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Alternatively Activated Macrophages Regulate Extracellular Levels of the Hormone Placental Lactogen via Receptor-Mediated Uptake and Transcytosis[1]

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Alternatively activated (M2) macrophages regulate immune responses and tissue remodelling. In many tissues including placenta, M2 express stabilin-1, a multidomain protein that exerts a dual role as a scavenger receptor for acetylated low density lipoprotein (acLDL) and SPARC (secreted protein acidic and rich in cysteine) and as an intracellular cargo carrier for SI-CLP. Using yeast two-hybrid screening, we identified the developmental hormone placental lactogen (PL) as a novel ligand of stabilin-1. In Chinese hamster ovary-stabilin-1 cells and M2, FACS and confocal microscopy demonstrated that stabilin-1 mediates internalization and endosomal sorting of PL. In M2 macrophages, PL was partially degraded in lysosomes; part of PL escaped degradation and was delivered to novel PL^+ storage vesicles lacking endosomal/lysosomal markers. During formation, PL^+ vesicles underwent transient interaction with the *trans*-Golgi network (TGN). Upon placement of PL-loaded M2 into PL-free medium, PL was secreted into the supernatant. Leupeptin, an inhibitor of lysosomal hydrolases, reduced PL degradation, enhanced sorting of PL into the TGN/storage vesicle pathway and increased PL secretion. Thus, processing of PL in M2 macrophages occurs either by the classical lysosomal pathway or by a novel TGN-associated *trans*-secretory pathway. Macrophages isolated from human placental villi efficiently endocytosed PL-FITC and transported it to the storage vesicles. Our data show that extracellular PL levels are determined by uptake, degradation, storage, and release in M2. During pregnancy PL concentration reaches 10 μg/ml in maternal circulation and stays below 0.5 μg/ml in fetal circulation. We propose that stabilin-1-positive macrophages determine the difference in PL levels between maternal and fetal circulation. ***The Journal of Immunology,*** **2008, 180: 3028–3037.**

Macrophages are responsible for the maintenance of tissue homeostasis and for orchestration of defense responses and healing processes in adult tissues (1–3). During developmental processes, placental macrophages produce substances important for pregnancy and parturition and they influence the balance of local immune reactions and angiogenesis in placenta (4, 5). One of the major regulatory activities of macrophages is clearance of exogenous nonself as well as unwanted-self ligands from the extracellular environment (6, 7). The selectivity of ligand recognition is achieved by the highly specific pattern of scavenger receptors expressed by resident tissue macrophages and newly immigrating monocytes undergoing differentiation to macrophages in the local cytokine context. Alternatively activated (M2) macrophages are involved in Th2 immune responses, anti-inflammatory tissue remodeling and maintenance of tissue homeostasis (2, 8, 9). The scavenger receptor stabilin-1, which we previously identified (10, 11), is a marker for alternative macrophage activation (8, 12, 13); stabilin-1 is expressed by specialized tissue macrophages in placenta, skin, and gut, as well as by sinusoidal endothelial cells in liver, spleen, bone marrow, and lymph nodes (10, 11, 14, 15).

Stabilin-1 mediates endocytosis of acetylated low density lipoprotein (acLDL),[4] one of the classical ligands of scavenger receptors (16–18). Stabilin-1-mediated intracellular trafficking of acLDL along the endocytic pathway requires PI3K activity (17). We have recently demonstrated that stabilin-1 mediates uptake and targeting for lysosomal degradation of the matricellular glycoprotein SPARC (secreted protein acidic and rich in cysteine) (14). SPARC is a soluble, nonstructural component of the extracellular matrix that regulates developmental processes, tissue remodeling, angiogenesis, wound healing and tumor-stromal cell interactions (19–23). We showed that stabilin-1-positive alternatively activated macrophages internalize and partially degrade SPARC in lysosomes. We proposed that alternatively activated macrophages can actively regulate SPARC content in the extracellular matrix

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[4] Abbreviations used in this paper: acLDL, acetylated low density lipoprotein; CHO, Chinese hamster ovary; PL, placental lactogen; hPL, human PL; TGN, *trans*-Golgi network; SI-CLP, stabilin-1 chitinase-like protein.

and adjust SPARC levels to the changing physiological needs of the organism. In pathological situations, SPARC activity may also be controlled by macrophages, which have a flexible phenotype and constitute the major regulatory immunological component of a tissue (14, 18, 24).

In addition to endocytosis/recycling, stabilin-1 is involved in trafficking between early/sorting endosomes and the *trans*-Golgi network (TGN) in human macrophages (25). Shuttling of stabilin-1 between the biosynthetic and endosomal/lysosomal compartments is mediated by GGA (Golgi-localized, γ-ear-containing, adenosine 5′-diphosphate-ribosylation factor-binding) adaptors, which bind to the DDSLL motif in the cytoplasmic tail of stabilin-1. GGA adaptors are highly specialized and responsible for TGN/endosomal shuttling of CI-MPR and sortilin that combine endocytic and intracellular sorting functions (26–31). In search for an intracellular cargo sorted by stabilin-1, we identified a novel stabilin-1 chitinase-like protein (SI-CLP) as an interacting protein. SI-CLP expression was strongly up-regulated in macrophages by IL-4 and dexamethasone in parallel with the expression of stabilin-1. Using stably transfected cells as well as stabilin-1 small interfering RNA-treated macrophages, we demonstrated that stabilin-1 is involved in delivery of newly synthesized SI-CLP from the TGN to the secretory pathway (32).

Stabilin-1 is a type I transmembrane receptor with a highly complex extracellular part containing clusters of epidermal growth factor-like and fasciclin domains. Apparently, the large extracellular domain of stabilin-1 is involved in numerous unknown tissue-specific interactions. Thus, abundant expression of stabilin-1 in placenta suggests its active participation in developmental processes (5). In this study, we performed yeast two-hybrid screening and identified the hormone placental lactogen (PL) as an extracellular ligand for stabilin-1. Stabilin-1 mediates the internalization and delivery of PL to the endocytic pathway in stably stabilin-1-transfected Chinese hamster ovary (CHO) cells. Stabilin-1-positive, alternatively activated (M2) macrophages stimulated by IL-4 in combination with dexamethasone ($M2_{IL\text{-}4/dex}$) endocytose PL efficiently, in contrast to stabilin-1-negative macrophages stimulated with only IL-4 or IFN-γ. Part of PL was degraded via the classical lysosomal pathway. A portion of the endocytosed PL, however, escaped degradation and was targeted to the TGN and further to PL "storage" vesicles. $M2_{IL\text{-}4/dex}$ were able to secrete stored PL back into the culture medium when placed in PL-free conditions. Our data indicate that stabilin-1 in alternatively activated macrophages is involved in the endocytosis of PL, which is followed by complex processing. Thus, alternatively activated macrophages use a novel combination of intracellular trafficking pathways, i.e., lysosomal degradation and TGN-related secretion, to determine extracellular PL levels. We propose that alternatively activated macrophages use stabilin-1-mediated endocytosis and intracellular sorting for the regulation of PL-dependent fetal growth and placental angiogenesis.

Materials and Methods

Primary macrophages, cell lines, and placenta tissue

Human monocytes/macrophages were isolated and cultivated as described (25). The cells were purified from individual buffy coats by density gradient centrifugations. Monocyte-enriched fractions were subjected to positive $CD14^+$ Magnetic Cell Sorting using CD14 magnetic beads (Miltenyi Biotec) resulting in 90–98% monocyte purity as confirmed by flow cytometry. Macrophages were cultivated in X-VIVO 10 Serum-free medium (Cambrex) at a concentration of 1×10^6 cells/ml with the following stimulants: IFN-γ at 1000 U/ml; IL-4 at 10 ng/ml (TEBU); and dexamethasone at 1×10^{-7} M (Sigma-Aldrich). The detailed protocol for monocyte isolation is available online (http://www.methods.info/).

Human placenta tissue was obtained from the Department of Obstetrics and Gynaecology (Medical University of Graz, Graz, Austria) following vaginal deliveries and abortions due to socioeconomic reasons. Usage of these materials was approved by the Ethics Committee of the Medical University of Graz. Term placenta tissue was used for immunohistochemical analysis. For isolation of Hofbauer cells, both term placenta following vaginal deliveries as well as abortion material from terminations of healthy pregnancies (weeks 7–10) was used. The fetal part of the placenta was mechanically separated, homogenized, subjected to treatment with collagenase type IV (catalog no. C5138; Sigma-Aldrich) at a final concentration 0.035% for 1 h at 37°C. Digested tissue was passed through a steel mesh and filtered through a nylon 70-μm filter. Cells were washed with PBS and subjected to density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich). In case of term placenta tissue, $CD14^+$ Magnetic Cell Sorting was performed. After isolation, Hofbauer cells were used for preparation of cytospins for direct analysis or incubated in X-VIVO 10 Serum-free medium (Cambrex) overnight to perform functional endocytosis assays.

CHO cells stably transfected with the pLP-IRESneo vector (BD Clontech) and with the expression construct pLP-IRESneo-hstabilin-1 have been described (17). Cells were propagated in F12 medium supplemented with 10% FCS, penicillin/streptomycin, and G418.

Yeast two-hybrid screening

The yeast two-hybrid screen was performed using the Matchmaker Gal4 Two-Hybrid system (BD Clontech). For bait generation, a fragment of stabilin-1 cDNA corresponding to aa 2302–2570 was amplified by PCR and cloned into the pDNR2 vector followed by subcloning into pLP-GBKT7 using the BD Creator cloning kit (BD Clontech). The bait protein was characterized according to the manufacturer's instruction; it was expressed on a high level, did not activate transcription in yeast and did not affect the mating efficiency. The pretransformed human placenta Matchmaker cDNA library in the pGADT7-Rec vector, the Yeastmaker Yeast Transformation kit, and plasmid isolation kits were purchased from BD Clontech. Selection conditions of the highest stringency were used.

Endocytosis assay

Human purified PL (hPL; Affiland), recombinant human produced in Sf9 cells and purified by anion exchange chromatography (33, 34) and purified mouse monoclonal MS-1 Ab (15) were labeled with FITC using FluoroTag FITC Conjugation kit (Sigma-Aldrich). FITC labeled IgG1κ, an isotype control for MS-1 was purchased from eBioscience. Fluorescently labeled proteins were used as ligands for the endocytosis assay. Endocytosis assay with stably transfected CHO-K1 cells was performed as follows: cells were grown until 70–80% confluent in 24- or 6-well plates. For immunofluorescence experiments, CHO-K1 cell lines were grown on glass coverslips. Directly before ligand uptake, the complete medium was removed, cells were washed once with PBS without Ca^{2+} and Mg^{2+} (Invitrogen Life Technologies), and F12 medium (without supplements but containing fluorescent ligands) was added. For macrophages, fluorescent ligands were added directly to the conditioned X-VIVO medium. Incubation with fluorescent ligands was performed at 37°C. Durations of ligand exposure and ligand concentrations are indicated in each experiment and in *Results*. Cessation of endocytosis was achieved by placing cells on ice or by immediate fixation in paraformaldehyde as previously described (25). For FACS analysis, both macrophages and CHO cells were incubated in culture dishes on ice for 15 min, and gently recovered in ice cold PBS. For immunofluorescent analysis, CHO cells were quickly washed three times with PBS without Ca^{2+} and Mg^{2+}, and then subjected to the fixation in paraformaldehyde. To prepare macrophage samples for immunofluorescence, we placed plastic dishes with macrophages on ice for 15 min, gently removed the cells by scraping, and prepared cytospins followed by fixation in paraformaldehyde as described.

FACS analysis

Quantification of bound/internalized fluorescent ligands was performed with FACSCalibur (BD Biosciences) according to standard protocols. Data were visualized and analyzed with WinMDI 2.8 software.

Treatment with leupeptin

Leupeptin 10-mM stock solution was prepared in H_2O and stored at −20°C in aliquots. Leupeptin was added to the macrophage culture medium 30 min before the endocytosis. In case of pulse-chase endocytosis, leupeptin was also added to the freshly exchanged medium. Concentration of leupeptin in macrophage medium was 0.33 mM.

Immunofluorescence and confocal microscopy

Cell fixation and staining procedures were performed as described (25). Frozen placenta sections were fixed in paraformaldehyde as described (35). The following primary Abs were used: rabbit polyclonal anti-stabilin-1 F4 (11) or RS1; mouse monoclonal anti-EEA1 and anti-p62^{lck} (BD Biosciences); rabbit polyclonal anti-Lamp3 (Santa Cruz Biotechnology); mouse monoclonal anti-human CD163 and sheep polyclonal anti-TGN46 (Serotec); rabbit polyclonal anti-human PL (Biozol). Corresponding secondary Abs, conjugated with Cy2, Cy3 and Cy5 were (Dianova). Confocal microscopy was performed using a Leica TCS SP2 laser scanning spectral confocal microscope, equipped with a 63× 1.32 objective. Excitation was with an argon laser emitting at 488 nm, a krypton laser emitting at 568 nm, and a helium/neon laser emitting at 633 nm. Data were acquired and analyzed with Leica confocal software. All two- and three-color images were acquired using a sequential scan mode.

Secretion assay and ELISA

Macrophages were exposed to nonlabeled hPL (Affiland) at a concentration 10 μg/ml for 30 min and for 3 h continuously. To stop endocytosis and intracellular ligand processing macrophage were replaced on ice for 15 min, harvested in sterile conditions in sterile Eppendorf tubes, sedimented at 2000 rpm at 4°C, washed twice with cold PBS, resuspended in fresh X-VIVO medium and replaced in culture dishes. Macrophages were incubated for 16 h at 37°C to allow accumulation of secreted products in culture medium. Supernatants of macrophages cultures were collected before and after ligand exposure at time pointes indicated in each experiment and *Results*. Supernatants were centrifuged at 2000 rpm to remove remaining cells, replaced into new tubes, and stored at −80°C. To control washing efficiency, last PBS washing solutions were also frozen at −80°C and analyzed in parallel with macrophage supernatants. ELISA quantification was performed using commercially available hPL ELISA kit (IBL) according to the protocol of the manufacturer.

Statistical analysis

Paired two-tailed *t* test was performed using the TTEST function of Excel program in Microsoft Office 2000 Pro.

Results

Identification of PL as an endocytic ligand of stabilin-1

Stabilin-1 is a type I transmembrane protein composed of a short cytoplasmic tail that interacts with GGA adaptors and a large extracellular part with a highly complex structure comprising seven fasciclin and multiple epidermal growth factor-like domains potentially involved in various protein-protein interactions (11). We applied yeast two-hybrid screening to identify proteins interacting with stabilin-1. The stabilin-1 bait fragment P9 did not activate transcription and was expressed at a high level in yeast. The P9 fragment contains one epidermal growth factor-like domain, the final fasciclin domain (F7), the transmembrane region and the cytoplasmic tail (Fig. 1*A*). Screening of a human placental cDNA library under conditions of highest stringency resulted in identification of PL as an interacting partner of stabilin-1. High concentrations of PL are produced by trophoblast cells in placenta, and concentration of PL in the maternal circulation reaches values of 5–10 μg/ml (reviewed in Anthony et al. (36)). At the same time, various subpopulations of placental macrophages express high amounts of stabilin-1 (5, 15). To verify whether PL is an endocytic ligand of stabilin-1, we used CHO cells stably transfected with stabilin-1. We have previously demonstrated that stabilin-1 functions as an efficient endocytic receptor for acLDL and SPARC in this cell line (14, 17). FITC-labeled PL at a final concentration of 10 μg/ml was added to the serum-free culture medium for 30 min at 37°C, and uptake of PL-FITC was quantified by FACS. A strong PL-FITC signal was observed in CHO-stabilin-1 cells, but not in control CHO cells stably transfected with empty vector (Fig. 1*B*). The strength of the PL-FITC signal was similar to that of anti-stabilin-1 mouse mAb MS-1 labeled with FITC. A nonspecific increase of scavenging activity in CHO-stabilin-1 cells was deemed negligible due to similar levels of transferrin-Alexa Fluor 546 endocytosis in CHO-stabilin-1 and CHO vector cells (17). Therefore, the increased PL-FITC signal in CHO-stabilin-1 cells was due to the presence of stabilin-1. Thus, we concluded that PL is a novel ligand for stabilin-1.

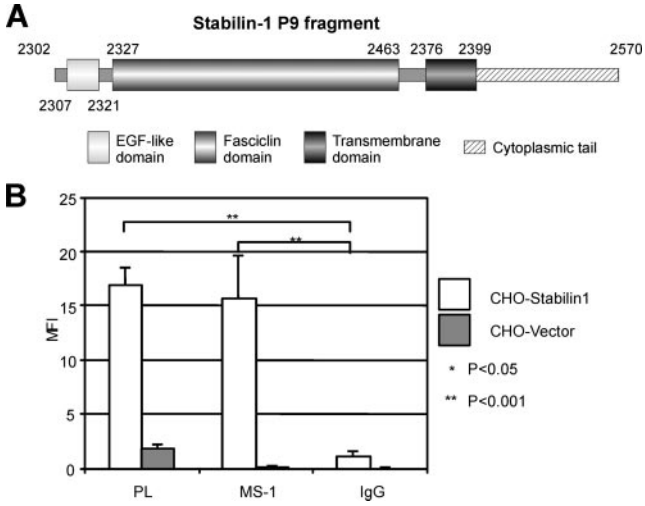

FIGURE 1. Identification of PL as a ligand for stabilin-1. *A*, Schematic representation of the stabilin-1 fragment used as a bait in yeast two-hybrid screening. *B*, FACS analysis of PL-FITC, MS-1-FITC, and IgGκ-FITC uptake in CHO-stabilin-1 cells (□) and CHO vector control cells (▨). Data indicate mean ± SD. Incubation with ligand was performed continuously for 30 min. PL-FITC, MS-1-FITC, and IgGκ-FITC were added at a concentration of 10 μg/ml. CHO-stabilin-1 cells, but not CHO vector cells, efficiently endocytose PL-FITC and anti-stabilin-1 mAb MS1-FITC, but not isotype control Ab.

Stabilin-1-mediated trafficking of PL in stably transfected CHO cells requires PI3K activity

We investigated whether stabilin-1 is responsible for the trafficking of PL along the endocytic pathway. As the enhanced PL-FITC signal detected by FACS could result not only from receptor-mediated ligand internalization, but also from surface-bound ligand retention, we examined the capacity of stabilin-1 to mediate internalization of PL-FITC by immunofluorescence and confocal microscopy as shown in Fig. 2. After 30 min of continuous endocytosis, high amounts of PL-FITC were delivered to the EEA1-positive early/sorting endosomal compartment in CHO-stabilin-1 cells, but not in CHO vector control cells (Fig. 2, *A* and *E*). We obtained similar results for two other endocytic ligands of stabilin-1, i.e., acLDL and SPARC (14, 17), indicating that stabilin-1 mediates PL-FITC trafficking to the endosomal pathway. Next we investigated whether stabilin-1–mediated trafficking of PL-FITC functions via the classical PI3K-dependent mechanism. PI3K activity can be specifically inhibited by wortmannin (17). Previously, we have shown that wortmannin arrests stabilin-1-mediated trafficking of acLDL in early/sorting endosomes (17). Here, CHO-stabilin-1 cells were pretreated with wortmannin for 30 min and exposed to PL-FITC. Wortmannin treatment induced formation of enlarged swollen endosomes in which PL-FITC was accumulating after 30 min of endocytosis (compare Fig. 2, *A* to *B*). After 1 h of endocytosis, the transfer of PL-FITC and stabilin-1 into the peripheral late endosomal compartment was almost abrogated (compare Fig. 2, *C* to *D*); stabilin-1 and PL-FITC both remained arrested in EEA1-positive endosomes concentrated in the perinuclear area of the cells. These results were in accordance with previously published data on stabilin-1-mediated trafficking of

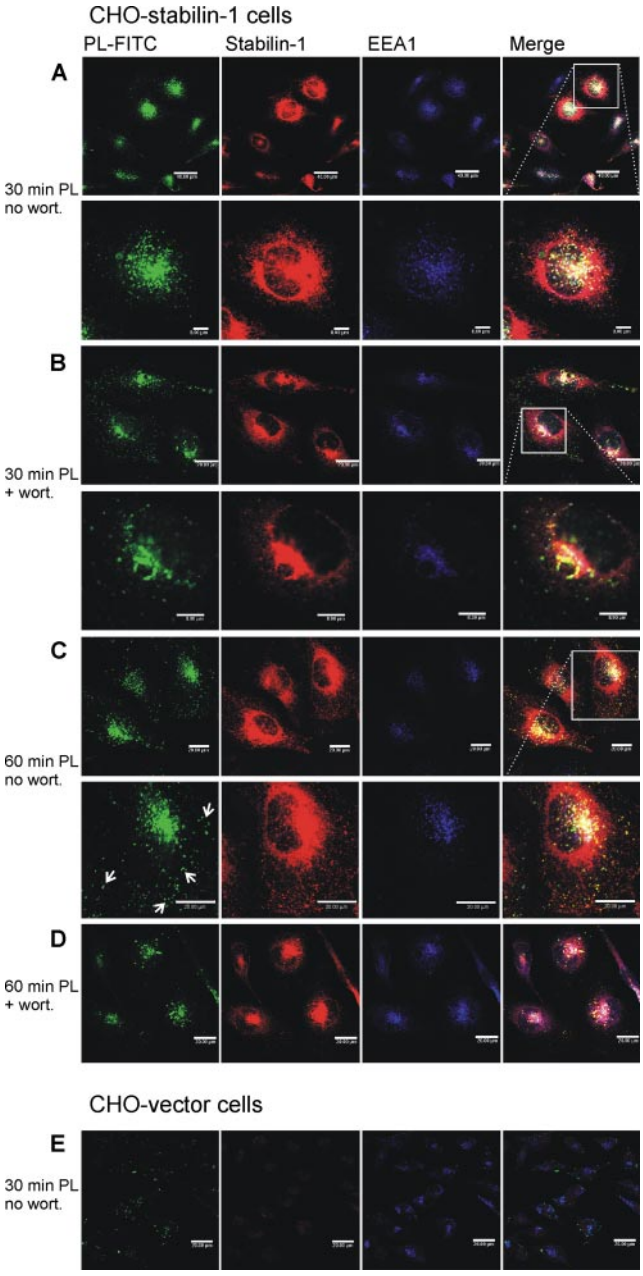

FIGURE 2. Stabilin-1 mediates endocytosis and PI3K-dependent trafficking of PL in stably transfected CHO cells. Immunofluorescence and confocal microscopy of PL-FITC internalization in CHO-stabilin-1 and CHO vector control cells. PL-FITC is shown in green, stabilin-1 is shown in red, and the early/sorting endosome marker EEA1 is shown in blue. Yellow indicates colocalization of PL-FITC and stabilin-1 (merge of green and red). Colocalization of PL-FITC, stabilin-1, and EEA-1 (merge of green, red, and blue) is shown at open arrow. Cells were pretreated with 1 μM wortmannin for 30 min before the start of endocytosis and continuously during the endocytosis experiment (*B* and *D*). PL-FITC was added at a concentration of 10 μg/ml. Incubation with PL-FITC was performed continuously for 30 min (*A* and *B*) and for 1 h (*C* and *D*). *A*, In nontreated cells, PL-FITC colocalizes with stabilin-1 in round-shaped small EEA-1-positive endosomes. *B*, In the presence of wortmannin, PL-FITC$^+$stabilin-1$^+$EEA1$^+$ structures are enlarged and swollen. *C*, In nontreated cells, PL-FITC$^+$stabilin-1$^+$EEA1$^-$ round-shaped late endosomal vesicles are distributed on the cell periphery (open arrows). *D*, No peripheral PL-FITC$^+$stabilin-1$^+$EEA1$^-$ late endocytic vesicles are formed in the presence of wortmannin. *E*, Very low signal of PL-FITC is detectable in small EEA1-negative vesicles in control CHO vector cells. Scale bar corresponds to 40 μm (*top panels*) and 8 μm (*bottom panels*) in *A*; 20 μm (*top panels*) and 8 μm (*bottom panels*) in *B*; and 20 μm in *C–E*.

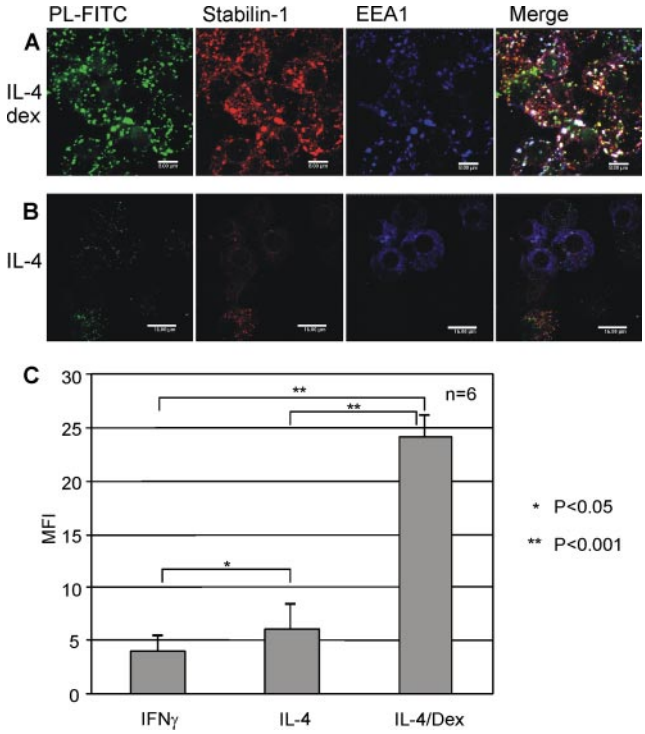

FIGURE 3. Stabilin-1 is required for the endocytosis of PL by human macrophages. Differentially stimulated macrophages were incubated continuously with 10 μg/ml purified hPL-FITC for 30 min. *A* and *B*, Immunofluorescence and confocal microscopy of PL-FITC endocytosis. PL-FITC is shown in green, stabilin-1 is shown in red, and the early/sorting endosome marker EEA1 is shown in blue. Yellow indicates colocalization of PL and stabilin-1 (merge of green and red). White indicates colocalization of PL, stabilin-1, and EEA-1 (merge of green, red, and blue). *A*, PL-FITC and stabilin-1 colocalize in the endosomal pathway in macrophages stimulated with IL-4 and dexamethasone. *B*, Very low amounts of PL-FITC are detectable in macrophages stimulated with IL-4. Scale bar corresponds to 8 μm (*A*) and 16 μm (*B*). *C*, FACS quantification of PL-FITC endocytosis by macrophages. A value for p was determined by paired two-tailed t test ($n = 6$ individuals). Data represent mean $\pm$ SD. MFI, Mean fluorescence intensity. Only stabilin-1-positive macrophages stimulated with IL-4 and dexamethasone show a strong signal both in immunofluorescence and FACS.

acLDL and indicated that PI3K activity is required for stabilin-1-mediated transfer of PL-FITC from early/sorting endosomes to the late endocytic pathway. Taken together, stabilin-1 mediates targeting of PL to the endocytic pathway via the classical PI3K-dependent mechanism.

Stabilin-1-positive alternatively activated macrophages efficiently endocytose PL

Expression of stabilin-1 by macrophages requires stimulation by IL-4 and dexamethasone (11, 25). We examined the endocytosis of PL-FITC by stabilin-1 using immunofluorescence and confocal microscopy. After 30 min of continuous endocytosis, PL-FITC was efficiently internalized by stabilin-1-positive macrophages. Most of the PL-FITC colocalized with stabilin-1, and this colocalization occurred preferentially in EEA1-positive early/sorting endosomes (Fig. 3*A*). We also asked whether stabilin-1 is required for the efficient endocytosis of PL and whether macrophages differentiated under other conditions and not expressing stabilin-1 were able to endocytose PL. Macrophages differentiated in the presence of IFN-γ (prototypic Th1 cytokine) do not express detectable amounts of stabilin-1. Macrophages stimulated by IL-4

(prototypic Th2 cytokine) in the absence of glucocorticoids express barely detectable levels of stabilin-1 in a small fraction of the cells (11). Monocyte-derived macrophages from six healthy individuals, cultured for 6 days in the presence of IFN-γ, IL-4, and IL-4/dexamethasone, were incubated with PL-FITC for 30 min. PL-FITC binding/internalization was subsequently analyzed using FACS and quantified. IFN-γ- and IL-4-stimulated macrophages exhibited low signals, whereas IL-4- and dexamethasone-stimulated macrophages demonstrated a strong FITC signal (Fig. 3*C*). Immunofluorescence analysis confirmed that PL-FITC was efficiently internalized and delivered to early endosomes only in macrophages stimulated with IL-4 and dexamethasone in combination. Barely detectable levels of PL-FITC were detected in EEA-1-negative small vesicles in macrophages stimulated with IFN-γ (data not shown). IL-4-stimulated macrophages internalized more PL-FITC in comparison to IFN-γ-stimulated macrophages; however, PL-FITC was rarely detected in EEA1-positive endosomes (Fig. 3*B*). In conclusion, we confirmed in this experiment by confocal microscopy that PL was internalized via a receptor-mediated pathway in stabilin-1-positive macrophages. In the absence of stabilin-1, macrophages were not able to internalize PL-FITC efficiently and did not deliver it to the endocytic pathway. The presence of minimal amounts of PL-FITC in EEA1-positive endosomes in macrophages stimulated with IL-4 suggests the presence of an alternative, stabilin-1-independent, receptor-mediated mechanism for the endocytosis of PL. Thus, we deduced that stabilin-1 is actively involved in internalization of PL in macrophages.

PL is partially degraded in lysosomes and partially sorted into a novel type of vesicles in $M2_{IL\text{-}4/dex}$

Upon receptor-mediated internalization, many ligands are subsequently sorted into late endosomes and lysosomes for degradation (37). To define the trafficking pathway of internalized PL in macrophages, we incubated macrophages with PL-FITC for different time periods and assessed its intracellular localization by immunofluorescence and confocal microscopy. After 30 min of continuous endocytosis, PL-FITC, as described, preferentially localized to stabilin-1-positive early endosomes (Fig. 3*A*), but a small portion of PL-FITC was translocated to the $p62^{lck}$-positive late endosomal compartment (data not shown). To examine whether PL-FITC is targeted to lysosomes for degradation, pulse-chase endocytosis was performed. PL-FITC was added to the macrophage culture medium for 35 min to allow efficient intracellular accumulation of the ligand. Thereafter, ligand-containing medium was exchanged with fresh X-VIVO medium without ligand, and continued incubation at 37°C allowed further processing of the intracellular PL-FITC. At 1 and 3 h after the beginning of endocytosis, the amount of intracellular PL-FITC in early endosomes was decreased compared with the beginning of the chase period (compare Fig. 3, *A* to Fig. 4*B*, *top*). PL-FITC was only rarely detected in stabilin-1-positive vesicles and the major portion of the nondegraded PL was concentrated in small round-shaped vesicles (Fig. 4, *A* and *B*, *top panels*). Western blot analysis confirmed that the total amount of intracellular PL-FITC was also significantly decreased (data not shown). To examine whether PL-FITC is degraded in lysosomes, leupeptin, an inhibitor of lysosomal enzymes, was used. Leupeptin efficiently blocked PL-FITC degradation beginning after 1 h of the start of endocytosis, and the effect was more pronounced after 3 h (Fig. 4*A*). Both in the presence and absence of leupeptin, PL-FITC-positive vesicles did not colocalize with lysosomal markers Lamp1 and Lamp3 (Fig. 4*A* and data not shown). In the absence of leupeptin, after 3 h of pulse-chase endocytosis most of PL-FITC was transported from EEA1-positive

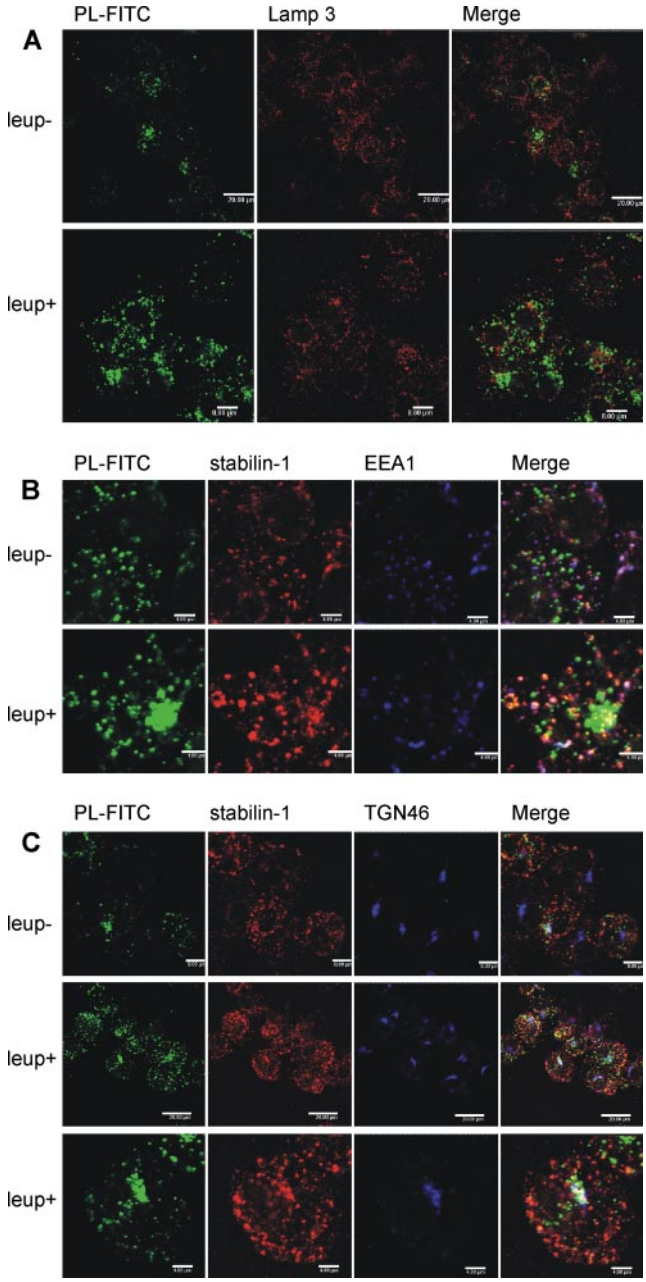

FIGURE 4. Identification of novel PL storage vesicles in alternatively activated macrophages. $M2_{IL4/dex}$ were allowed to endocytose PL-FITC (10 μg/ml) for 35 min at 37°C; then the medium was exchanged to ligand-free X-VIVO, and the cells were incubated for an additional 2 h 25 min. Leupeptin was added at a concentration of 0.33 mM for 30 min before the start of endocytosis and was continuously present during 3 h of endocytosis and ligand processing (leup+). PL-FITC is visualized in green; Lamp3 is shown in red (*A*), stabilin-1 is shown in red (*B* and *C*); EEA1 is shown in blue (*B*); and TGN46 is shown in blue (*C*). Yellow indicates merge of green and red; pink indicates merge of blue and red; and white indicates merge of green, red, and blue. *A*, Leupeptin blocks degradation of PL-FITC, but does not increase its concentration in Lamp3-lysosomes. *B*, Leupeptin induces both accumulation of PL-FITC in stabilin-1^+EEA^+ endosomes, but does not block formation of PL^+ storage vesicles (single green color in merge). *C*, PL-FITC colocalizes with stabilin-1 in TGN both in the presence (+) and in the absence (−) of leupeptin. Scale bar indicates 20 μm (*top panels*) and 8 μm (*bottom panels*) in *A*; 4 μm in *B*; and 8 μm (*upper row*), 20 μm (*middle row*), and 4 μm (*lower row*) in *C*.

stabilin-1-positive early endosomes to vesicles that were negative for stabilin-1, EEA-1, the late endosomal marker p62^{lck}, and the lysosomal markers Lamp1 and Lamp3 (Fig. 4, *A* and *B*, *top panels* and data not shown). Leupeptin treatment resulted both in accumulation of PL-FITC in enlarged positive EEA-1 stabilin-1 endosomes and an increase of the PL-FITC amount in PL$^+$ vesicles negative for EEA-1 and stabilin-1 (Fig. 4*B*). In summary, our data indicate that a significant portion of PL-FITC was degraded via the classical lysosomal pathway, whereas nondegraded PL-FITC was sorted into a novel vesicular compartment (PL$^+$"storage" vesicles, see schematic presentation in Fig. 6*B*) distinct from the endosomal/lysosomal compartment.

PL$^+$ vesicles transiently associate with the TGN

Searching for the trafficking pathway leading to the formation of PL$^+$ storage vesicles, we found that in ~5–10% of the cells PL-FITC-positive vesicles were concentrated in areas closely adjacent or overlapping with vesicular structures positive for TGN46, a marker for the TGN. PL-FITC, TGN46, and stabilin-1 were frequently found to be colocalized, suggesting that stabilin-1 mediates sorting of PL-FITC into the TGN. Leupeptin treatment resulted in an increase of the association of PL-FITC-positive vesicles with the TGN (Fig. 4), suggesting that blocking PL degradation in lysosomes redirects PL trafficking into the alternative pathway. After 6 h of endocytosis, the amount of PL-FITC localized in the TGN was reduced, and after 24 h no PL-FITC was found in the TGN (data not shown). To test whether the escape from degradation and sorting into storage vesicles are processes specific for PL, we performed a control experiment with the anti-stabilin-1 mouse FITC-labeled mAb MS-1 (MS-1-FITC) and SPARC-FITC, endocytic ligand of stabilin-1 (14). Initial efficiencies for the uptake of PL-FITC, MS-1-FITC, and SPARC-FITC were similar (compare 30 min time points in Fig. 5). After 24 h, however, numerous round-shaped PL$^+$ vesicles, which were negative for stabilin-1, were still observed (Fig. 5*A*). At the same time degradation of MS-1 was almost completed (Fig. 5*B*), whereas nondegraded SPARC was mainly accumulated in enlarged polymorphic endosomes (Fig. 5*C*). In summary, stabilin-1 seems to direct part of the endocytosed PL into a novel, nonlysosomal pathway that transiently communicates with the TGN and leads to the accumulation of endocytosed PL in PL$^+$ storage vesicles.

Secretion of endocytosed exogenous PL by M2$_{IL\text{-}4/dex}$

Next we asked what is the physiological function of the PL$^+$ storage vesicles. One of the major functions of the TGN is sorting of newly synthesized proteins into the secretory pathway (38). Vesicles formed in the late Golgi compartment possess surface determinants, which are necessary for the commitment of these vesicles to constitutive secretion. Because PL$^+$ vesicles transiently communicated with the TGN, we hypothesize that PL$^+$ vesicles might acquire some of the characteristics of secretory vesicles. We examined whether endocytosed PL that has escaped degradation could be secreted back into the extracellular space. M2$_{IL\text{-}4/dex}$ were exposed to PL at a concentration of 10 μg/ml for 3 h to ensure efficient uptake and transport of PL into storage vesicles. After 3 h of endocytosis, cells were harvested, extensively washed, and placed in X-VIVO medium that did not contain PL. Sixteen hours later, secretion of PL into the culture medium was quantified using ELISA. Indeed, PL was secreted back into the supernatant. The concentration of PL in the medium was ~250 ng/ml (Fig. 6*A*), and was within the range of the PL concentrations measured in fetal blood (4–500 ng/ml) (36). Lower concentrations of PL were detected in supernatants of macrophages, which had endocytosed PL for only 30 min, indicating that the amount of secreted PL depends on its intracellular concentration (data not shown). Absence of endogenous production of PL in macrophages was confirmed by the absence of ELISA signal in macrophage-conditioned medium in samples where no PL was added (Fig. 6*A*). Because we showed before that leupeptin inhibits degradation of PL and redirects PL to the storage PL$^+$ vesicles, we analyzed whether leupeptin affects

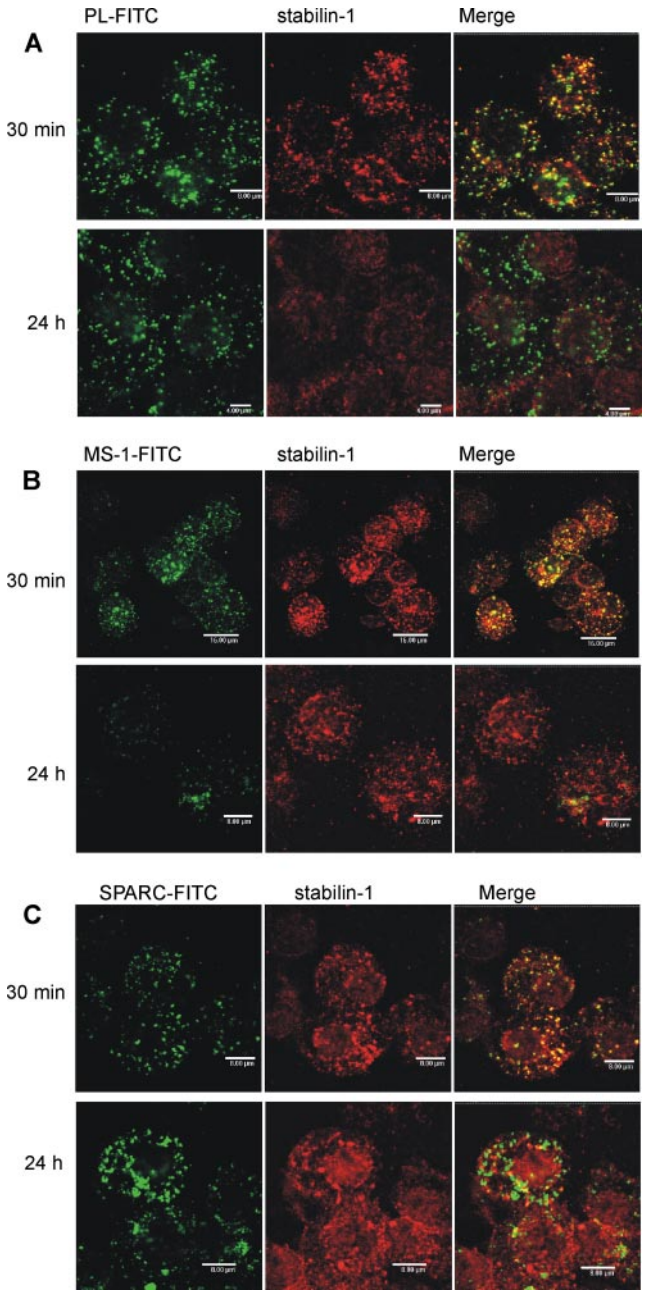

FIGURE 5. Long-term storage of endocytosed PL in alternatively activated (M2) macrophages. M2$_{IL4/dex}$ were allowed to endocytose PL-FITC (*A*), MS-1-FITC (*B*), or SPARC-FITC (*C*) for 30 min (*top panels*) or for 24 h to allow ligand processing (*bottom panels*). PL-FITC, SPARC-FITC, and MS-1-FITC are shown in green, stabilin-1 is shown in red, and merge of green and red is shown as yellow. After 30 min all three ligands were endocytosed with similar efficiency. *A*, After 24 h of continuous endocytosis PL-FITC was localized in small round-shaped stabilin-1-negative vesicles. *B*, Most of MS-1-FITC was degraded. *C*, Nondegraded SPARC-FITC was mainly accumulated in enlarged polymorphic endosomes partially positive for stabilin-1. Scale bar indicates 8 μm (*top panels*) and 4 μm (*bottom panels*) in *A*; 16 μm (*top panels*) and 8 μm (*bottom panels*) in *B*; and 8 μm in *C*.

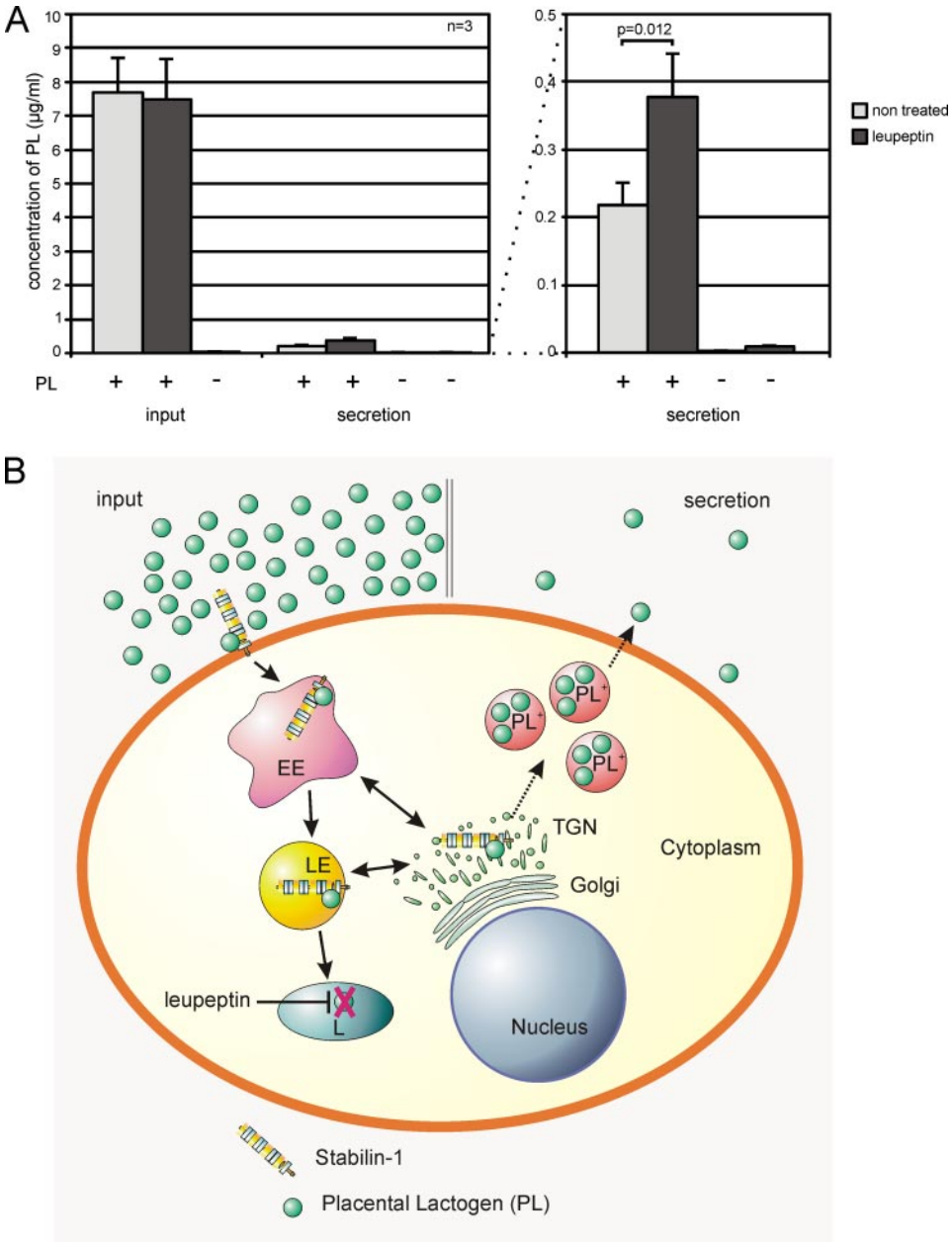

FIGURE 6. Secretion of endocytosed PL by alternatively activated (M2) macrophages. *A*, $M2_{IL4/dex}$ were allowed to endocytose PL continuously for 3 h. No PL was added to control $M2_{IL4/dex}$. Next the cells were harvested, washed, and replaced in fresh X-VIVO medium. After another 16 h, secretion of PL was measured by ELISA. Leupeptin was added at a concentration of 0.33 mM for 30 min before the start of endocytosis and was continuously present during 3 h of endocytosis and ligand processing, as performed in Fig. 4. Three minutes after addition of PL, medium aliquots were taken to detect the starting concentration of PL; absence of PL in conditioned medium of PL-nontreated $M2_{IL4/dex}$ was controlled in parallel (termed input). Absence of PL in the last washing step was controlled by ELISA (data not shown). Leupeptin-treated macrophages secreted twice more PL than nontreated macrophages. In PL-nontreated samples, no PL was detected in conditioned medium of $M2_{IL4/dex}$. *B*, Schematic representation of PL endocytosis, sorting, and secretion in stabilin-1-positive $M2_{IL4/dex}$. At high concentrations, PL is endocytosed via stabilin-1 and delivered to the $EEA1^+$ early endosomal/sorting compartment (EE). Part of PL is targeted to late endosomes (LE) and consequently to lysosomes (L) for degradation. Another part of PL is transiently targeted to the TGN, and is then sorted into PL^+ storage vesicles, which are negative for stabilin-1 as well as for endosomal and for lysosomal markers. Endocytosed PL is secreted into the extracellular space in the absence of extracellular PL. Leupeptin inhibits degradation of PL in lysosomes, redirects PL sorting into storage vesicles, and increases the amount of secreted PL.

secretion of PL. Indeed, leupeptin treatment resulted in a statistically significant 2-fold increase of PL secretion (Fig. 6*A*). In summary, we propose that at high extracellular concentrations PL is endocytosed via stabilin-1 in alternatively activated macrophages and then partially degraded in lysosomes and partially sorted into storage vesicles. In the absence of extracellular PL, its secretion back to the extracellular space is observed. A schematic representation of PL trafficking in stabilin-1-positive $M2_{IL-4/dex}$ as suggested by the findings presented in this experiment is given in Fig. 6*B*.

Placental lactogen in macrophages isolated from human placental villi (Hofbauer cells)

Next we asked whether macrophages in human placenta are able to process exogenous PL. First we analyzed, whether stabilin-1-positive macrophages are localized closely to PL-producing trophoblast cells in villi of human placenta. Because Abs recognizing stabilin-1 and PL are both raised in rabbit, we first showed, that all stabilin-1-positive Hofbauer cells are recognized by mouse anti-CD163 Ab (Fig. 7*A*). Next we demonstrated, that $CD163^+$ Hofbauer cells are localized in close proximity to the trophoblast cells that produce high amount of PL (Fig. 7*B*). One side of the trophoblast layer is directly exposed to the maternal circulation (Fig. 7*B*, dotted areas), whereas Hofbauer cells are localized between the trophoblast layer and the fetal circulation. We isolated Hofbauer cells from human placental villi and analyzed their capacity to endocytose exogenously added PL as well as SPARC, another ligand of stabilin-1 we previously described (14). Both PL-FITC and SPARC-FITC were efficiently endocytosed; however, their trafficking was different (Fig. 7*C*). During continuous endocytosis nondegraded SPARC was accumulated in polymorphic enlarged endosomes, similar to SPARC-positive endosomes formed after 24 h of continuous endocytosis in monocyte-derived $M2_{IL4/dex}$ (Fig. 5*C*). In contrast PL was sorted into small round-shaped vesicles, morphologically highly similar to the vesicles detected in $M2_{IL4/dex}$ (compare Fig. 7*C* to Fig. 4*B* and Fig. 5*A*). Analysis of freshly isolated macrophages from villi also showed that PL is present in round shaped intracellular vesicles (Fig. 7*D*). Together

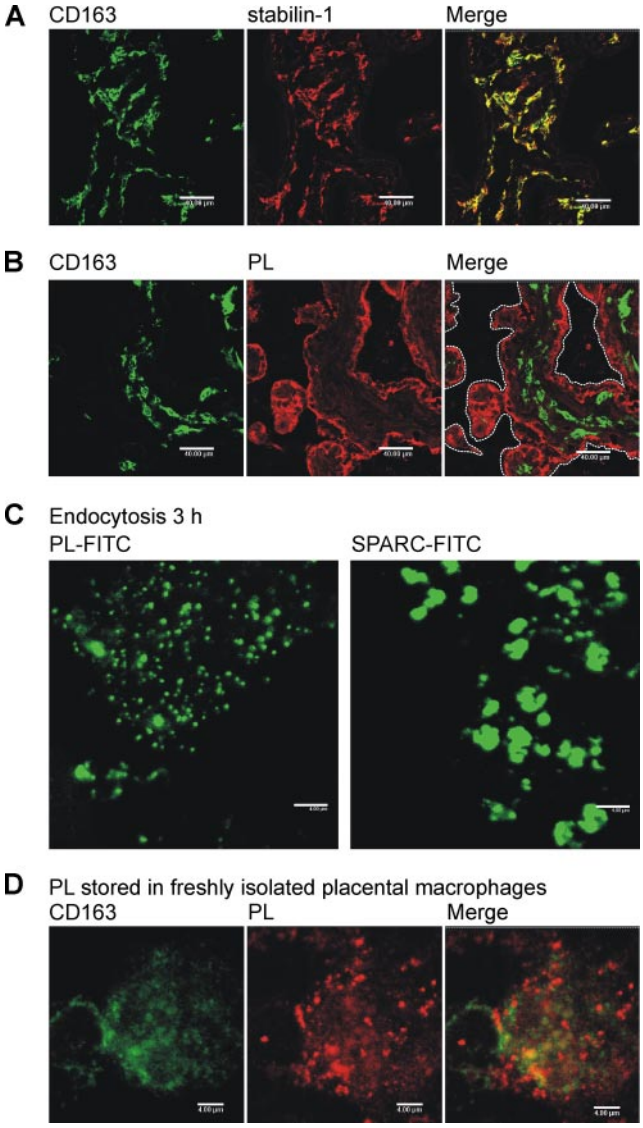

FIGURE 7. Placental macrophages process PL. *A*, Human term placenta frozen tissue sections were analyzed by double immunofluorescence and confocal microscopy. Both stabilin-1 and CD163 are expressed in the same Hofbauer cells. *B*, Hofbauer cells are localized inside villi closely to the PL-producing trophoblast cell layer (red), whereas another side of the trophoblast layer is exposed to the maternal circulation (dotted line indicates border between the trophoblast layer and maternal circulation). *C*, Hofbauer cells isolated from human villi endocytose both PL-FITC and SPARC-FITC. PL-FITC is sorted into secretory-like vesicles. SPARC is accumulated in enlarged endosomes. Hofbauer cells immediately after isolation from human placental villi contain PL in intracellular vesicles (visualized in red). Abs used are the following: rabbit anti-stabilin-1 RS1; rabbit anti-PL, mouse anti-CD163, Cy2-labeled anti-mouse, and Cy3-labeled anti-rabbit. Scale bar indicates 40 μm in *A* and *B* and 4 μm in *C* and *D*.

these data support our hypothesis that placental villi macrophages, i.e., Hofbauer cells mediate the uptake and processing of PL secreted by the trophoblast cells.

Discussion

Stabilin-1 is a multifunctional receptor that mediates endocytosis of extracellular factors such as acLDL and SPARC as well as intracellular sorting of cargo proteins such as the lysosomal protein SI-CLP (18). Stabilin-1 is abundantly expressed in various subpopulations of M2 macrophages, including placental macrophages (5, 8, 10, 15, 18), but placenta-specific ligands of stabilin-1 have not been described. In this study, we have shown that the hormone PL produced by trophoblast cells is an endocytic ligand for stabilin-1. We further demonstrated that stabilin-1-positive macrophages are able to efficiently internalize PL and to degrade part of it within lysosomes. We found, however, that intracellular sorting of endocytosed PL is not restricted to the endosomal/lysosomal pathways. Part of PL transiently enters the TGN and is delivered to long-living PL storage vesicles. Placement of PL-fed macrophages containing endocytosed and nondegraded PL into PL-free medium leads to the secretion of intracellularly stored PL. Thus, alternatively activated macrophages that express stabilin-1 are able to regulate extracellular levels of PL via a complex mechanism that includes uptake followed by degradation or sorting to storage vesicles, and secretion (Fig. 6*B*).

PL belongs to the growth hormone/prolactin family of polypeptide regulators of pregnancy, postnatal growth and lactogenesis, acting both as a circulating hormone and as a local paracrine/autocrine factor (36). Primates produce a single PL involved in fetal growth regulation and mammogenesis (36). Human PL has 96% similarity to growth hormone and is produced by cytotrophoblast and syncytiotrophoblast cells. Human PL is detectable in the placenta by day 18 of pregnancy and can be detected in the maternal circulation by the third week. The concentration of hPL increases exponentially in the maternal circulation during the first trimester and gradually until term reaching values of 5–10 μg/ml. Investigation of a variety of growth factors and other mediators did not reveal the regulatory mechanism for hPL secretion. Currently, PL synthesis and secretion in placenta are thought to be constitutive. In contrast to the high concentration of hPL in maternal blood, hPL concentration in fetal blood ranges from 4 to 500 ng/ml between weeks 12 and 20 of gestation, and is only 20–30 ng/ml at term (36). There is no direct transfer of hPL from the maternal to the fetal circulation, and separate routes must exist for hPL to enter the maternal and fetal vascular compartments. However the mechanism by which hPL is routed either into maternal or fetal vessels is unknown.

In vivo stabilin-1 is expressed in Hofbauer cells, which are localized between the PL-producing trophoblast cells and the fetal vessels. We have provided evidence that Hofbauer cells are able to endocytose PL and sort it into secretory-like vesicles. Our findings indicate that stabilin-1-positive macrophages are able to internalize and degrade PL thereby decreasing the extracellular concentration of PL. Moreover, stabilin-1-positive macrophages are able to direct endocytosed PL into storage vesicles, and to secrete PL into the extracellular space. Thus, stabilin-1 positive macrophages possess two mechanisms for the regulation of extracellular concentrations of PL. We assume that there are multiple factors, including anatomical location, neighboring cells as well as soluble mediators that might contribute to the balance of these two intracellular processing pathways for PL in macrophages. We hypothesize that the multiple layers of decidual and villous macrophages in placenta create a buffering system for the regulation of PL levels in the maternal and fetal circulation.

In addition, PL was suggested to regulate reorganization and growth of maternal and fetal blood vessels (39). A precise balance of pro- and antiangiogenic factors in placenta is an absolute prerequisite for the development of a healthy infant. Both decreased concentrations of PL and impaired placental angiogenesis are associated with intrauterine growth restriction, which is a significant cause of infant mortality and morbidity (40, 41). Moreover, intrauterine growth restriction infants have an increased risk of developing coronary heart disease, type-2 diabetes, hypertension, and stroke. Previously, we have shown that alternatively activated

macrophages use stabilin-1 for the endocytosis and degradation of SPARC, a complex regulator of angiogenesis and tissues remodelling (14). Taken together, stabilin-1-positive macrophages determine extracellular levels of PL and of SPARC, and may thus play a complex role in optimization of angiogenic regulators.

Stabilin-1 seems to play a central role in PL processing by macrophages. Being expressed on the cell surface, it serves as a sensor for the actual concentration of PL in the extracellular space. We have shown that stabilin-1 is able to direct endocytic ligands into the lysosomal degradation pathway (14, 18). At the same time, stabilin-1 shuttles between endosomes and the TGN (25), and we found in this study that stabilin-1 is colocalized with PL in the TGN. In the TGN, PL^+ vesicles may undergo exchange of membrane components and acquire surface determinants necessary for the secretion. Thus, we suggest that stabilin-1 is an important regulatory protein in deciding the amount of PL targeted for degradation and the amount of PL delivered to the TGN. Thus the ability of stabilin-1 to target ligands to the TGN points toward the existence of a novel mechanism of transcytosis of extracellular ligands. Transcytosis of macromolecules seems not to be restricted to the stabilin-1-PL receptor-ligand pair. For example placental macrophages have been shown to endocytose and store extracellular human chorionic gonadotropin (42). Secretion of stored human chorionic gonadotropin, however, was not investigated, and the receptor responsible for intracellular trafficking of human chorionic gonadotropin has still to be identified (43). Another example of transcytosis has been reported in fully differentiated peripheral blood neutrophils. In contrast to granulocyte precursors during differentiation, mature neutrophils do not express haptoglobin mRNA or protein; however, they internalize, store and resecrete haptoglobin (44, 45). In this experiment, we show for the first time that transcytosis involves a step of transient interaction of ligand-containing vesicles with the TGN.

In summary, we have identified the hormone PL as a novel extracellular protein ligand of stabilin-1. In alternatively activated macrophages, stabilin-1 directs PL into alternative intracellular sorting pathways followed by degradation in lysosomes or storage and secretion. We suggest that the combination of these two pathways allows macrophages to regulate extracellular levels of PL and to create a buffering barrier for PL between the maternal and fetal circulation that is important for normal fetal development and for preparation of efficient lactation.

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Disclosures

The authors have no financial conflict of interest.

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