Ym1 in alveolar macrophages from *Hpse*^{-/-} mice

Next, we sought to identify the macrophage-associated crystalline material.

Alveolar macrophages constitutively express Ym1, a chitinase-like protein that previously has been demonstrated to exhibit a marked tendency to spontaneously form crystals *in vitro* (Chang et al., 2001). Moreover, Ym1 is known to form needle-shaped intracellular crystals (Harbord et al., 2002; Nio et al., 2004) that closely resemble the crystals found in *Hpse*^{-/-} alveolar macrophages (Fig. 3).

Hence, one plausible scenario is that the crystalline bodies seen in *Hpse*^{-/-} alveolar macrophages contain Ym1. To address this possibility, we applied immunocytochemistry to stain for Ym1 in alveolar and peritoneal macrophages from *WT* and *Hpse*^{-/-} mice. As shown in Fig. 4A and B, alveolar macrophages from both *WT* and *Hpse*^{-/-} mice stained positively for Ym1 whereas peritoneal macrophages were negative, in agreement with the reported absence of Ym1 in unstimulated peritoneal macrophages (Hung et al., 2002). However, *Hpse*^{-/-} macrophages stained considerably stronger for Ym1 than did corresponding *WT* cells, indicating that *Hpse* deficiency indeed results in accumulation of cell-associated Ym1. Noteworthy, the staining showed a patchy, intracellular appearance, compatible with the presence of Ym1 within membrane-enclosed organelles (Fig 4A).

Although Ym1 is a likely candidate to explain the altered morphology of *Hpse*^{-/-} alveolar macrophages, there are other known crystal-forming proteins to consider.

Of particular interest are immunoglobulin (Ig) light and heavy chains, which are known to occasionally form needle-shaped intracellular crystals in tissue macrophages of patients with lymphoproliferative diseases (Lebeau et al., 2002). However, the alveolar macrophages stained weakly and non-patchy for IgG, with no differential extent of staining in *WT* versus *Hpse*^{-/-} cells (Fig. 4C), suggesting that the crystalline bodies seen in *Hpse*^{-/-} macrophages do not originate from accumulated IgG.

To further substantiate the association between intracellular Ym1 and crystalline formation, we evaluated Ym1 protein levels in both the cell- and fluid- fraction of BAL from 13-17 months old mice, using immunoblot analysis. At this age there is a significant difference between *WT* and *Hpse*^{-/-} mice in the visual appearance of intracellular crystalline material, although a low degree of granulation is also found in *WT* mice (Fig 1C). As shown in figure 4D, high amounts of cell-associated Ym1 were apparent in three out of four samples collected from *Hpse*^{-/-} mice, and only in one of three samples collected from *WT* mice. Notably, the *Hpse*^{-/-} sample with low levels of Ym1 protein was also given low scores for intracellular granulation (Fig 4E). In fact, this sample was given the lowest scores within the group of 13-17 months old *Hpse*^{-/-} mice (Fig 1C) and is therefore not a representative sample.

When analyzing samples from both genotypes, there was a statistically significant correlation between intracellular Ym1 levels and the amount of crystalline material in BAL cells (Fig 4E). In contrast, extracellular Ym1 levels did not correlate with intracellular crystals (Fig 4F) and the levels of Ym1 in cell-free BAL fluid did not differ between *Hpse*^{-/-} and *WT* mice (Fig 4D). Collectively, these results strongly suggest that heparanase deficiency results in accumulation of Ym1 in airway

macrophages and that Ym1 is the crystal-forming unit in *Hpse*^{-/-} alveolar macrophages.

3.5. Non-degraded HS chains in bone marrow derived *Hpse*^{-/-} macrophages

The results described above indicate that the absence of heparanase affects the homeostasis of alveolar macrophages. To study the possible involvement of macrophage HSPGs in this effect, biosynthetic labeling experiments were carried out. Because of the low number of macrophages that can be recovered from BAL fluid, the biosynthetic labeling experiments were instead performed using macrophages derived by *in vitro* differentiation of bone marrow precursors (bone marrow-derived macrophages; BMMØs). As shown in Fig. 5A, cells strongly positive for the macrophage marker CD11b were obtained by *in vitro* differentiation of bone marrow precursors in the presence of L929-conditioned medium. The numbers of CD11b-positive cells and the intensity of CD11b staining did not differ between *WT* and *Hpse*^{-/-} cells, indicating that absence of heparanase does not influence macrophage differentiation (Fig 5A). Moreover, staining with May-Grünwald/Giemsa did not reveal any obvious morphological difference between BMMØs from *WT* and *Hpse*^{-/-} mice (not shown). In contrast to the BAL macrophages, no signs of intracellular granulation were seen in *Hpse*^{-/-} BMMØs. However, as this phenotype appears to be age-dependent (see Fig. 1B and C), its absence in BMMØs after 10 days of culture is not unexpected.

Having confirmed the macrophage phenotype of BMMØs (Fig 5A), we metabolically labeled the cells with ³⁵S-sulfate, followed by purification of cell-associated and secreted HS. Gel filtration analysis showed that HS chains

recovered from both the cell-associated and secreted HS isolated from *Hpse*^{-/-} BMMØs are longer than HS extracted from *WT* BMMØs (Fig. 5B), indicating that lack of heparanase leads to accumulation of non-degraded HS chains in *Hpse*^{-/-} BMMØs.

4. Discussion

In the present study we establish that heparanase decisively affects macrophage homeostasis. This was demonstrated by the findings that macrophages lacking heparanase contain non-degraded HS chains and that alveolar macrophages from *Hpse*^{-/-} mice exhibit an age-dependent increase in granule-like structures, identified as electron dense crystalline bodies surrounded by intracellular membranes. Moreover, the altered morphology of *Hpse*^{-/-} alveolar macrophages was associated with an increased patchy staining of intracellular Ym1, a protein known to form intracellular crystals (Nio et al., 2004).

Heparanase expression has been detected in a variety of leukocyte populations, including monocytes/macrophages, dendritic cells, neutrophils, CD4⁺ T cells, mast cells and eosinophils (Benhamron et al., 2006; Temkin et al., 2004; Vlodaysky et al., 1992). Interestingly, heparanase expressed on the surface of dendritic cells and macrophages has been shown to promote migration of these cells, by degrading ECM barriers (Benhamron et al., 2006; Sasaki et al., 2004). Moreover, heparanase can enhance cell adhesion independent of its enzymatic activity, and thereby contribute to cell motility (Goldshmidt et al., 2003; Sotnikov et al., 2004). These findings suggest a role for heparanase in regulating leukocyte migration processes. We therefore hypothesized that absence of heparanase may lead to defective leukocyte migration, possibly resulting in a reduced number of leukocytes in the BAL. We show, however, that the absence of heparanase does not influence these leukocyte populations adversely. Thus, it appears that under normal conditions heparanase expression is not essential for migration of leukocytes in to the

airways. However, it remains to be elucidated whether heparanase influences migration of the corresponding cell types during an inflammatory reaction.

A striking and novel finding in this study was the appearance of granulated alveolar macrophages in aged mice lacking heparanase, as detected by May Grünwald/Giemsa staining. This phenomenon was exclusively seen in the BAL macrophage population, and was totally absent in other airway leukocytes and in peritoneal macrophages. A closer examination of the macrophages by TEM revealed that the granule-like structures had a distinct electron dense needle-shaped crystalline appearance. Moreover, the TEM analysis showed that the intracellular crystalline bodies were enclosed by membranes. One possible interpretation of these findings is that the crystalline bodies are located in lysosomes, and that they arise as a consequence of impaired regulation of compounds accumulating in the lysosome due to the absence of heparanase. In line with such a possibility, heparanase has previously been identified in intracellular compartments, including late endosomes and lysosomes (Goldshmidt et al., 2002a) in which proheparanase is processed and activated (Zetser et al., 2004). Moreover, heparanase activity is optimal at pH5-6 (Gilat et al., 1995), further supporting a physiological function of heparanase restricted to cellular compartments having acidic pH, such as lysosomes.

An obvious possibility would be that the crystalline bodies in *Hpse*^{-/-} macrophages contain HSPGs that accumulate in lysosomes as a result of heparanase-deficiency. However, since the absence of heparanase did not result in a substantial accumulation of cell-associated HSPGs as judged by biosynthetic labeling

experiments and by basic-dye cell staining, it appears less likely that HSPGs are predominant components of the crystals. Another possibility is that accumulation of crystalline bodies is linked to the marked increase in HS chain length shown to accompany the absence of heparanase. Although we cannot precisely explain how the increased HS chain length could account for the accumulation of crystalline material, one potential scenario could be that abnormally long HS chains may serve as a support that accumulates multiple crystal-forming molecules thus facilitating crystal formation. This would be in some analogy with the proposed contribution of HSPGs to amyloid formation (Stevens and Kisilevsky, 2000).

A crucial issue is to identify the crystal-forming unit in *Hpse*^{-/-} alveolar macrophages. Here we present several observations suggesting that the chitinase-like protein Ym1 is a main candidate. Most importantly, we demonstrate a statistically significant correlation between the extent of granule-like structures in alveolar macrophages and the amounts of intracellular Ym1 in the BAL cell fraction. Moreover, there was a markedly increased Ym1 immunostaining in *Hpse*^{-/-} alveolar macrophages as compared to *WT* counterparts, suggesting an intracellular accumulation of Ym1 protein in the absence of heparanase. Notably, staining of Ym1 was irregular with several intensely stained regions surrounded by non-stained areas, compatible with the presence of Ym1 within membrane-surrounded organelles. Ym1 exhibits several known features that are in agreement with this notion. Firstly, Ym1 is known to easily form crystals *in vitro* (Chang et al., 2001) and intracellular crystalline bodies *in vivo* (Nio et al., 2004), in particular in alveolar macrophages from aged immunodeficient mice (Guo et al., 2000; Harbord et al., 2002). Secondly, since HS and heparin are physiological ligands to

Ym1 (Chang et al., 2001), it may be speculated that changes in HS turnover affect Ym1 aggregation and crystallization. Finally, Ym1 is selectively expressed by alveolar macrophages and immature neutrophils under normal conditions (Nio et al., 2004) and is only expressed by peritoneal macrophages upon LPS-stimulation (Hung et al., 2002) or differentiation into alternatively activated macrophages (Kreider et al., 2007; Rauh et al., 2005). The selective expression of Ym1 in alveolar, but not peritoneal macrophages is well in line with the appearance of granule-like structures only in the former cell type. Collectively, these findings strongly suggest that Ym1 is the crystal-forming unit in *Hpse*^{-/-} alveolar macrophages.

Ym1 belongs to a group of chitinase-like proteins, including Ym2 and BRP-39 in mice, and YKL-40 in humans (Bussink et al., 2007). Increased expression of these proteins has been associated with inflammatory diseases, in particular with allergic asthma (reviewed in (Lee et al., 2008; Shuhui et al., 2009)). The physiological function of Ym1 is not clear, but a role in eosinophil chemotaxis (Owhashi et al., 2000) and promotion of cytokine production (Cai et al., 2009) has been suggested. Interestingly, Ym1 has been reported to exhibit a weak ability to cleave *N*-acetylglucosamine units (Guo et al., 2000; Harbord et al., 2002), suggesting that Ym1 may not only bind to HS but can possibly also cleave HS, and thus function in a manner similar to heparanase. However, the ability of Ym1 to process HS remains to be established.

In conclusion, the present study indicates that heparanase has a physiological role in airway cell homeostasis, by regulating the morphology of alveolar macrophages

and by controlling the levels of intracellular Ym1. Considering the findings presented here and the implication of heparanase in inflammatory processes in general (Li and Vlodaysky, 2009), it may be anticipated that heparanase has a role in the regulation of airway inflammatory diseases.

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Figure captions

Fig. 1. Effect of heparanase deficiency on airway leukocyte composition and morphology. Bronchoalveolar lavage (BAL) cells were collected from *Hpse*^{-/-} and *WT* mice of different ages and stained with May-Grünwald/Giemsa. (A) Differential counts of BAL leukocytes from 7 months old mice (n=11; pooled from 3 independent experiments): tot, total cells; mac, macrophages; lym, lymphocytes; neu, neutrophils; eos, eosinophils. The graph shows the mean total number of cells ± SEM. (B) Representative images showing the presence of granule-like material in *Hpse*^{-/-} alveolar macrophages from 3 (n=3), 7 (n=11; pooled from 3 independent experiments) and 13-17 (n=6; pooled from 2 independent experiments) months old mice. The extent of granulation was quantified by blinded scoring and the results are presented as mean scores ± SEM (C). Statistical differences were determined by Mann-Whitney U test: ***, *P* < 0.001; *, *P* < 0.05; not significant (ns), *P* > 0.05.

Fig 2. Basic dye staining of *Hpse*^{-/-} and *WT* macrophages. Alveolar macrophages (AM) and peritoneal macrophages (PM) from 7 months old mice (n=3-4) were stained with Alcian blue (A) or Toluidine blue (B) dye. Stained peritoneal mast cells (MC) are shown as positive controls. Representative images are shown. ND, not done. 400x original magnification.

Fig 3. Transmission electron microscopy (TEM) analysis of alveolar macrophages. Upper panels: Representative micrographs showing the morphology of *WT* and *Hpse*^{-/-} macrophages. Lower panels: Higher magnification of selected areas marked

by squares in the upper panels. Note the abundance of electron dense crystalline material surrounded by membranes (arrows) in the *Hpse*^{-/-} macrophage. N, nucleus; L, lysosome. Original magnification x 9,000.

Fig. 4. Heparanase deficiency results in increased levels of Ym1 in alveolar macrophages. BAL and peritoneal cells were collected from 7 months old *WT* and *Hpse*^{-/-} mice (A-C). Cytospin preparations of BAL cells (A, C) and peritoneal cells (B) were stained with anti-Ym1 (A, B), anti-IgG heavy and light chains (C), or isotype specific control antibodies. Images are representatives of 6 (A) or 2 (B and C) independent experiments. BAL cells and cell-free BAL fluid were collected from 13-17 months old mice and subjected to SDS-PAGE followed by immunoblotting with anti-Ym1 antibodies (D). Band intensities (counts/mm²) were measured and the results are expressed as normalized densitometry values, i.e. ratios between samples and the corresponding β -actin loading control (E, F). Amounts of intracellular granule-like structures were analyzed by blinded scoring of May-Grünwald/Giemsa-stained cells and were plotted against corresponding densitometry values (E, F). The degree of granulation was significantly associated with intracellular Ym1 levels (E) but not with extracellular Ym1 levels (F) in BAL, as determined by Spearman correlation test ($P < 0.05$).

Fig. 5. Effects of heparanase deficiency on the molecular size of HS isolated from bone marrow-derived macrophages (BMMØs). (A) Flow cytometry analysis demonstrating that *WT* and *Hpse*^{-/-} BMMØs show equal expression of the macrophage marker CD11b. (B) Gel chromatography on Superose 6 was applied

to analyze biosynthetically ^{35}S -sulfate-labeled secreted (upper panel) and cell-associated (lower panel) HS from *WT* and *Hpse*^{-/-} BMMØ.

Fig.1

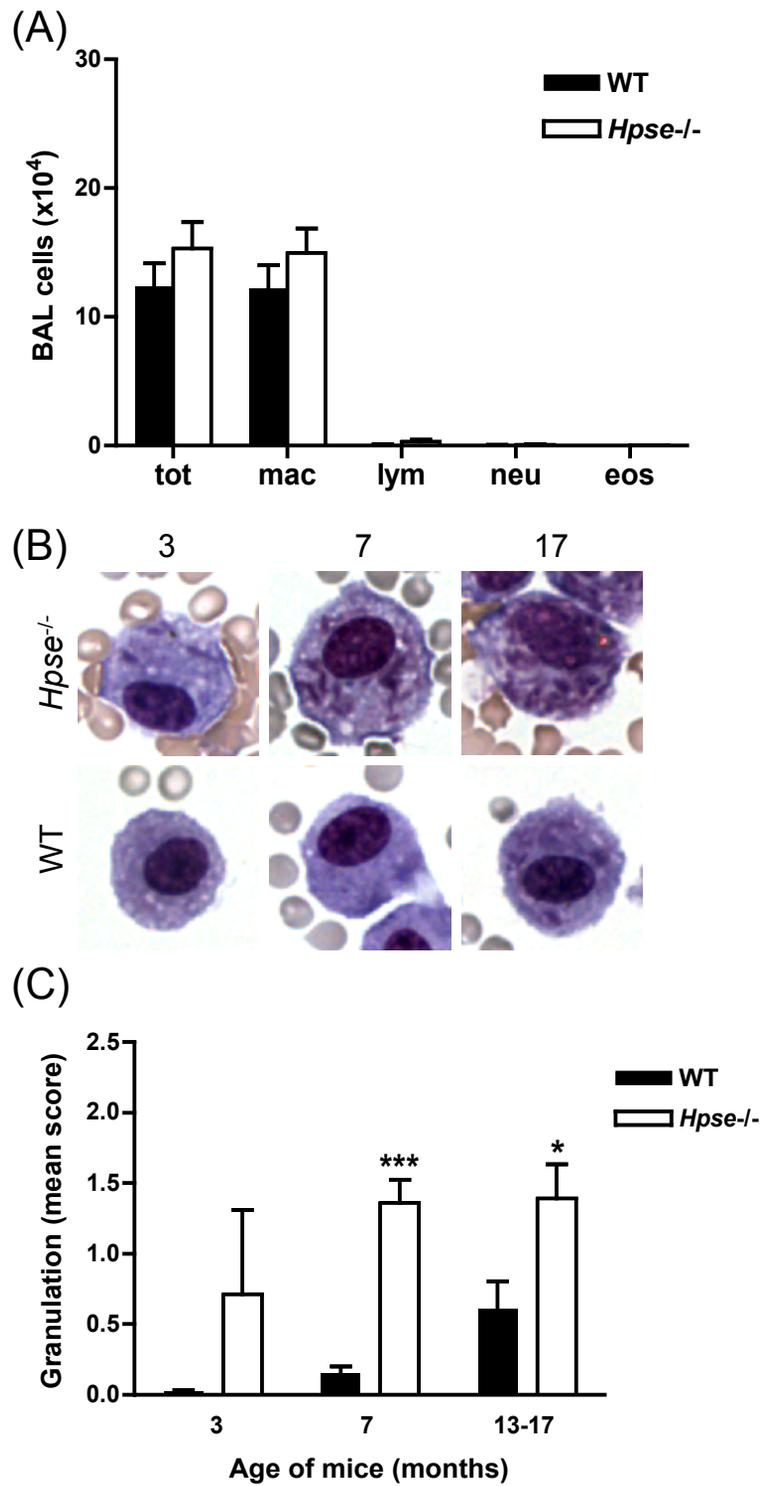


Fig.2

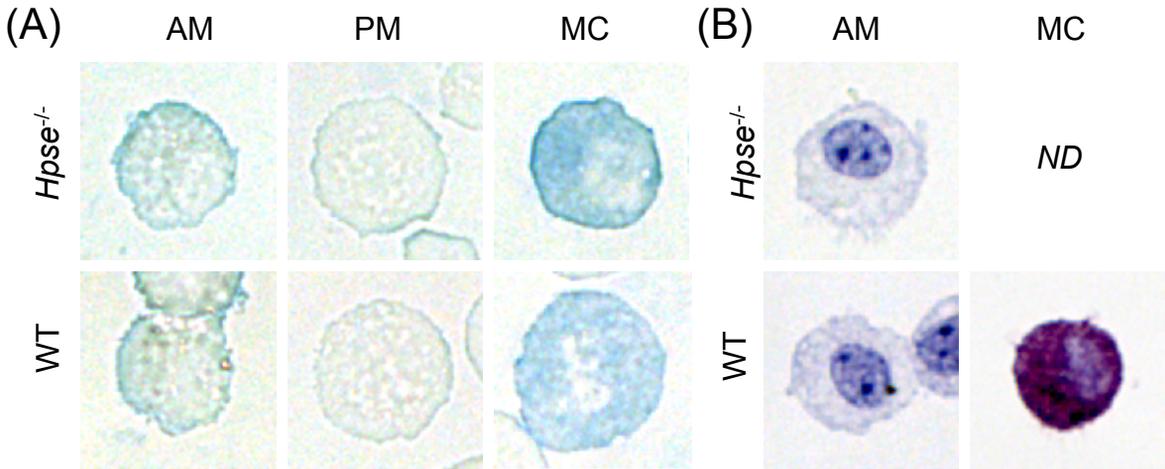


Fig.3

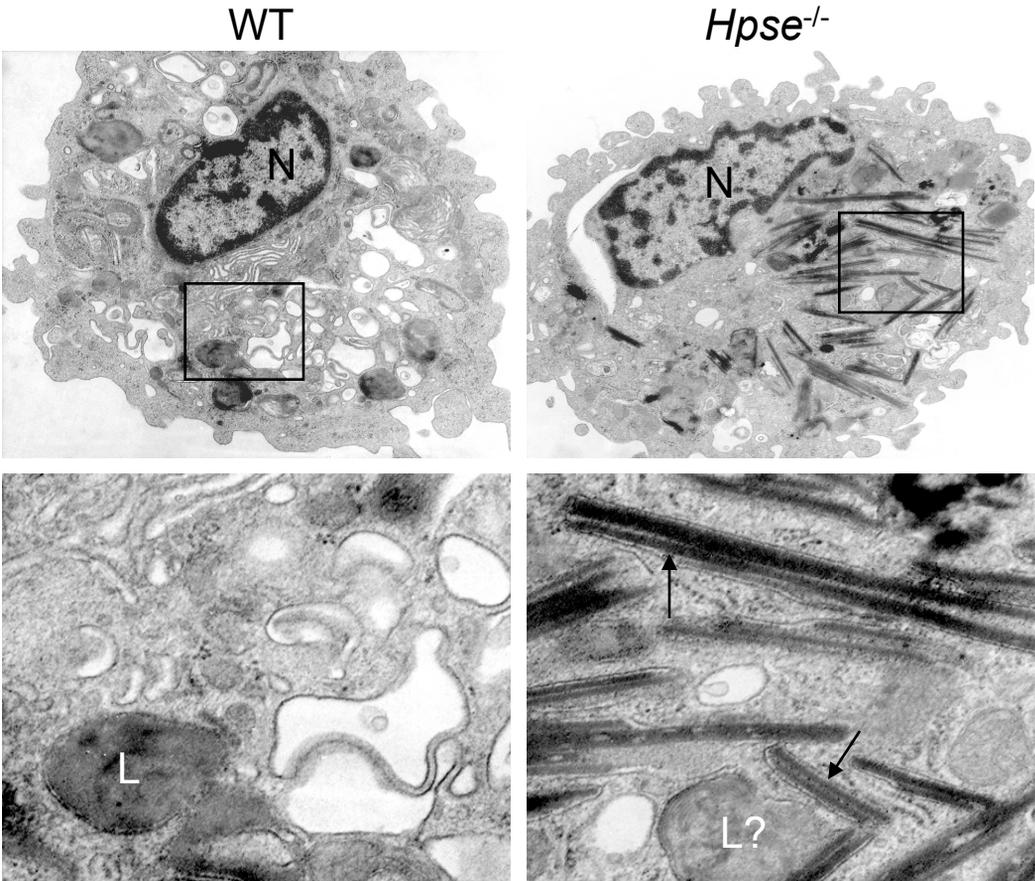


Fig.4

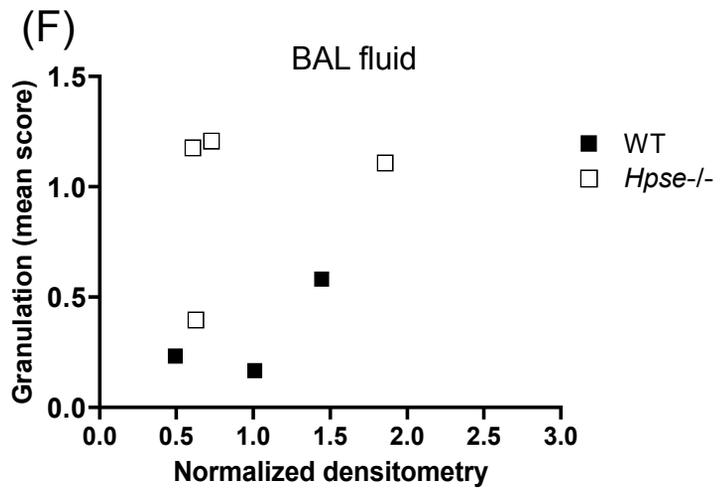
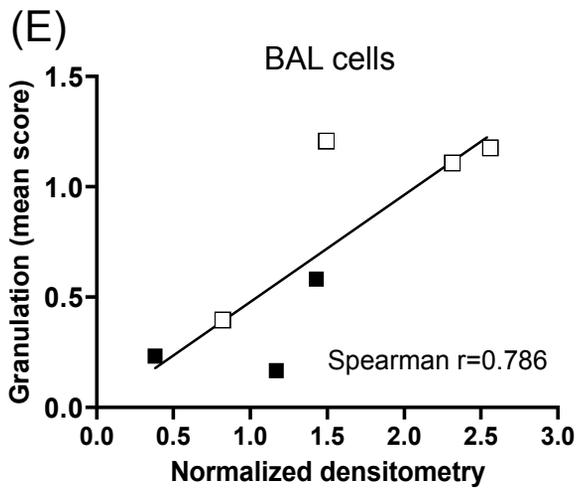
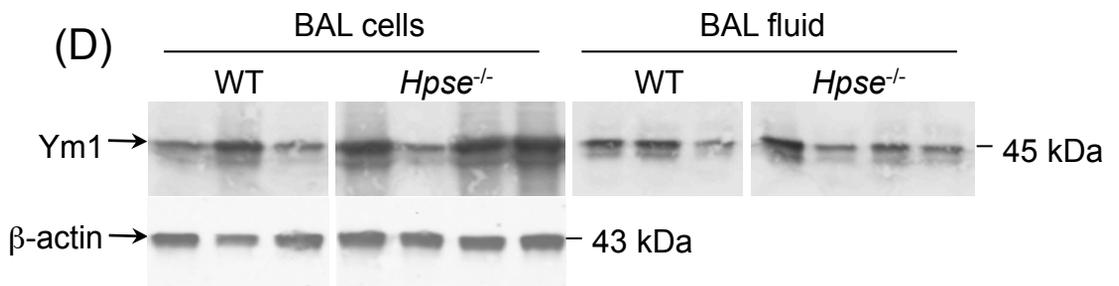
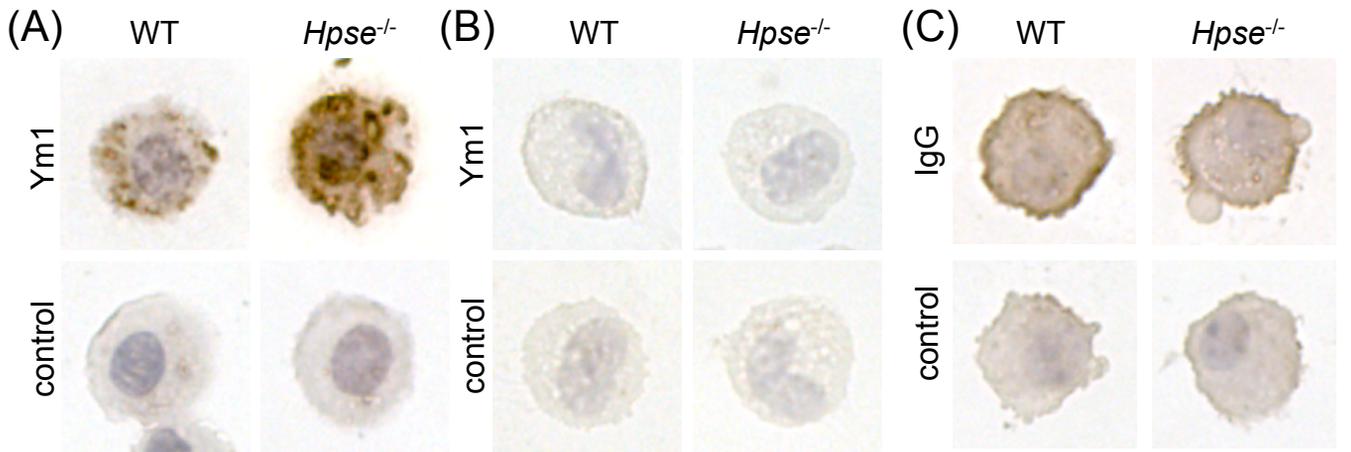


Fig.5

