LRP1 controls TNF release via the TIMP-3/ADAM17 axis in endotoxin-activated macrophages.

Running title: LRP1 regulates macrophage TNF release

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Abbreviations used in this article: ADAM, a disintegrin and metalloproteinase; HSPG, heparan sulfate proteoglycan; LRP1, low-density lipoprotein receptor-related protein 1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; RAP, receptor-associated protein; TACE, TNF-α-converting enzyme; TIMP-3, tissue inhibitor of metalloproteinases 3.

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Abstract: The metalloproteinase ADAM17 plays a pivotal role in initiating inflammation by releasing TNF from its precursor. Prolonged TNF release causes many chronic inflammatory diseases, indicating that tight regulation of ADAM17 activity is essential for resolution of inflammation. Here, we report that the endogenous ADAM17 inhibitor TIMP-3 inhibits ADAM17 activity only when it is bound to the cell surface, and that cell surface levels of TIMP-3 in endotoxin-activated human macrophages are dynamically controlled by the endocytic receptor LRP1. Pharmacological blockade of LRP1 inhibited endocytic clearance of TIMP-3, leading to an increase in cell surface levels of the inhibitor that blocked TNF release. Following LPS stimulation, TIMP-3 levels on the surface of macrophages increased 4-fold within 4 hours and continued to accumulate at 6 h, before a return to baseline levels at 8 h. This dynamic regulation of cell surface TIMP-3 levels was independent of changes in TIMP-3 mRNA levels, but correlated with shedding of LRP1. These results shed light on the basic mechanisms that maintain a regulated inflammatory response and ensure its timely resolution.

Introduction

A disintegrin and metalloproteinase 17 (ADAM17) regulates paracrine and autocrine immune signalling, cell proliferation, tissue regeneration and cancer progression through its ability to cleave or 'shed' the transmembrane precursors of multiple cytokine and growth factor proteins and receptors. Key ADAM17 substrates include the precursor form of the inflammatory cytokine TNF, giving the enzyme its alternative name of TNF-α-converting enzyme (TACE) (1-4). While the transmembrane TNF precursor has biological activity, *in vivo* studies indicate that the soluble form of the cytokine plays indispensable roles in initiating paracrine signalling events central to acute inflammatory responses (5, 6).

ADAM17 activity is rapidly induced in response to LPS or PMA stimulation, without any increase in its cell surface expression. Many mechanisms have been proposed to explain this phenomenon, including compartmentalisation of the enzyme in membrane micro-domains (7, 8) and conformational changes in the ADAM17 ectodomain (9, 10), postulated to involve protein disulfide isomerase (10, 11) and/or interaction of juxta-membrane regions of ADAM17 with the lipid bilayer (12). Phosphorylation of the intracellular domain of ADAM17 affects activity in some cases (13-17), but not all (9). ADAM17 activity may also be regulated by substrate availability, either by localisation (7), dimerization (18), intracellular domain phosphorylation (19) or glycosylation (20). Regulation of ADAM17 intracellular trafficking by iRhoms (21-23) and the sorting protein PACS2 (24) also contribute to the inter-related network of molecular mechanisms regulating ADAM17 activation.

Prolonged or increased TNF release is associated with numerous inflammatory conditions, such as septic shock (25), rheumatoid arthritis (26), colitis (27, 28) and Crohn's disease (29), indicating that ADAM17 activity must be tightly regulated. Although activation of ADAM17 has been studied in detail, mechanisms controlling resolution of ADAM17 activity are less well understood.

Tissue inhibitor of metalloproteinases 3 (TIMP-3) inhibits ADAM17 activity by forming a high affinity 1:1 stoichiometric complex with the enzyme (30). *Timp3*-null mice exhibit increased TNF release (5, 31-33) and increased susceptibility to TNF-associated tissue damage in models of diabetes (31), atherosclerosis (34), kidney fibrosis (33), lung injury (35) and liver regeneration (5). These studies indicate that TIMP-3 is the primary physiological inhibitor of ADAM17 and that TIMP-3 is critical for termination of TNF release.

We have previously demonstrated that extracellular levels of TIMP-3 in chondrocytes are primarily regulated post-translationally, by endocytosis via the endocytic scavenger receptor low-density lipoprotein receptor-related protein 1 (LRP1) and subsequent degradation in lysosomes (36-38). Since LRP1 is also highly expressed in macrophages (39, 40), we investigated whether LRP1 also controls TIMP-3 levels in these cells and whether LRP1 has an impact on the duration of ADAM17 activity and TNF release. By studying primary human macrophages stimulated with LPS, we were able to gain insight into physiological TIMP-3 and ADAM17 regulation that has not been observed in previous studies using macrophage-like cell lines (e.g. THP-1 or U937) or non-physiological stimuli (e. g. PMA). We found that LPS-induced shedding of LRP1 from activated macrophages led to an accumulation of TIMP-3 on the cell surface, and consequent inhibition of TNF release. LRP1 thus controls the duration of ADAM17 activity by regulating the abundance of its inhibitor, and contributes to the regulated resolution of inflammation.

Materials and methods

Materials

FCS was from Life Technologies, RPMI-1640 medium from PAA Laboratories, recombinant human GM-CSF, M-CSF and IL-4 were from Peprotech. Ultra-pure LPS from *E. coli* 0111:B4 was from Invivogen. Recombinant human TIMP-3, TIMP-3 blocking antibody (catalogue number MAB973, clone number 183551) and IL-6 receptor ELISA kit were from R&D Systems. For immunoblotting, the TIMP-3 antibody (AB6000) was from Merck Millipore, and the actin antibody (ab3280) from Abcam. LRP1 blocking antibody (01-05) was purchased from BioMac. Porcine mucosal heparin, PMA and ionomycin were from Sigma-Aldrich (St Louis, MO, USA). Receptor associated protein (RAP) was expressed in *E. coli* and purified as previously described (41), with addition of a Triton X-114 washing step to remove LPS (42). Removal of LPS was confirmed by the Limulus Amebocyte Lysate assay (Pierce).

Cell isolation and culture

Enriched populations of human monocytes and lymphocytes were isolated from the blood of healthy donors by elutriation. Macrophages were differentiated from monocytes by 5 days of culture in RPMI-1640 medium supplemented with 50 ng/ml GM-CSF and 20 % FCS, or 100 ng/ml M-CSF and 5 % FCS. Dendritic cells were differentiated from monocytes by 6 days of culture in RPMI-1640 medium supplemented with 5 % FCS, 50 ng/ml GM-CSF and 10 ng/ml IL-4. CD4+ T cells were obtained from enriched lymphocytes populations by negative magnetic separation (Miltenyi Biotec) and cultured in RPMI-1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. CD4+ T cells were stimulated with 20 ng/ml PMA and 0.5 µg/ml ionomycin for 4 h. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega) according to the manufacturer's instructions.

LRP1 blockade

Cells were treated for 16 h with RAP (1 µM), heparin (200 µg/ml, Sigma-Aldrich), LRP1 blocking antibody (10 µg/ml, BioMac), TIMP-3 antibody (10 µg/ml, catalogue number 183551, R&D

Systems) or matching isotype controls (10 µg/ml) in RPMI-1640 supplemented with 10 % FCS for 16 h. Cells were then stimulated with LPS (100 ng/ml) for 4 h.

Measurement of cytokine production

Cells were stimulated with LPS (100 ng/ml) in RPMI-1640 supplemented with 10 % FCS for 1 - 48 h. Media were harvested and TNF secretion quantified using the OptEIA ELISA kit for human TNF (BD Bioscience). The rate of TNF release per hour was estimated from cumulative TNF release using the formula: $\Delta\mathrm{TNF/hour} = \frac{[\mathrm{TNF}]x - [\mathrm{TNF}]y}{x-y}$, where x and y represent successive time points.

Flow cytometry

Macrophages were detached by incubating in 2 mM EDTA/PBS for 10 min at 37 °C. Fc receptors were blocked using 10% human AB serum. Cells were stained with fixable viability dye (eBioscience), conjugated antibodies against ADAM17 (FAB9301F, R&D Systems), LRP-1 α-chain (12-0919-42, eBioscience), LRP1 β-chain (FAB6360A, R&D Systems), membrane TNF (HM2024F, Hycult Biotech) or matching isotope controls for 30 min at 4 °C, washed and fixed. The antibody against the LRP1 β-chain recognises the intracellular portion of the β-chain, and cells were thus permeabilised using the Cytofix/Cytoperm Kit (BD Bioscience) according to manufacturer's instructions. Samples were then analyzed on an LSRII (BD Bioscience) and viable, single cells were analyzed with FlowJo software (TreeStar).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells with the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions, chromosomal DNA was eliminated using DNase I digestion (Qiagen), then cDNA was synthesized from 500 ng total RNA with a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Gene expression was measured by the change-in-threshold (ΔΔCt) method, based on quantitative real-time PCR in a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with TaqMan primer sets for human TNF, TIMP-3, LRP1, and RPLP0 (Applied Biosystems) and 5 µl of a 1:5 dilution of the 20 µl cDNA reaction.

Surface biotinylation and TIMP-3 immunoblotting

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154 $1x10^7$ macrophages were washed 3 times in ice-cold PBS, and incubated in 1 mg/ml Sulfo-NHS-
155 LC-Biotin (Thermo Fisher Scientific) for 30 min on ice. To quench excess unbound biotin, cells
156 were washed 3 times in 100 mM glycine in PBS. Cells were lysed in RIPA buffer [50 mM Tris-
157 HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, containing
158 complete protease inhibitor cocktail (Roche) and 10 mM 1,10-phenanthroline (Sigma-Aldrich)] and
159 total protein concentration was determined by DC-Protein Assay (Bio-Rad). Biotinylated surface
160 proteins were isolated from 500 µg of total cell lysate by adding high capacity avidin agarose beads
161 (Pierce) to the lysates (overnight, 4 °C). The avidin agarose beads were washed 3 times with RIPA
162 buffer containing 300 mM NaCl, and bound proteins eluted with SDS sample buffer (50 µl). For
163 analysis of TIMP-3 in conditioned medium, cells were treated in serum-free medium and proteins
164 concentrated by addition of 5 % trichloroacetic acid to the harvested medium before
165 immunoblotting. Samples were resolved on a NuPage 4-20% Tris-glycine gel (Invitrogen) and
166 transferred to PVDF using a TransBlot Turbo blotting system (BioRad). Blots were blocked in 5%
167 milk powder in PBS, and TIMP-3 detected using a rabbit polyclonal antibody (AB6000, Merck
168 Millipore).

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170 *Statistical analysis*

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172 Statistical analysis was carried out with GraphPad Prism 6.0. Data are shown as mean + SEM.
173 Mean values were compares using two-tailed student's *t*-test or Mann-Whitney tests as appropriate.
174 *P* values of < 0.05 were considered statistically significant.

Results

Blockade of LRP1 endocytic pathway increases cell surface TIMP-3

To evaluate if LRP1 regulates TIMP-3 levels in macrophages, we treated GM-CSF-differentiated primary human macrophages with recombinant RAP, an antagonist of ligand binding to LRP receptors, and measured levels of TIMP-3 on the cell surface and in the conditioned medium. Cell surface levels of TIMP-3 increased after 4 h of LPS stimulation, and were further elevated by blockade of the LRP1 pathway with RAP (Fig. 1A). RAP had no effect on cell surface TIMP-3 levels in unstimulated cells (Fig. 1A) or on levels of TIMP-3 in the conditioned medium (Fig. 1B).

TIMP-3 has been shown to bind to heparan sulfate proteoglycans and can be solubilised from these binding sites by heparin (43). We found that heparin reduced levels of TIMP-3 on the cell surface and greatly increased levels of the inhibitor in the medium of GM-CSF-differentiated primary human macrophages (Fig. 1A, B). This suggests that heparin redistributes TIMP-3 from the cell surface into the medium, and in so doing, blocks its endocytosis by LRP1 and increases total TIMP-3 levels.

The increase in cell surface TIMP-3 upon RAP treatment correlated with a marked inhibition of TNF release in response to LPS (Fig. 2A). Blockade of LRP1 with RAP similarly inhibited TNF release in primary human monocytes, M-CSF-differentiated macrophages and dendritic cells (Suppl. Fig. 1). RAP also inhibited ADAM17-dependent shedding of the IL-6 receptor (Fig. 2B). Heparin had no effect on ADAM17 shedding of TNF (Fig. 2A) or IL-6R (Fig. 2B), indicating that TIMP-3/heparin complexes in the medium are unable to inhibit cell surface ADAM17 activity.

RAP and heparin had no effect on cell viability (Fig. 2C), or on cell surface levels of proTNF (Fig. 2D) or ADAM17 (Fig. 2E, F).

The effects of RAP could be recapitulated by treating cells with an LRP1 blocking antibody, which dose-dependently inhibited TNF release (Fig. 3A). Conversely, the ability of RAP (Fig. 3B) and the LRP1 blocking antibody (Fig. 3C) to inhibit TNF release were abrogated by co-treatment with a TIMP-3 blocking antibody.

LRP1 blockade thus increased levels of TIMP-3 on the surface of LPS-stimulated macrophages and inhibited ADAM17-mediated release of TNF.

TIMP-3 accumulation on the cell surface correlates with the drop in TNF release

We next investigated the dynamics of TIMP-3 regulation in LPS-stimulated macrophages. Stimulation of GM-CSF-differentiated primary human macrophages with LPS induced release of TNF from the cells, with maximal levels of the cytokine accumulating in the medium 8 hours after stimulation (Fig. 4A). From these data, the rate of TNF release per hour was calculated, and found to peak 2 h after addition of LPS (Fig. 4B).

Flow cytometry analysis indicated that cell surface levels of ADAM17 were unchanged by LPS stimulation (Fig. 4C). Using surface biotinylation, we observed that the amount of TIMP-3 on the surface of activated macrophages increased 4-fold within 4 hours of stimulation, and returned to baseline levels by 8 h after LPS addition (Fig. 4D). This dynamic regulation within the first 8 hours after stimulation was not accompanied by any change in TIMP-3 mRNA levels, although reduced mRNA expression was observed 24 hours after stimulation (Fig. 4E).

The anti-inflammatory cytokine IL-10 has previously been shown to inhibit TNF release by both TIMP-3-dependent and -independent mechanisms (44). We found that IL-10 and RAP inhibited TNF release additively (Fig. 4F), suggesting they act through different mechanisms.

LRP1 is shed from the cell surface during resolution of inflammation.

LRP1-mediated endocytosis can be regulated by shedding of the receptor, as it reduces the endocytic capacity of the cell and the released LRP1 ectodomains act as decoy receptors (36, 45, 46). Shedding of LRP1 is mediated by proteases that cleave in the juxta-membrane region of the short transmembrane β-chain of LRP1, releasing the large ligand-binding α-chain from the cell.

We found that LPS stimulation decreased levels of the LRP1 α-chain on the surface of LPS-stimulated macrophages (Fig. 5A, B), while levels of the LRP1 β-chain were not affected (Fig. 5C). The α- and β-chain are generated by cleavage of a single polypeptide chain, and mRNA expression of this LRP1 precursor was similarly not affected by LPS treatment (Fig. 5D). This is consistent with previous reports showing that LPS induces metalloproteinase-dependent shedding of the LRP1 ectodomain (46-49).

Regulation of TIMP-3 by LRP1 is cell type-dependant

We examined whether LRP1 regulates TIMP-3 in other primary human immune cell types. Expression of LRP1 was high in monocytes, dendritic cells, GM-CSF- and M-CSF-differentiated macrophages (Fig. 6A), with lower expression in CD4+ T-cells (Fig. 6A). The pattern of TIMP-3

241 expression was similar, being highest in GM-CSF- and M-CSF-differentiated macrophages, and
242 lowest in CD4+ T-cells (Fig. 6B). Treatment of CD4+ T-cells with PMA/ionomycin stimulated
243 them to release TNF, and this was inhibited by addition of 100 nM recombinant TIMP-3 (Fig. 6C),
244 confirming that ADAM17 is responsible for the release. However, RAP had no effect on TNF
245 release from CD4+ T-cells stimulated with PMA/ionomycin (Fig. 6D) or anti-CD3/anti-CD28 (not
246 shown). Similarly, TNF release from CD4+ T-cells was not inhibited by an anti-LRP1 blocking
247 antibody (Fig. 6E) or an anti-TIMP-3 antibody (Fig. 6F). Equivalent results were observed in CD8+
248 T-cells (not shown). This suggests that LRP1 regulates TIMP-3 levels in myeloid lineages but not
249 in lymphoid lineages.

Discussion

Prolonged TNF release is associated with multiple chronic inflammatory conditions, indicating that mechanisms modulating ADAM17 activity are important for maintaining a regulated inflammatory response.

TIMP-3 is a negative regulator of ADAM17, with *Timp3*-null mice showing increased TNF release and susceptibility to TNF-dependent tissue damage in a variety of models, such as chronic hepatic inflammation (5), vasculitis (31), cardiac remodelling after myocardial infarction (32), interstitial nephritis and fibrosis (33), atherosclerosis in ApoE-null mice (34), and bleomycin-induced lung injury (35). Here, we have shown that the endocytic receptor LRP1 dynamically regulates cell surface levels of TIMP-3 in activated macrophages, and that LRP1 contributes to the timely resolution of inflammatory responses. This temporal regulation is achieved by progressive shedding of LRP1 from the cell surface.

Our findings are in agreement with previous murine studies that indicated an anti-inflammatory role for LRP1 in macrophages. Targeted deletion of LRP1 in macrophages increased LPS-stimulated TNF release (39, 48) and increased susceptibility to atherosclerosis (39, 50-53). LRP1-deficient cells exhibit increased NF-κB activation (54), consistent with prolonged TNF release, and LRP1-deficient macrophages show an increased inflammatory phenotype (55). Our results have demonstrated that the underlying molecular mechanism for the anti-inflammatory role of LRP1 is its ability to regulate cell surface levels of TIMP-3 and thus to modulate TNF release.

We observed no decrease in cell surface levels of ADAM17 following LPS stimulation, in agreement with previous studies on LPS-stimulated monocytes (1) and THP-1 cells (56). PMA stimulation has been shown to stimulate endocytosis of ADAM17 in THP-1 cells (56), but PMA is a non-physiological activator that acts via protein kinase C, and not via the TLR4 pathway stimulated by LPS. To most closely recapitulate ADAM17 regulation in a physiological context, we thus restricted our study to LPS-stimulated primary human macrophages.

Blocking LRP1 had no effect on cell surface levels of TIMP-3 in the absence of LPS activation, suggesting that TIMP-3 does not bind to LRP1 in the resting state. Xu *et al*. (15) proposed that TIMP-3 associates with ADAM17 in an inactive complex under basal conditions, and that the TIMP-3/ADAM17 complex dissociates upon activation. We propose that the released TIMP-3 binds to LRP1 and is endocytosed during the phase of peak TNF release (Fig. 7). With a slower kinetic, LPS also induces LRP1 shedding, leading to an increase in cell surface levels of TIMP-3 by 4 h after stimulation, with consequent inhibition of ADAM17 activity and resolution of TNF release. TIMP-3 is known to bind to heparan sulfate proteoglycans (HSPG) (43), and can be

283 released from cells by heparinase treatment (57), indicating that it may accumulate on cell surface
284 HSPG once LRP1 is shed. Alterations in the sulfation of HSPG may thus affect the efficacy with
285 which TIMP-3 is retained and could be of particular importance in chronic inflammatory conditions
286 (58-60).

287 Shedding of LRP1 reduces the rate of ligand uptake both by reducing cell surface levels of the
288 receptor, and by the shed receptor acting as a decoy receptor for LRP1 ligands (36, 45, 46). LPS
289 was previously shown to stimulate shedding of LRP1 in macrophages, with shedding detected
290 within 30 minutes of the LPS treatment (46-48). Various LRP1 sheddases have been proposed,
291 including ADAM17 (46, 61) and other metalloproteinases such as ADAM10 (61), ADAM12 (62)
292 and MT1-MMP (62), the serine proteinase tissue plasminogen activator (tPA) (63) and the aspartyl
293 proteinase β-secretase (β site of amyloid precursor protein-cleaving enzyme, BACE) (64). It is
294 unclear whether different sheddases act in different tissues, and differences between constitutive
295 and induced shedding of LRP1 have not been systematically investigated. Gorovoy *et al*. (46)
296 reported that ADAM17 is responsible for LPS-induced shedding of LRP1 in RAW 264.7 cells,
297 suggesting that in macrophages, ADAM17 may initiate its own temporal regulation via LRP1 and
298 TIMP-3.

299 Our model in Fig. 7 also illustrates the importance of the LRP1/TIMP-3 axis, since disturbance
300 of this process would lead to sustained TNF release and prolonged inflammation. Increased
301 expression of LRP1, which may impair TIMP-3 accumulation, has been observed in chronic
302 inflammatory conditions such as atherosclerosis (65, 66) and multiple sclerosis (67). Increased
303 levels of shed LRP1 have also been reported in chronic inflammatory states such as atherosclerosis
304 (68), rheumatoid arthritis (46), systemic lupus erythematosus (46), acute respiratory distress
305 syndrome (69), and liver disease (45). We postulate that release of TNF is prolonged in these
306 conditions due to chronic exposure to activating stimuli, which leads to uninhibited ADAM17
307 activity on the cell surface. Such active ADAM17 may shed LRP1, and may lead to an increase of
308 cell surface TIMP-3, but at a level that is insufficient to block elevated ADAM17 activity. We have
309 recently engineered TIMP-3 mutants that are resistant to LRP1-mediated endocytosis and that have
310 a longer half-life in tissues (38). These mutants may help restore baseline levels of ADAM17
311 activity and promote resolution of inflammation in such conditions.

312 LRP1 does not universally regulate TIMP-3 levels and TNF release. Our results indicate that
313 LRP1 expression is low in T cells, and LRP1 blockade had no effect on their TNF release. TNF
314 expressed in different cell types is thought to have distinct and non-redundant functions, with
315 macrophage-derived TNF playing a dominant role in host defence, and both macrophage- and T-
316 cell-derived TNF contributing to tissue damage in an autoimmune hepatitis model (70, 71).

Understanding the cell-specific regulation of TNF release may enable us to develop strategies to block TNF release in a cell-selective rather than a systemic manner.

Resolution of inflammation is as important as its initiation, and dysregulated resolution is linked with numerous autoimmune diseases. In this study, we have demonstrated the new, previously unexplored concept that the endocytic receptor LRP1 contributes to resolution of inflammation by facilitating a dynamic increase in levels of TIMP-3 on the surface of activated macrophages, thereby terminating TNF release. These findings open new avenues for treating chronic inflammatory conditions.

Disclosures

The authors have no financial conflicts of interest.

References

1. Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, and D. P. Cerretti. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 385: 729-733.
2. Moss, M. L., S. L. Jin, M. E. Milla, D. M. Bickett, W. Burkhart, H. L. Carter, W. J. Chen, W. C. Clay, J. R. Didsbury, D. Hassler, C. R. Hoffman, T. A. Kost, M. H. Lambert, M. A. Leesnitzer, P. McCauley, G. McGeehan, J. Mitchell, M. Moyer, G. Pahel, W. Rocque, L. K. Overton, F. Schoenen, T. Seaton, J. L. Su, J. D. Becherer, and et al. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 385: 733-736.
3. Chalaris, A., N. Adam, C. Sina, P. Rosenstiel, J. Lehmann-Koch, P. Schirmacher, D. Hartmann, J. Cichy, O. Gavrilova, S. Schreiber, T. Jostock, V. Matthews, R. Hasler, C. Becker, M. F. Neurath, K. Reiss, P. Saftig, J. Scheller, and S. Rose-John. 2010. Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice. *J Exp Med* 207: 1617-1624.
4. Horiuchi, K., T. Kimura, T. Miyamoto, H. Takaishi, Y. Okada, Y. Toyama, and C. P. Blobel. 2007. Cutting edge: TNF-alpha-converting enzyme (TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock. *J Immunol* 179: 2686-2689.
5. Mohammed, F. F., D. S. Smookler, S. E. Taylor, B. Fingleton, Z. Kassiri, O. H. Sanchez, J. L. English, L. M. Matrisian, B. Au, W. C. Yeh, and R. Khokha. 2004. Abnormal TNF activity in Timp3-/- mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat Genet* 36: 969-977.
6. Black, R. A. 2004. TIMP3 checks inflammation. *Nat. Genet.* 36: 934-935.
7. Tellier, E., M. Canault, L. Rebsomen, B. Bonardo, I. Juhan-Vague, G. Nalbone, and F. Peiretti. 2006. The shedding activity of ADAM17 is sequestered in lipid rafts. *Exp Cell Res* 312: 3969-3980.

8. Ali, M., A. Saroha, Y. Pewzner-Jung, and A. H. Futerman. 2015. LPS-mediated septic shock is augmented in ceramide synthase 2 null mice due to elevated activity of TNFalpha-converting enzyme. *FEBS Lett* 589: 2213-2217.
9. Le Gall, S. M., T. Maretzky, P. D. Issuree, X. D. Niu, K. Reiss, P. Saftig, R. Khokha, D. Lundell, and C. P. Blobel. 2010. ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site. *J Cell Sci* 123: 3913-3922.
10. Dusterhoft, S., S. Jung, C. W. Hung, A. Tholey, F. D. Sonnichsen, J. Grotzinger, and I. Lorenzen. 2013. Membrane-proximal domain of a disintegrin and metalloprotease-17 represents the putative molecular switch of its shedding activity operated by protein-disulfide isomerase. *J Am Chem Soc* 135: 5776-5781.
11. Willems, S. H., C. J. Tape, P. L. Stanley, N. A. Taylor, I. G. Mills, D. E. Neal, J. McCafferty, and G. Murphy. 2010. Thiol isomerases negatively regulate the cellular shedding activity of ADAM17. *Biochem J* 428: 439-450.
12. Dusterhoft, S., M. Michalek, F. Kordowski, M. Oldefest, A. Sommer, J. Roseler, K. Reiss, J. Grotzinger, and I. Lorenzen. 2015. Extracellular Juxtamembrane Segment of ADAM17 Interacts with Membranes and Is Essential for Its Shedding Activity. *Biochemistry* 54: 5791-5801.
13. Schwarz, J., S. Schmidt, O. Will, T. Koudelka, K. Kohler, M. Boss, B. Rabe, A. Tholey, J. Scheller, D. Schmidt-Arras, M. Schwake, S. Rose-John, and A. Chalaris. 2014. Polo-like kinase 2, a novel ADAM17 signaling component, regulates tumor necrosis factor alpha ectodomain shedding. *J Biol Chem* 289: 3080-3093.
14. Killock, D. J., and A. Ivetic. 2010. The cytoplasmic domains of TNFalpha-converting enzyme (TACE/ADAM17) and L-selectin are regulated differently by p38 MAPK and PKC to promote ectodomain shedding. *Biochem J* 428: 293-304.
15. Xu, P., J. Liu, M. Sakaki-Yumoto, and R. Derynck. 2012. TACE activation by MAPK-mediated regulation of cell surface dimerization and TIMP3 association. *Sci Signal* 5: ra34.
16. Diaz-Rodriguez, E., J. C. Montero, A. Esparis-Ogando, L. Yuste, and A. Pandiella. 2002. Extracellular signal-regulated kinase phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: a potential role in regulated shedding. *Mol Biol Cell* 13: 2031-2044.
17. Zheng, Y., J. Schlondorff, and C. P. Blobel. 2002. Evidence for regulation of the tumor necrosis factor alpha-convertase (TACE) by protein-tyrosine phosphatase PTPH1. *J Biol Chem* 277: 42463-42470.
18. Hartmann, M., L. M. Parra, A. Ruschel, C. Lindner, H. Morrison, A. Herrlich, and P. Herrlich. 2015. Inside-out Regulation of Ectodomain Cleavage of Cluster-of-Differentiation-44 (CD44) and of Neuregulin-1 Requires Substrate Dimerization. *J Biol Chem* 290: 17041-17054.
19. Parra, L. M., M. Hartmann, S. Schubach, Y. Li, P. Herrlich, and A. Herrlich. 2015. Distinct Intracellular Domain Substrate Modifications Selectively Regulate Ectodomain Cleavage of NRG1 or CD44. *Mol Cell Biol* 35: 3381-3395.
20. Goth, C. K., A. Halim, S. A. Khetarpal, D. J. Rader, H. Clausen, and K. T. Schjoldager. 2015. A systematic study of modulation of ADAM-mediated ectodomain shedding by site-specific O-glycosylation. *Proc Natl Acad Sci U S A* 112: 14623-14628.
21. Adrain, C., M. Zettl, Y. Christova, N. Taylor, and M. Freeman. 2012. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. *Science* 335: 225-228.
22. McIlwain, D. R., P. A. Lang, T. Maretzky, K. Hamada, K. Ohishi, S. K. Maney, T. Berger, A. Murthy, G. Duncan, H. C. Xu, K. S. Lang, D. Haussinger, A. Wakeham, A. Itie-Youten, R. Khokha, P. S. Ohashi, C. P. Blobel, and T. W. Mak. 2012. iRhom2 regulation of TACE

controls TNF-mediated protection against Listeria and responses to LPS. *Science* 335: 229-232.

23. Issuree, P. D., T. Maretzky, D. R. McIlwain, S. Monette, X. Qing, P. A. Lang, S. L. Swendeman, K. H. Park-Min, N. Binder, G. D. Kalliolias, A. Yarilina, K. Horiuchi, L. B. Ivashkiv, T. W. Mak, J. E. Salmon, and C. P. Blobel. 2013. iRHOM2 is a critical pathogenic mediator of inflammatory arthritis. *J Clin Invest* 123: 928-932.
24. Dombernowsky, S. L., J. Samsoe-Petersen, C. H. Petersen, R. Instrell, A. M. Hedegaard, L. Thomas, K. M. Atkins, S. Auclair, R. Albrechtsen, K. J. Mygind, C. Frohlich, M. Howell, P. Parker, G. Thomas, and M. Kveiborg. 2015. The sorting protein PACS-2 promotes ErbB signalling by regulating recycling of the metalloproteinase ADAM17. *Nat Commun* 6: 7518.
25. Beutler, B., and A. Cerami. 1989. The biology of cachectin/TNF--a primary mediator of the host response. *Annu Rev Immunol* 7: 625-655.
26. Feldmann, M., F. M. Brennan, M. J. Elliott, R. O. Williams, and R. N. Maini. 1995. TNF alpha is an effective therapeutic target for rheumatoid arthritis. *Ann N Y Acad Sci* 766: 272-278.
27. Neurath, M. F., I. Fuss, M. Pasparakis, L. Alexopoulou, S. Haralambous, K. H. Meyer zum Buschenfelde, W. Strober, and G. Kollias. 1997. Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. *Eur J Immunol* 27: 1743-1750.
28. Targan, S. R., S. B. Hanauer, S. J. van Deventer, L. Mayer, D. H. Present, T. Braakman, K. L. DeWoody, T. F. Schaible, and P. J. Rutgeerts. 1997. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 337: 1029-1035.
29. van Dullemen, H. M., S. J. van Deventer, D. W. Hommes, H. A. Bijl, J. Jansen, G. N. Tytgat, and J. Woody. 1995. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* 109: 129-135.
30. Amour, A., P. M. Slocombe, A. Webster, M. Butler, C. G. Knight, B. J. Smith, P. E. Stephens, C. Shelley, M. Hutton, V. Knauper, A. J. Docherty, and G. Murphy. 1998. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett* 435: 39-44.
31. Federici, M., M. L. Hribal, R. Menghini, H. Kanno, V. Marchetti, O. Porzio, S. W. Sunnarborg, S. Rizza, M. Serino, V. Cunsolo, D. Lauro, A. Mauriello, D. S. Smookler, P. Sbraccia, G. Sesti, D. C. Lee, R. Khokha, D. Accili, and R. Lauro. 2005. Timp3 deficiency in insulin receptor-haploinsufficient mice promotes diabetes and vascular inflammation via increased TNF-alpha. *J Clin Invest* 115: 3494-3505.
32. Tian, H., M. Cimini, P. W. Fedak, S. Altamentova, S. Fazel, M. L. Huang, R. D. Weisel, and R. K. Li. 2007. TIMP-3 deficiency accelerates cardiac remodeling after myocardial infarction. *J Mol Cell Cardiol* 43: 733-743.
33. Kassiri, Z., G. Y. Oudit, V. Kandalam, A. Awad, X. Wang, X. Ziou, N. Maeda, A. M. Herzenberg, and J. W. Scholey. 2009. Loss of TIMP3 enhances interstitial nephritis and fibrosis. *J Am Soc Nephrol* 20: 1223-1235.
34. Stöhr, R., M. Cavalera, S. Menini, M. Mavilio, V. Casagrande, C. Rossi, A. Urbani, M. Cardellini, G. Pugliese, R. Menghini, and M. Federici. 2014. Loss of TIMP3 exacerbates atherosclerosis in ApoE null mice. *Atherosclerosis* 235: 438-443.
35. Gill, S. E., I. Huizar, E. M. Bench, S. W. Sussman, Y. Wang, R. Khokha, and W. C. Parks. 2010. Tissue inhibitor of metalloproteinases 3 regulates resolution of inflammation following acute lung injury. *Am. J. Pathol.* 176: 64-73.
36. Scilabra, S. D., L. Troeberg, K. Yamamoto, H. Emonard, I. Thogersen, J. J. Enghild, D. K. Strickland, and H. Nagase. 2013. Differential regulation of extracellular tissue inhibitor of metalloproteinases-3 levels by cell membrane-bound and shed low density lipoprotein receptor-related protein 1. *J Biol Chem* 288: 332-342.

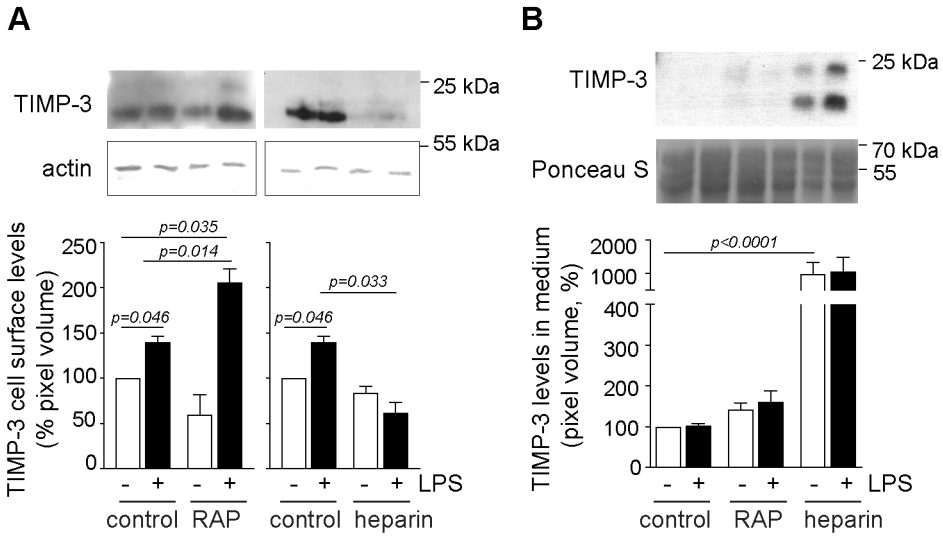
37. Troeberg, L., K. Fushimi, R. Khokha, H. Emonard, P. Ghosh, and H. Nagase. 2008. Calcium pentosan polysulfate is a multifaceted exosite inhibitor of aggrecanases. *FASEB J* 22: 3515-3524.
38. Doherty, C. M., R. Visse, D. Dinakarpandian, D. K. Strickland, H. Nagase, and L. Troeberg. 2016. Engineered Tissue Inhibitor of Metalloproteinases-3 Variants Resistant to Endocytosis Have Prolonged Chondroprotective Activity. *J Biol Chem* In press.
39. Overton, C. D., P. G. Yancey, A. S. Major, M. F. Linton, and S. Fazio. 2007. Deletion of macrophage LDL receptor-related protein increases atherogenesis in the mouse. *Circ Res* 100: 670-677.
40. Nilsson, A., L. Vesterlund, and P. A. Oldenborg. 2012. Macrophage expression of LRP1, a receptor for apoptotic cells and unopsonized erythrocytes, can be regulated by glucocorticoids. *Biochem Biophys Res Commun* 417: 1304-1309.
41. Yamamoto, K., L. Troeberg, S. D. Scilabra, M. Pelosi, C. L. Murphy, D. K. Strickland, and H. Nagase. 2013. LRP-1-mediated endocytosis regulates extracellular activity of ADAMTS-5 in articular cartilage. *FASEB J* 27: 511-521.
42. Reichelt, P., C. Schwarz, and M. Donzeau. 2006. Single step protocol to purify recombinant proteins with low endotoxin contents. *Protein Expr Purif* 46: 483-488.
43. Yu, W. H., S. Yu, Q. Meng, K. Brew, and J. F. Woessner, Jr. 2000. TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. *J Biol Chem* 275: 31226-31232.
44. Brennan, F. M., P. Green, P. Amjadi, H. J. Robertshaw, M. Alvarez-Iglesias, and M. Takata. 2008. Interleukin-10 regulates TNF-alpha-converting enzyme (TACE/ADAM-17) involving a TIMP-3 dependent and independent mechanism. *Eur J Immunol* 38: 1106-1117.
45. Quinn, K. A., P. G. Grimsley, Y. P. Dai, M. Tapner, C. N. Chesterman, and D. A. Owensby. 1997. Soluble low density lipoprotein receptor-related protein (LRP) circulates in human plasma. *J Biol Chem* 272: 23946-23951.
46. Gorovoy, M., A. Gaultier, W. M. Campana, G. S. Firestein, and S. L. Gonias. 2010. Inflammatory mediators promote production of shed LRP1/CD91, which regulates cell signaling and cytokine expression by macrophages. *J Leukoc Biol* 88: 769-778.
47. LaMarre, J., B. B. Wolf, E. L. Kittler, P. J. Quesenberry, and S. L. Gonias. 1993. Regulation of macrophage alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein by lipopolysaccharide and interferon-gamma. *J Clin Invest* 91: 1219-1224.
48. Zurhove, K., C. Nakajima, J. Herz, H. H. Bock, and P. May. 2008. Gamma-secretase limits the inflammatory response through the processing of LRP1. *Sci Signal* 1: ra15.
49. Yamamoto, K., S. Santamaria, K. A. Botkjaer, J. Dudhia, L. Troeberg, Y. Itoh, G. Murphy, and H. Nagase. 2017. Inhibition of shedding of low-density lipoprotein receptor-related protein 1 reverses cartilage matrix degradation in osteoarthritis. *Arthritis Rheumatol.* 69: 1246-1256.
50. Hu, L., L. S. Boesten, P. May, J. Herz, N. Bovenschen, M. V. Huisman, J. F. Berbee, L. M. Havekes, B. J. van Vlijmen, and J. T. Tamsma. 2006. Macrophage low-density lipoprotein receptor-related protein deficiency enhances atherosclerosis in ApoE/LDLR double knockout mice. *Arterioscler Thromb Vasc Biol* 26: 2710-2715.
51. Yancey, P. G., Y. Ding, D. Fan, J. L. Blakemore, Y. Zhang, L. Ding, J. Zhang, M. F. Linton, and S. Fazio. 2011. Low-density lipoprotein receptor-related protein 1 prevents early atherosclerosis by limiting lesional apoptosis and inflammatory Ly-6Chigh monocytosis: evidence that the effects are not apolipoprotein E dependent. *Circulation* 124: 454-464.
52. Lillis, A. P., S. C. Muratoglu, D. T. Au, M. Migliorini, M. J. Lee, S. K. Fried, I. Mikhailenko, and D. K. Strickland. 2015. LDL Receptor-Related Protein-1 (LRP1) Regulates Cholesterol Accumulation in Macrophages. *PLoS One* 10: e0128903.

53. Muratoglu, S. C., S. Belgrave, A. P. Lillis, M. Migliorini, S. Robinson, E. Smith, L. Zhang, and D. K. Strickland. 2011. Macrophage LRP1 suppresses neo-intima formation during vascular remodeling by modulating the TGF-beta signaling pathway. *PLoS One* 6: e28846.
54. Gaultier, A., S. Arandjelovic, S. Niessen, C. D. Overton, M. F. Linton, S. Fazio, W. M. Campana, B. F. Cravatt, 3rd, and S. L. Gonias. 2008. Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. *Blood* 111: 5316-5325.
55. May, P., H. H. Bock, and J. R. Nofer. 2013. Low density receptor-related protein 1 (LRP1) promotes anti-inflammatory phenotype in murine macrophages. *Cell Tissue Res* 354: 887-889.
56. Doedens, J. R., and R. A. Black. 2000. Stimulation-induced down-regulation of tumor necrosis factor-alpha converting enzyme. *J Biol Chem* 275: 14598-14607.
57. Koers-Wunrau, C., C. Wehmeyer, A. Hillmann, T. Pap, and B. Dankbar. 2013. Cell surface-bound TIMP3 induces apoptosis in mesenchymal Cal78 cells through ligand-independent activation of death receptor signaling and blockade of survival pathways. *PLoS One* 8: e70709.
58. Gowd, V., M. S. Sandeep, and D. N. Chilkunda. 2016. Effect of pathological conditions on peritoneal macrophage glycosaminoglycans: Impact on cytoadherence. *Int. J. Biol. Macromol.* 92: 1183-1190.
59. Papakonstantinou, E., I. Klagas, M. Roth, M. Tamm, and D. Stolz. 2016. Acute Exacerbations of COPD Are Associated With Increased Expression of Heparan Sulfate and Chondroitin Sulfate in BAL. *Chest* 149: 685-695.
60. Sabol, J. K., W. Wei, M. López-Hoyos, Y. Seo, A. Andaya, and J. A. Leary. Heparan sulfate differences in rheumatoid arthritis versus healthy sera. *Matrix Biol.* 40: 54-61.
61. Liu, Q., J. Zhang, H. Tran, M. M. Verbeek, K. Reiss, S. Estus, and G. Bu. 2009. LRP1 shedding in human brain: roles of ADAM10 and ADAM17. *Mol Neurodegener* 4: 17.
62. Selvais, C., L. D'Auria, D. Tyteca, G. Perrot, P. Lemoine, L. Troeberg, S. Dedieu, A. Noel, H. Nagase, P. Henriet, P. J. Courtoy, E. Marbaix, and H. Emonard. 2011. Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1 (LRP-1) and clearance function. *FASEB J* 25: 2770-2781.
63. Polavarapu, R., M. C. Gongora, H. Yi, S. Ranganthan, D. A. Lawrence, D. Strickland, and M. Yepes. 2007. Tissue-type plasminogen activator-mediated shedding of astrocytic low-density lipoprotein receptor-related protein increases the permeability of the neurovascular unit. *Blood* 109: 3270-3278.
64. von Arnim, C. A., A. Kinoshita, I. D. Peltan, M. M. Tangredi, L. Herl, B. M. Lee, R. Spoelgen, T. T. Hshieh, S. Ranganathan, F. D. Battey, C. X. Liu, B. J. Bacskai, S. Sever, M. C. Irizarry, D. K. Strickland, and B. T. Hyman. 2005. The low density lipoprotein receptor-related protein (LRP) is a novel beta-secretase (BACE1) substrate. *J Biol Chem* 280: 17777-17785.
65. Llorente-Cortés, V., M. Otero-Viñas, M. Berrozpe, and L. Badimon. 2004. Intracellular lipid accumulation, low-density lipoprotein receptor-related protein expression, and cell survival in vascular smooth muscle cells derived from normal and atherosclerotic human coronaries. *Eur. J. Clin. Investig.* 34: 182–190.
66. Luoma, J., T. Hiltunen, T. Särkioja, S. K. Moestrup, J. Gliemann, T. Kodama, T. Nikkari, and S. Ylä-Herttuala. 1994. Expression of alpha2-macroglobulin receptor/low density lipoprotein receptor-related protein and scavenger receptor in human atherosclerotic lesions. *J. Clin. Invest.* 93: 2014–2021.
67. Hendrickx, D. A., N. Koning, K. G. Schuurman, M. E. van Strien, C. G. van Eden, J. Hamann, and I. Huitinga. 2013. Selective upregulation of scavenger receptors in and around demyelinating areas in multiple sclerosis. *J. Neuropathol. Exp. Neurol.* 72: 106-118.

68. de Gonzalo-Calvo, D., A. Cenarro, M. Martínez-Bujidos, L. Badimon, A. Bayes-Genis, J. Ordonez-Llanos, F. Civeira, and V. Llorente-Cortés. 2015. Circulating soluble low-density lipoprotein receptor-related protein 1 (sLRP1) concentration is associated with hypercholesterolemia: A new potential biomarker for athereosclerosis. *Int J Cardiol* 201: 20-29.
69. Wygrecka, M., J. Wilhelm, E. Jablonska, D. Zakrzewicz, K. T. Preissner, W. Seeger, A. Guenther, and P. Markart. 2011. Shedding of low-density lipoprotein receptor–related protein-1 in acute respiratory distress syndrome. *Am J Respir Crit Care Med* 184: 438-448.
70. Grivennikov, S. I., A. V. Tumanov, D. J. Liepinsh, A. A. Kruglov, B. I. Marakusha, A. N. Shakhov, T. Murakami, L. N. Drutskaya, I. Forster, B. E. Clausen, L. Tessarollo, B. Ryffel, D. V. Kuprash, and S. A. Nedospasov. 2005. Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: protective and deleterious effects. *Immunity* 22: 93-104.
71. Winsauer, C., A. A. Kruglov, A. A. Chashchina, M. S. Drutskaya, and S. A. Nedospasov. 2014. Cellular sources of pathogenic and protective TNF and experimental strategies based on utilization of TNF humanized mice. *Cytokine Growth Factor Rev* 25: 115-123.

575 **FIGURE LEGENDS**

Figure 1



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578 **FIGURE 1.** LRP1 blockade increased levels of TIMP-3 on the cell surface of LPS-stimulated primary
579 human macrophages. Macrophages were differentiated *in vitro* from primary human monocytes by
580 treatment with GM-CSF (50 ng/ml) for 5 days and then treated with RAP (1 μM) or heparin (200
581 μg/ml) for 16 h, before stimulation with LPS (100 ng/ml) for 4 h. (**A**) Levels of TIMP-3 on the cell
582 surface were quantified by surface biotinylation and streptavidin pulldown, followed by
583 immunoblotting for TIMP-3. Actin levels in cell lysates were determined by immunoblotting of the
584 same samples. Blots show a representative experiment, and graph shows mean (+ SEM, n =3 donors)
585 TIMP-3 levels. (**B**) Levels of TIMP-3 in the conditioned medium (mean + SEM, n = 6 donors) were
586 quantified by immunoblotting. Blot shows representative experiment, with Ponceau S staining of the
587 same blot as a loading control.

Figure 2

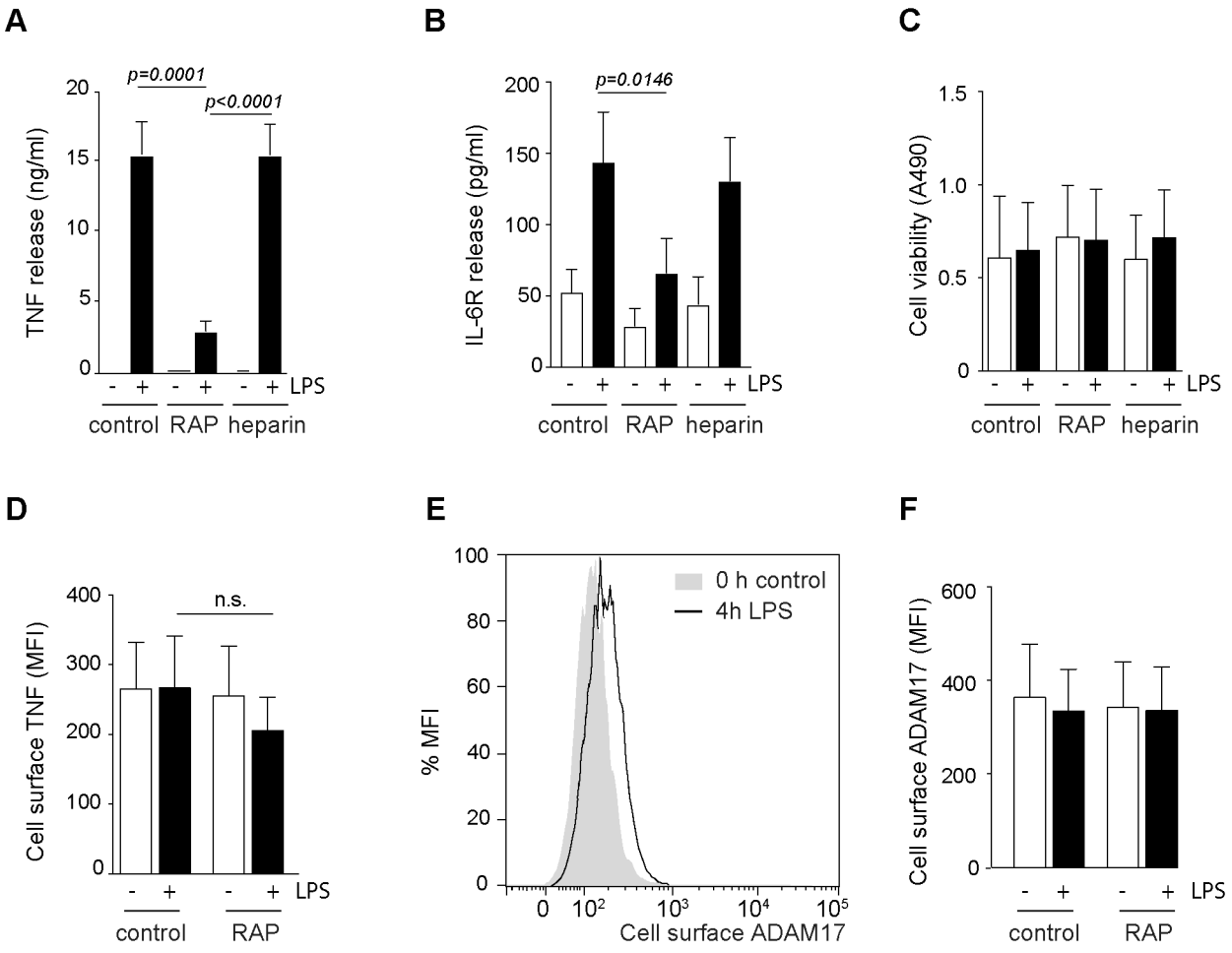


FIGURE 2. LRP1 blockade inhibited ADAM17 activity in LPS-stimulated macrophages. Macrophages were differentiated in vitro from primary human monocytes by treatment with GM-CSF (50 ng/ml) for 5 days and then treated with RAP (1 μM) or heparin (200 μg/ml) for 16 h, before stimulation with LPS (100 ng/ml) for 4 h. (**A**) TNF release was quantified by ELISA (mean + SEM, n = 7 donors). (**B**) IL-6 receptor (IL-6R) release was quantified by ELISA (mean + SEM, n = 8 donors). (**C**) Cell viability was determined by MTS assay (mean + SEM, n = 3 donors). (**D**) Levels of TNF on the cell surface (mTNF) were determined by flow cytometry, gated on single, viable cells (mean + SEM, n = 3 donors). (**E**) Representative experiment showing quantification of ADAM17 levels on the cell surface by flow cytometry. (**F**) Quantification of cell surface levels of ADAM17 by flow cytometry (mean + SEM, n = 3 donors).

Figure 3

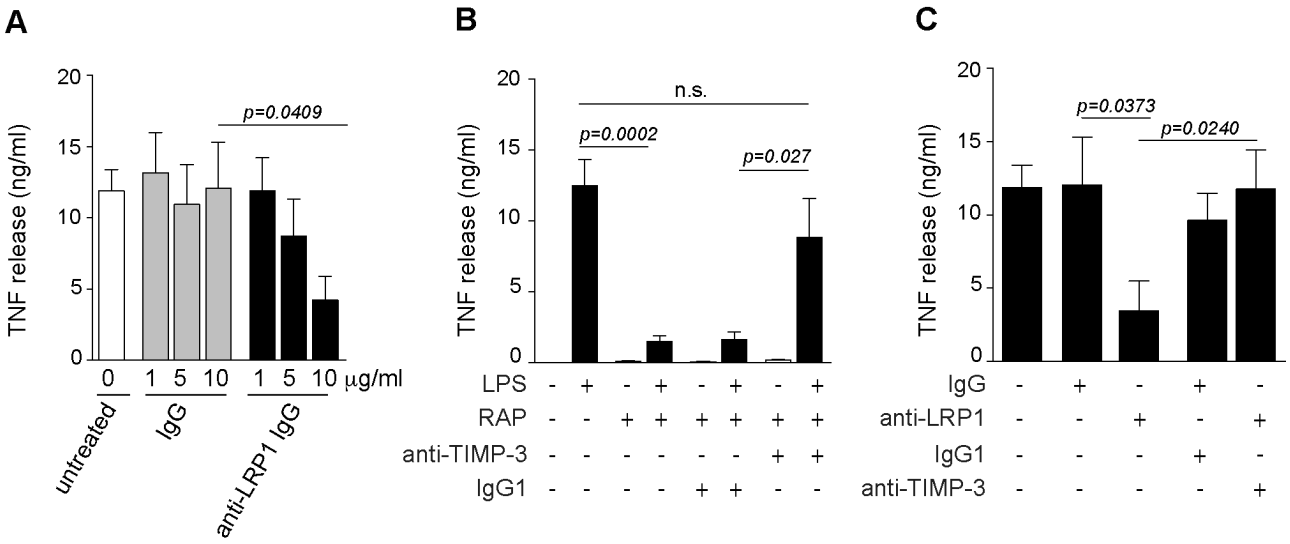


FIGURE 3. Effects of LRP1 blockade were abrogated by TIMP-3 blocking antibodies. Macrophages were differentiated in vitro from primary human monocytes by treatment with GM-CSF (50 ng/ml) for 5 days. (**A**) Macrophages were treated with anti-LRP-1 blocking IgG (1, 5 or 10 µg/ml) or the matching isotype control IgG (1, 5 or 10 µg/ml) for 16 h, and then treated with LPS (100 ng/ml) for 4 h. TNF release (mean + SEM, n = 3 donors) was quantified by ELISA. (**B**) Macrophages were treated with RAP (1 µM) and an anti-TIMP-3 antibody (10 µg/ml) or matching IgG1 isotype control for 16 h, and then treated with control medium or LPS (100 ng/ml) for 4 h. TNF release (mean + SEM, n = 6 donors) was quantified by ELISA. (**C**) Macrophages were treated for 16 h with anti-LRP1 blocking antibody, anti-TIMP-3 antibody (10 µg/ml) or matching isotype controls, and then treated with control medium or LPS (100 ng/ml) for 4 h. TNF release (mean + SEM, n = 3 donors) was quantified by ELISA.

Figure 4

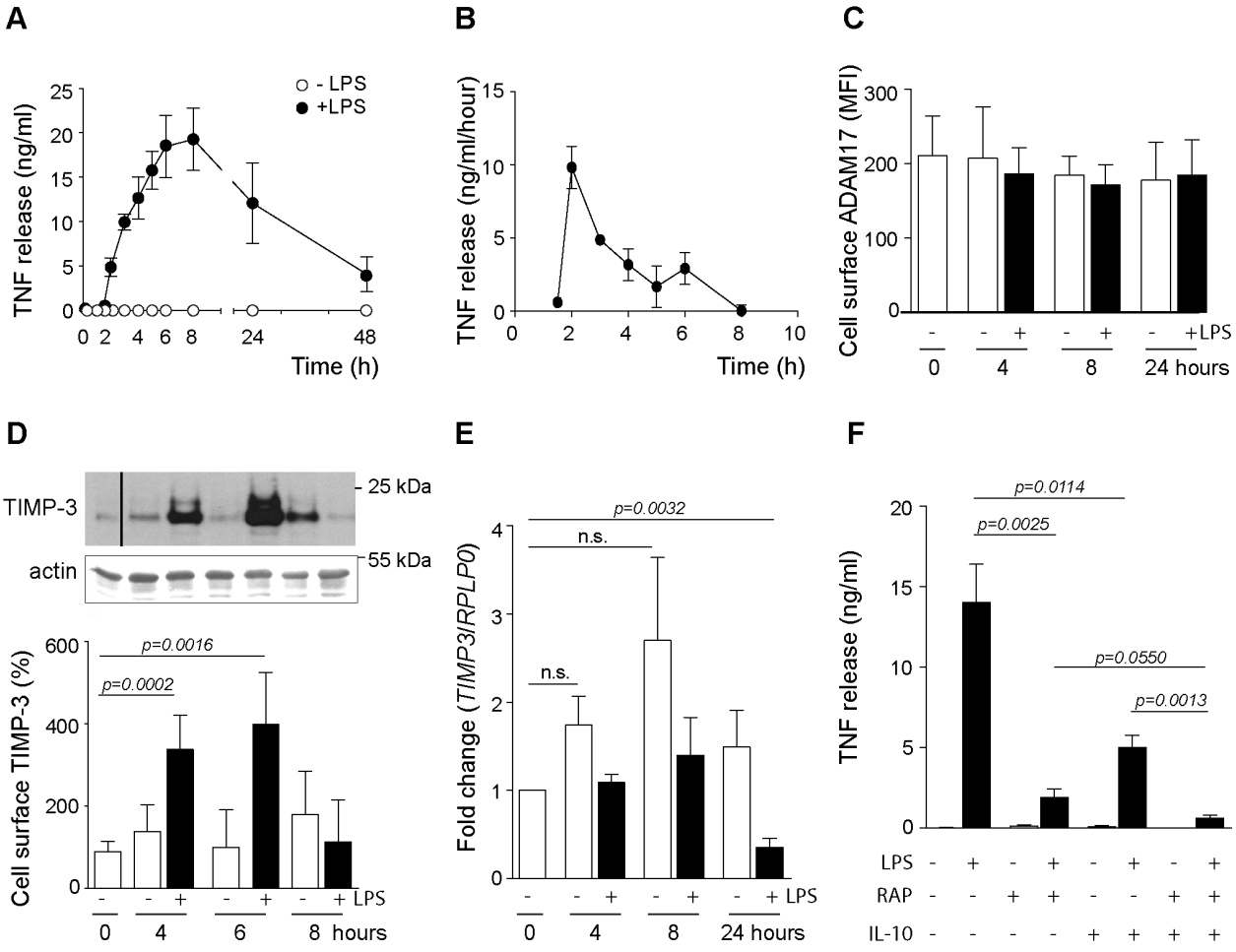


FIGURE 4. Time course of TNF release from LPS-stimulated macrophages. Macrophages were differentiated in vitro from primary human monocytes by treatment with GM-CSF (50 ng/ml) for 5 days. (**A** - **E**) Macrophages were treated with control medium or LPS (100 ng/ml) for the indicated time periods. (**A**) Cumulative TNF release (mean + SEM, n = 3 donors) was quantified by ELISA (❍, control; ●, LPS-treated). (**B**) From the data in **A**, the amount of TNF released per hour (mean + SEM, n = 3 donors) was calculated. **(C**) Cell surface levels of ADAM17 (mean + SEM, n = 3 donors) were determined by flow cytometry. (**D**) Levels of TIMP-3 on the cell surface were quantified by surface biotinylation and streptavidin pulldown, followed by immunoblotting for TIMP-3. Actin levels in cell lysates were determined by immunoblotting of the same samples. Blots show a representative experiment, and graph shows mean (+ SEM, n =3 donors) TIMP-3 levels, with TIMP-3 blot spliced as indicated. (**E**) *TIMP-3* mRNA expression was determined by RT-PCR, and expressed relative to *RPLP0* (mean + SEM, n = 3 donors). (**F**) Macrophages were treated with RAP (1 μM) and/or IL-10 (100

ng/ml) for 16 h, and then stimulated with control medium or LPS (100 ng/ml) for 4 h. TNF release (mean + SEM, n = 4 donors) was qualified by ELISA.

Figure 5

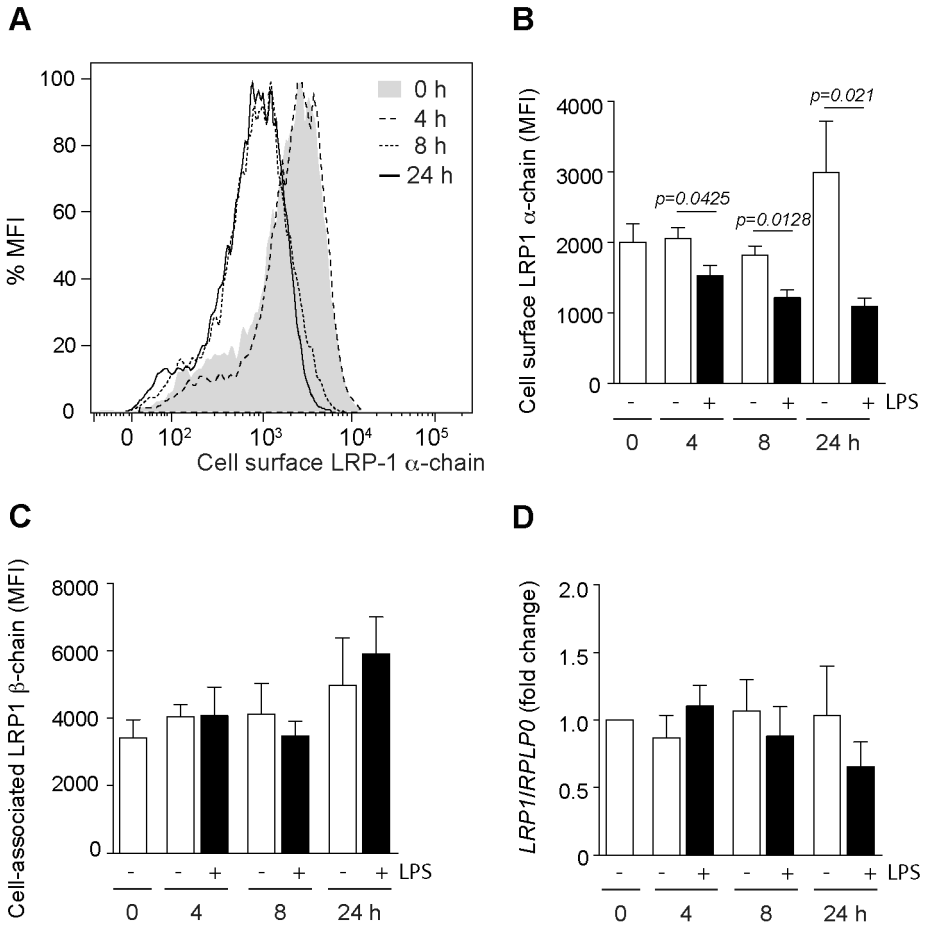


FIGURE 5. LPS induced shedding of LRP1 from macrophages. Macrophages were differentiated in vitro from primary human monocytes by treatment with GM-CSF (50 ng/ml) for 5 days, and then treated with control medium or LPS (100 ng/ml) for the indicated time periods and analyzed as described below. (**A**) Representative experiment showing flow cytometry analysis of LRP1 ectodomain (α-chain) on the macrophage cell surface. (**B**) LRP1 α-chain expression on the cell surface (mean + SEM, n = 5 donors) was quantified by flow cytometry. (**C**) LRP1 β-chain expression on the cell surface (mean + SEM, n = 4 donors) was quantified by flow cytometry. **(D**) *LRP1* expression (mean + SEM, n = 3 donors) was quantified by RT-PCR analysis and normalised to *RPLP0*.

Figure 6

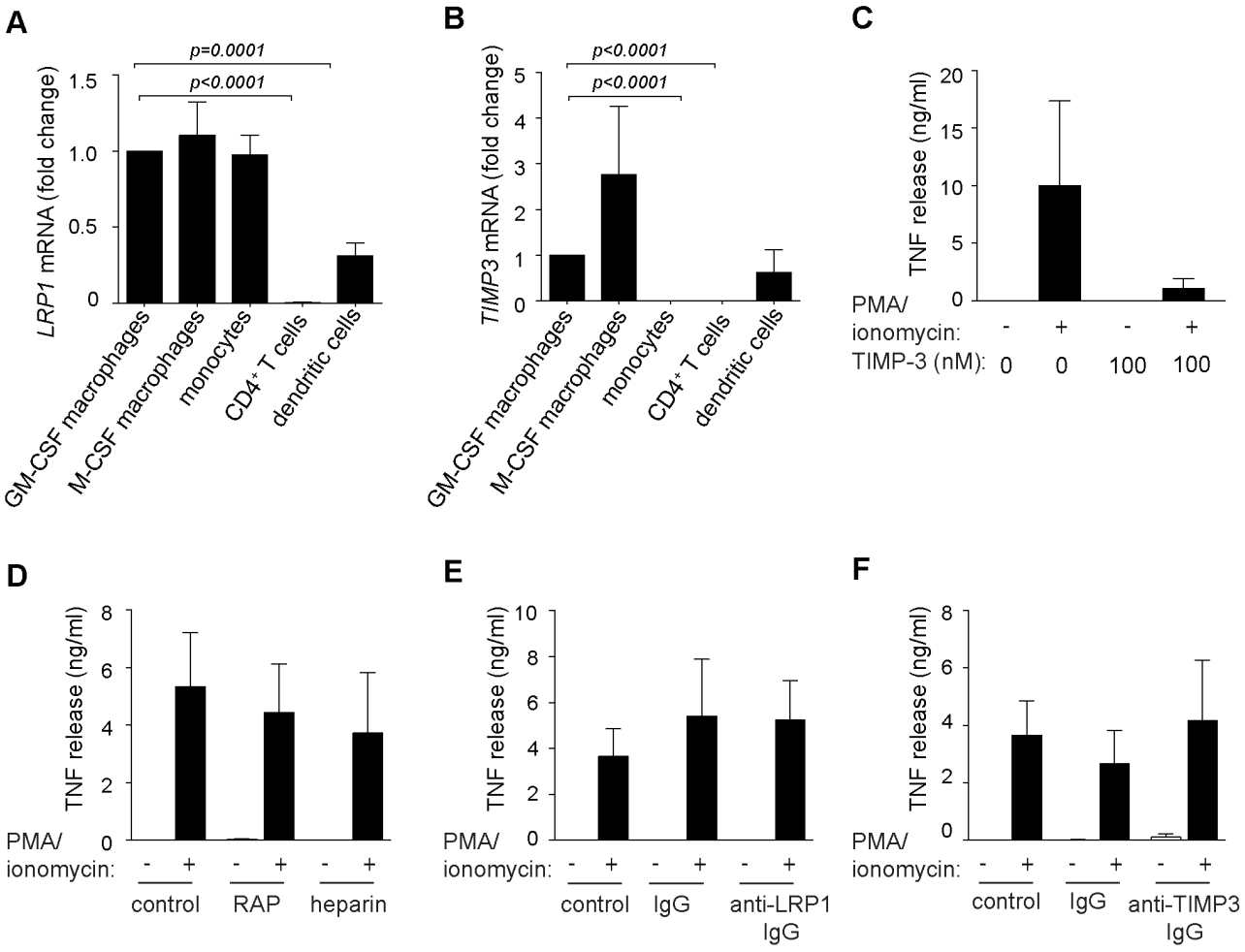


FIGURE 6. LRP1 blockade had no effect on TNF release from CD4+ T cells. CD4+ T cells were obtained from enriched lymphocytes populations by negative magnetic separation. (**A**) *LRP1* mRNA expression (mean + SEM, n = 4 donors) in GM-CSF macrophages (mean Ct = 21.62), M-CSF macrophages (mean Ct = 20.37), monocytes (mean Ct = 22.00), CD4+ T cells (mean Ct = 27.86) and dendritic cells (mean Ct = 22.28) was determined by RT-PCR and normalised to *RPLP0* (mean Ct values of 18.45 to 19.86). Results are shown relative to GM-CSF-differentiated macrophages, set as 1. (**B**) *TIMP-3* mRNA expression (mean + SEM, n = 4 donors) in GM-CSF macrophages (mean Ct = 20.29), M-CSF macrophages (mean Ct = 20.39), monocytes (mean Ct = 33.95), CD4+ T cells (mean Ct = 31.75) and dendritic cells (mean Ct = 22.27) was determined by RT-PCR and normalised to *RPLP0* (mean Ct values of 18.45 to 19.86). Results are shown relative to GM-CSF-differentiated macrophages, set as 1. (**C**) CD4+ T cells were stimulated for 4 h with PMA (20 ng/ml) and ionomycin (0.5 µg/ml) in the presence of purified recombinant TIMP-3 (0 or 100 nM, added 30 min after stimulation). TNF release (mean + SEM, n = 3 donors) was quantified by ELISA. (**D**) CD4+ T cells

were treated with RAP (1 μM) or heparin (0.2 μg/ml) for 16 h, and then stimulated with PMA (20 ng/ml) and ionomycin (0.5 μg/ml) for 4 h. TNF release (mean + SEM, n = 4 donors) was quantified by ELISA. (**E**) CD4+ T cells were treated with an anti-LRP1 blocking antibody or the matching isotype control (10 μg/ml, 16 h), and then stimulated with PMA (20 ng/ml) and ionomycin (0.5 μg/ml) for 4 h. TNF release (mean + SEM, n = 3 donors) was quantified by ELISA. (**F**) CD4+ T cells were treated with an anti-TIMP-3 blocking antibody or the matching isotype control (10 μg/ml, 16 h), and then stimulated with PMA (20 ng/ml) and ionomycin (0.5 μg/ml) for 4 h. TNF release (mean + SEM, n = 3 donors) was quantified by ELISA.

Figure 7

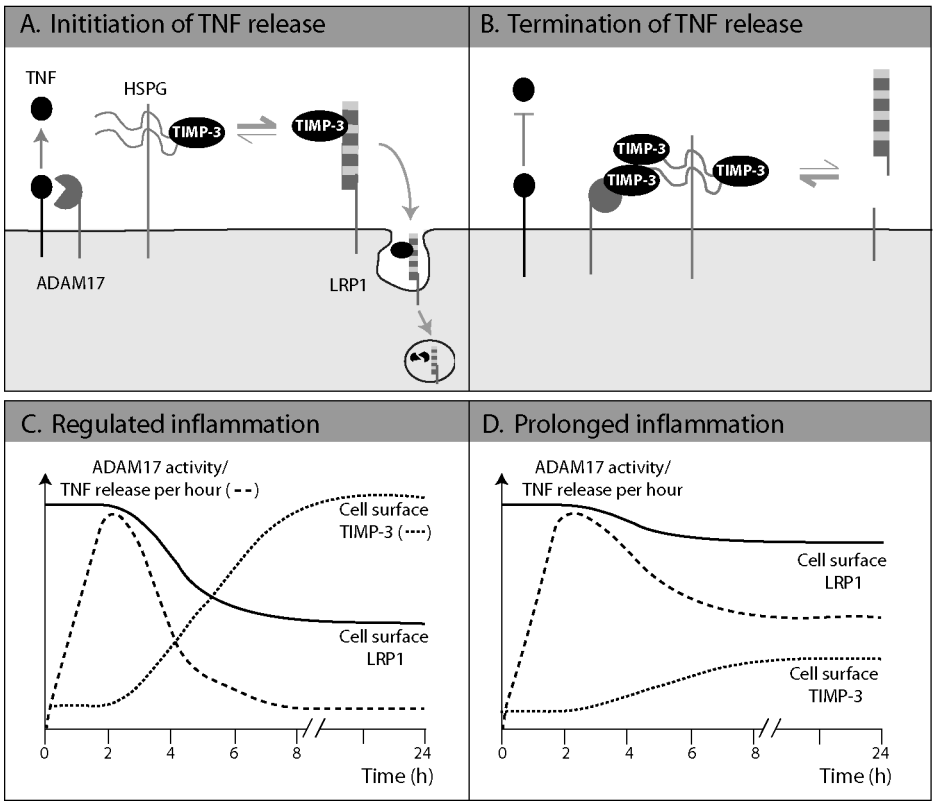
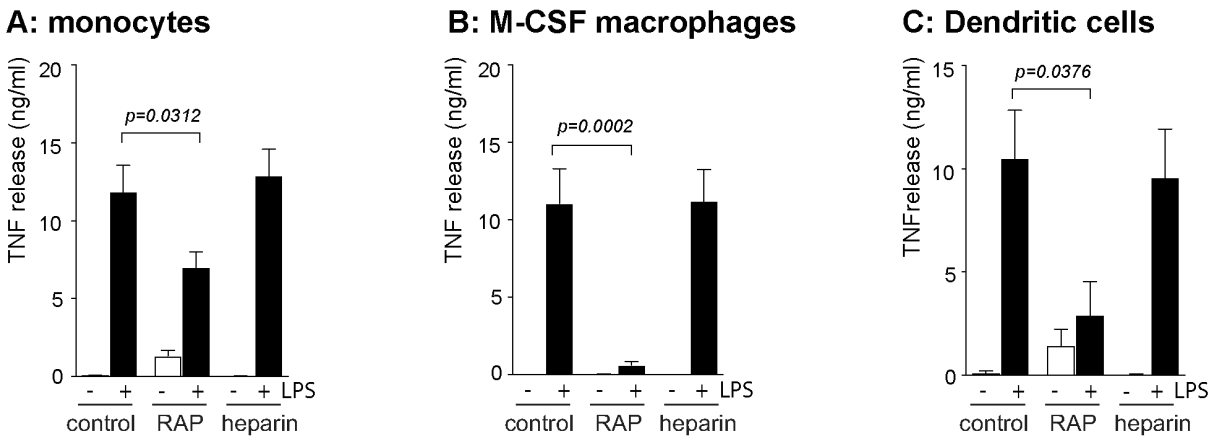


FIGURE 7. Proposed model of LRP1 regulation of ADAM17 activity in macrophages. (**A**) Upon LPS stimulation, ADAM17 activity increases rapidly and TNF is released (0 - 2 h after stimulation). Cell surface levels of TIMP-3 are determined by the equilibrium between its binding to heparan sulfate proteoglycans (HSPG) and its endocytosis by LRP1. (**B**) With a slower kinetic (2 - 4 h after stimulation), LPS also induces shedding of LRP1, possibly by stimulating expression of an LRP1 'sheddase'. This leads to a reduction in TIMP-3 endocytosis by LRP1 and consequent accumulation of TIMP-3 on the cell surface, where it inhibits ADAM17 activity. (**C**) Timely resolution of the inflammatory response requires shedding of LRP1 to allow TIMP-3 to accumulate on the cell surface. (**D**) Inflammation is likely to be prolonged in conditions where LRP1 expression is increased or its shedding is impaired, with reduced accumulation of TIMP-3 leading to prolonged ADAM17 activity and TNF release.



SUPPLEMENTAL FIGURE 1. LRP-1 blockade inhibits TNF release from LPS-stimulated primary human myeloid cells. Primary human monocytes were obtained from peripheral blood mononuclear cells by elutriation. (**A)** Primary human monocytes were treated with RAP (1 μM) and heparin (200 μg/ml) and with LPS (100 ng/ml) for 4 h. TNF release (mean + SEM, n = 7 donors) was quantified by ELISA. (**B)** Macrophages were differentiated *in vitro* from primary human monocytes by treatment with M-CSF (100 ng/ml) for 5 days, treated with RAP (1 μM) or heparin (200 μg/ml) for 16 h, and then stimulated with LPS (100 ng/ml) for 4 h. TNF release (mean + SEM, n = 4 donors) was quantified by ELISA. (**C)** Dendritic cells were differentiated *in vitro* from primary human monocytes by treatment with GM-CSF (50 ng/ml) plus IL-4 (10 ng/ml) for 5 days, treated with RAP (1 μM) or heparin (200 μg/ml) for 16 h, and then stimulated with LPS (100 ng/ml) for 4 h. TNF release (mean + SEM, n = 4 donors) was quantified by ELISA.