Treatment of Dermal Fibroblasts with GPI-Anchored Human TIMP-1 Protein Moderates Processes Linked to Scar Formation

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Tissue inhibitors of metalloproteinases exhibit diverse physiological/biological functions including moderation of the proteolytic processing of growth factors and turnover of extracellular matrix. These various biological activities are linked in part to the stoichiometry of tissue inhibitor of metalloprotein/matrix metalloprotein (TIMP/MMP)/surface protein interactions. TIMP-1, a secreted protein, can be detected on the cell surface only through its interaction with surface-bound proteins. Proteins anchored by glycosylphosphatidylinositol (GPI), when purified and added to cells or tissues, are efficiently incorporated into their surface membranes. A GPI anchor was fused to TIMP-1 to focus defined concentrations of the inhibitory protein independently on the surface of primary dermal fibroblast cells. Exogenously added recombinant TIMP-1-GPI effectively inserted into the cell membrane of fibroblasts blocked the secretion of MMPs and markedly altered the stoichiometry of MMP association with the cell surface. TIMP-1-GPI treatment resulted in inhibition of fibroblast-reduced proliferation, and transiently reduced expression of fibrosis-associated genes. These effects were dose dependent. Treated cells also showed a more proapoptotic phenotype based on apoptotic assays and western blot analysis for apoptosis-associated protein expression. GPI-anchored TIMP-1 may represent a more effective version of the protein for use in therapeutic approaches to help control fibrosis and scar formation.

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INTRODUCTION

During wound healing, fibroblasts regulate the formation and contraction of granulation tissue. They are recruited to the site of the wound where they proliferate and undergo activation to myofibroblasts. During normal wound healing, the myofibroblasts eventually undergo apoptosis. In pathological situations, sustained fibroblast activation and reduced apoptosis can lead to myofibroblast overproliferation, excessive extracellular matrix production (ECM), overcontraction, and eventually hypertrophic or keloid scar formation (Clark, 1993; Schaffer *et al.*, 1997; Fattman, 2008).

In tissue remodeling, the production of ECM is generally counterbalanced by its turnover. The catabolism is mediated by various enzyme families that include the matrix metalloproteinases (MMPs) (Bonewald, 1999; Crosby and Waters, 2010; Mirastschijski *et al.*, 2010). In humans, there are 23 MMPs subgrouped by their substrate specificity and cellular localization. They include the collagenases, gelatinases, stromelysins, and membrane-type MMPs (Sternlicht and Werb, 2001; Morrison *et al.*, 2009; Fanjul-Fernandez *et al.*, 2010). The catalytic activity of MMPs is generally low in normal tissue, but are increased during inflammation and wound repair (Overall and Lopez-Otin, 2002). Importantly, these enzymes do more than metabolize ECM. Many membrane-bound cytokines, receptors, and adhesion molecules are also processed, or released from the cell surface, by the action of MMPs. Thus, this enzyme family can be seen as controlling diverse aspects of tissue homeostasis and wound repair, and therefore represents a potential target for therapeutic intervention in some pathologic settings.

Although MMP expression can be controlled transcriptionally by cytokines and growth factors, their catalytic activity is also regulated by four endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) (Brew *et al.*, 2000; Overall and Lopez-Otin, 2002). The balance between MMP/TIMP expression is essential for the regulation of tissue homeostasis and is linked to various pathogenic processes (Brew *et al.*, 2000; Ra and Parks, 2007; Mirastschijski *et al.*, 2010).

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Abbreviations: 7-AAD, 7-aminoactiomycin D; ECM, extracellular matrix production; GPI, glycosylphosphatidylinositol; MMPs, matrix metalloproteinases; PLC, phospholipase C; TIMP, tissue inhibitors of metalloproteinases; TNF-α, tumor necrosis factor-α

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The bioactivity of TIMP-1 can be altered by changing its subcellular distribution (Djafarzadeh *et al.*, 2004, 2006, 2009, 2012; Raggi *et al.*, 2009). Human TIMP-1 protein was modified by fusion to a glycosylphosphatidylinositol (GPI) anchor. GPI-anchored proteins can be efficiently transferred from one cell to another through a process called cell painting (Medof *et al.*, 1996; Djafarzadeh *et al.*, 2004). Recombinant TIMP-1-GPI is efficiently incorporated into cell membranes where it results in a shift in the proteolytic microenvironment at the cell surface. This can result in enhanced and new cellular behavior (Djafarzadeh *et al.*, 2004, 2006, 2009, 2012; Raggi *et al.*, 2009).

The effect of GPI-anchored TIMP-1 treatment on dermal fibroblast biology was evaluated. Application of TIMP-1-GPI led to markedly reduced matrix gene expression and fibroblast proliferation, and rendered cells more sensitive to apoptosis. Treatment with TIMP-1-GPI may show efficacy for the suppression of scar formation.

RESULTS

Exogenously added TIMP-1-GPI incorporates into surface membranes of primary dermal fibroblasts

GPI-anchored TIMP-1 protein was generated and isolated as previously described (Djafarzadeh *et al.*, 2004). The incorporation of purified TIMP-1-GPI protein into the surface membrane of primary dermal fibroblasts was demonstrated by incubation of purified 14 ng ml^{-1} TIMP-1-GPI or recombinant human TIMP-1 (rhTIMP-1) control protein for 30 minutes at 37 °C (see Materials and Methods). Surface-associated TIMP-1 protein was measured using a human TIMP-1-specific antibody and FACS (Figure 1a). Incubation with control human recombinant TIMP-1 or vehicle did not result in a fluorescence shift; however, addition of GPI-anchored TIMP-1 resulted in a strong surface signal for TIMP-1. The necessity of the GPI anchor was verified by digestion of TIMP-1-GPI-treated fibroblasts with phospholipase C (PLC) (120 ng ml^{-1}), which specifically cleaves GPI anchors. PLC treatment led to a loss of TIMP-1 signal measured by FACS. The efficiency of incorporation was determined by measuring the resultant free TIMP-1 protein left in the supernatant after incorporation by TIMP-1-specific ELISA. As previously reported (Djafarzadeh *et al.*, 2006), the efficiency of incorporation was generally between 30 and 60% of input protein (data not shown).

The effect of surface-bound TIMP-1 on the secretion of MMP-2 and MMP-9 by fibroblasts was tested using gelatinase zymography (Djafarzadeh *et al.*, 2006) (Figure 1b). Recombinant hTIMP-1 protein added at 14 ng ml^{-1} had limited effects on the secretion of MMP-2 or MMP-9. In contrast, when added at a concentration of 7 or 14 ng ml^{-1}, TIMP-1-GPI led to a reduction in the secretion of both proMMP-2 and proMMP-9. Heat-denatured TIMP-1-GPI (TIMP-1-GPI) did not lead to reduced secretion of the gelatinases. This reduction in secretion was then quantified using MMP-2- and MMP-9-specific ELISAs performed on growth media from the treated cells (Figure 1c).

To establish whether the reduction in gelatinase secretion was permanent, the GPI anchor was again cleaved from the TIMP-1-GPI-treated cells using PLC (120 ng ml^{-1}) 24 hours after treatment. After an additional 24 hours, the cell culture supernatants were assayed for the presence of MMP-2 and MMP-9 by gelatinase zymography. The results show that following GPI digestion (lane 7) the fibroblast cells re-secrete the two gelatinase enzymes at approximately the same level seen in the buffer and rhTIMP-1 controls (lane 4) (Figure 1d).

Treatment with TIMP-1-GPI leads to an increase in the surface expression of MMPs

TIMP-1 binds to all active forms of MMPs with the exception of MMP-14. On the basis of the reduced secretion of the gelatinases observed, TIMP-1-GPI treatment may lead to a sequestering of MMPs on the cell surface (Djafarzadeh *et al.*, 2006). Following incubation of the fibroblasts with 14 ng ml^{-1} of TIMP-1-GPI protein for 24 hours, FACS analyses was performed using MMP-1-, MMP-2-, MMP-9-, and MMP-13-specific antibodies, which showed an increase in mean channel fluorescence for each MMP studied (Figure 2) (Brew *et al.*, 2000). To further verify that this was not a nonspecific effect, the surface expression of collagen and tumor necrosis factor binding protein 2 was evaluated in parallel. Surface expression of these molecules was not effected by TIMP-1-GPI treatment (Figure 2).

GPI-anchored TIMP-1 suppresses serum and tumor necrosis factor-α (TNF-α) induced fibroblast proliferation

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide stainings were performed to assess the effect of TIMP-1-GPI on the proliferation of activated dermal fibroblasts. Serum is a potent stimulator of fibroblast proliferation. TNF-α at low concentrations can also stimulate fibroblast proliferation (Hetzel *et al.*, 2005). The effect of TIMP-1-GPI on proliferation of primary dermal fibroblast cells was determined in response to serum, and in combination with different concentrations of TNF-α (Figure 3). TIMP-1-GPI-treated fibroblasts showed a dose-dependent reduction in proliferation (at 48 hours) to low concentrations of TNF-α (400 pg ml^{-1}) or serum stimulation (Figure 3). Heat-denatured TIMP-1-GPI or rhTIMP-1 had no effect. Higher TNF-α reduced serum-stimulated proliferation and TIMP-1-GPI completely abolished proliferation of TNF-α-stimulated fibroblasts.

TIMP-1-GPI leads to increased apoptosis of dermal fibroblast cells and shifts the expression of proapoptic and antiapoptotic proteins

Fibroblast apoptosis is crucial to the resolution of fibrosis (Gabbiani, 2003; Fattman, 2008). Reduced fibroblast apoptosis is associated with pathological scar formation (Fattman, 2008). A FACS-based assay using Annexin V/7-aminoactiomycin D staining was used to assess the effect of TIMP-1-GPI treatment on the apoptosis of fibroblasts. TIMP-1-GPI treatment, but not rhTIMP-1, was found to increase the level of apoptosis in dermal fibroblasts (Figure 4a).

The cellular mechanisms involved in fibroblast apoptosis are not well understood, but are linked to the expression of the Bcl-2 family of proteins (Gabbiani, 2003). This family includes members that can suppress (Bcl-2 and Survivin) or increase

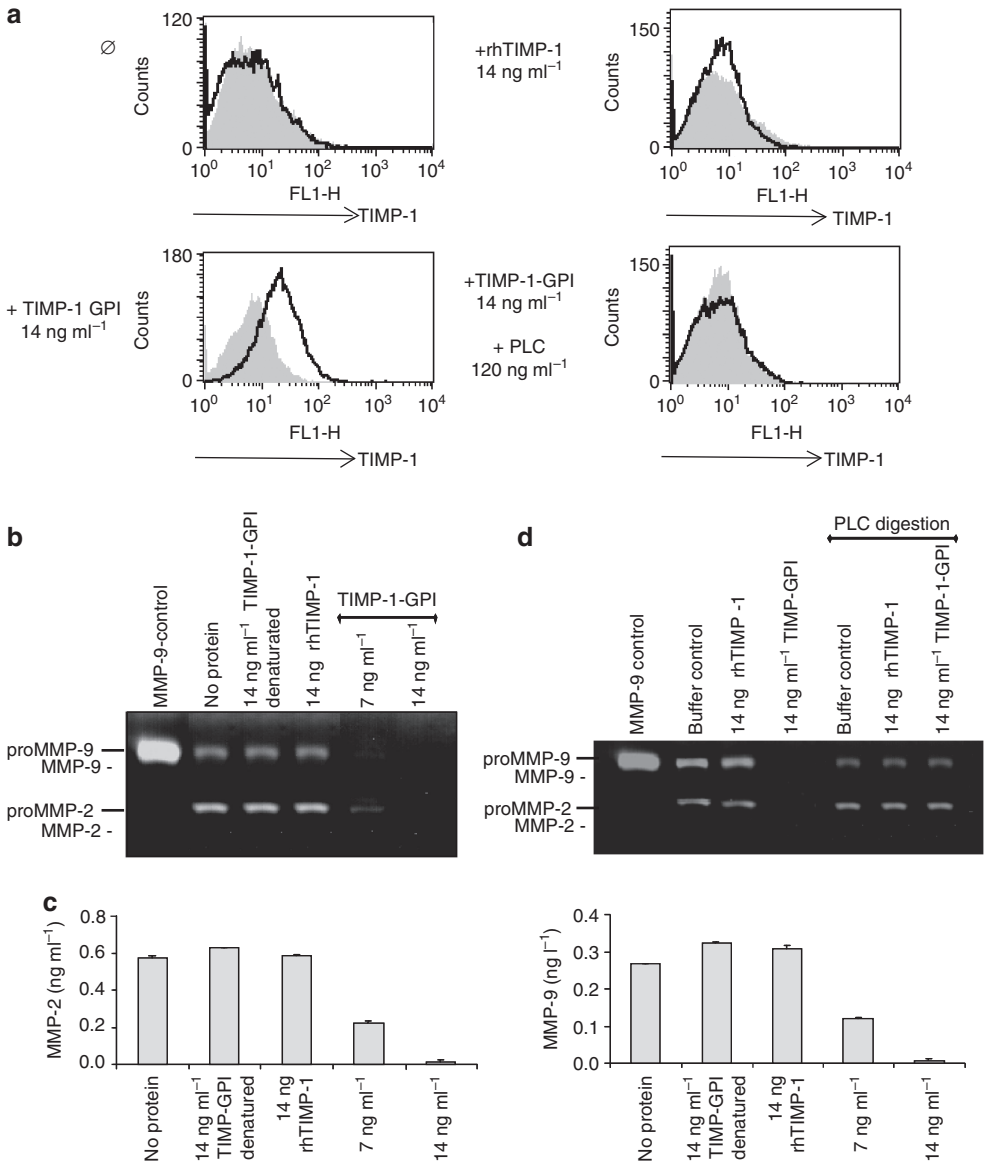


Figure 1. Tissue inhibitor of metalloproteinases-1-glycosylphosphatidylinositol (TIMP-1-GPI) is efficiently reincorporated into the surface membranes of fibroblasts and reduces the secretion of matrix metalloprotein (MMP)-2 and MMP-9. (**a**) Purified TIMP-1-GPI or control recombinant human (rh) TIMP-1 was added to primary dermal fibroblasts and TIMP-1 detected by FACS analysis. Gray histograms represent isotype controls and solid-line histograms represent TIMP-1 antibody staining. To demonstrate GPI linkage, treated cells were incubated with 120 ng ml^{-1} PLC and then subjected to FACS analysis. (**b**) TIMP-1-GPI treatment reduced MMP-2 and MMP-9 release into the growth media as seen by gelatine zymography. (**c**) ELISA was used to quantify MMP-2 and MMP-9 proteins. (**d**) The reduced gelatinase secretion seen with TIMP-1-GPI treatment recovered following PLC treatment. Fibroblasts were pretreated with vehicle, rhTIMP-1, or TIMP-1-GPI for 24 hours, and then treated by phospholipase C (PLC) digestion. After an additional 24 hours, the cell culture supernatants were assayed by gelatinase zymography. rhTIMP-1, recombinant human TIMP-1.

(Bax and Apaf-1) the level of apoptosis. The balance between pro and antiapoptotic Bcl-2 proteins helps dictate the ability of cells to undergo apoptosis.

Bcl-2, Bax, Survivin, and Apaf-1 protein expression was analyzed following treatment. Dermal fibroblasts were pre-incubated with TIMP-1-GPI or rhTIMP-1 for 24 hours. Proteins were then extracted from whole-cell lysates and western blot analysis was performed (see Materials and Methods) (Figure 4b). TIMP-1-GPI led to a reduction in cellular levels of antiapoptotic Bcl-2 and Survivin, but showed a corresponding increase in levels of the proapoptotic Apaf-1 and Bax proteins, potentially explaining the proapoptotic effect of TIMP-1-GPI treatment seen.

TIMP-1-GPI treatment transiently reduces messenger RNA (mRNA) expression of fibrosis-associated genes

A quantitative assessment of the mRNA expression of a select group of genes linked to fibrosis and components of ECM was carried out using TaqMan reverse transcriptase PCR (RT-PCR) analysis. Primary dermal fibroblasts were left untreated, or

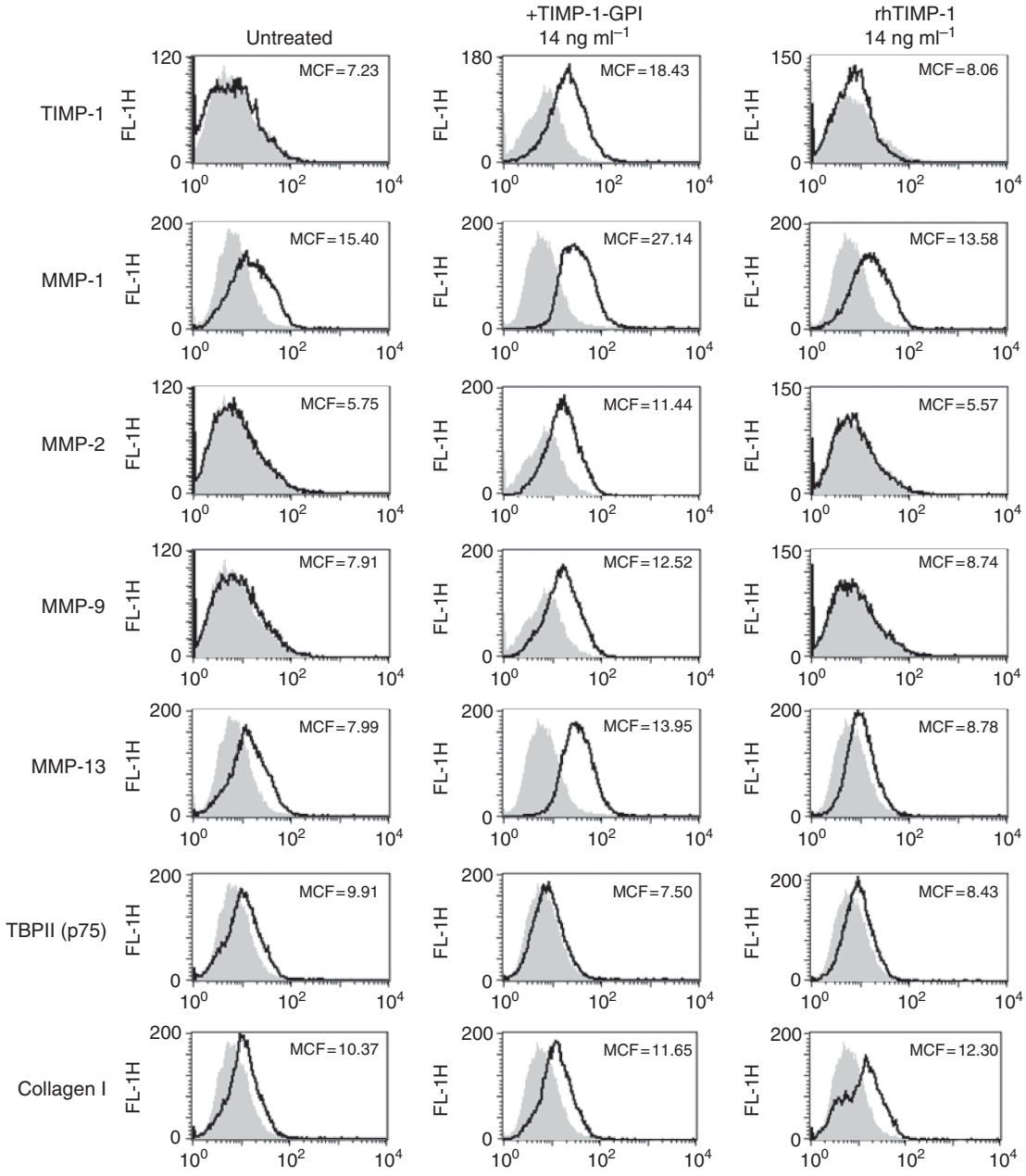


Figure 2. Tissue inhibitor of metalloproteinases-1-glycosylphosphatidylinositol (TIMP-1-GPI) treatment led to increased surface expression of matrix metalloproteinases (MMPs). Following incubation of primary dermal fibroblasts with 14 ng ml of purified recombinant human (rh) TIMP-1-GPI protein for 24 hours, FACS analyses using MMP-1-, MMP-2-, MMP-9-, and MMP-13-specific antibodies were performed. Shown are increased mean channel fluorescence (MCF) signals for TIMP-1 after cell treatment with TIMP-GPI, as well as increased fluorescence of MMP-1, MMP-2, MMP-9, and MMP-13. Gray histograms represent isotype controls, and solid-line histograms represent respective antibody stainings. TBP is tumor necrosis factor binding protein II. Shown are representative histograms of repeated experiments with similar results. rhTIMP-1, recombinant human TIMP-1.

treated with 7 ng ml^{-1} TIMP-1-GPI, 14 ng ml^{-1} TIMP-1-GPI, 14 ng ml^{-1} of heat-denatured TIMP-1-GPI, or 14 ng ml^{-1} of rhTIMP-1. After 30 minutes, the cells were washed with medium and then stimulated with increasing concentrations of serum (0, 1, 5, and 10% fetal calf serum), with or without the presence of 10 ng ml^{-1} TNF-α. After 48 hours, mRNA was isolated and subjected to analysis using TaqMan RT-PCR. The genes analyzed were collagen 1A1, collagen 4A2, fibronectin, collagen 16A1, connective tissue growth factor, and transforming growth factor beta 1 (tgf-β1). In each instance, a dose-dependent increase in mRNA expression was seen in response to serum stimulation (Figure 5a–f). In some instances, TNF-α at 10 ng ml^{-1} costimulation led to an increased level of mRNA expression over that seen with serum stimulation alone. TIMP-1-GPI treatment was found to suppress the induced expression of each of the genes.

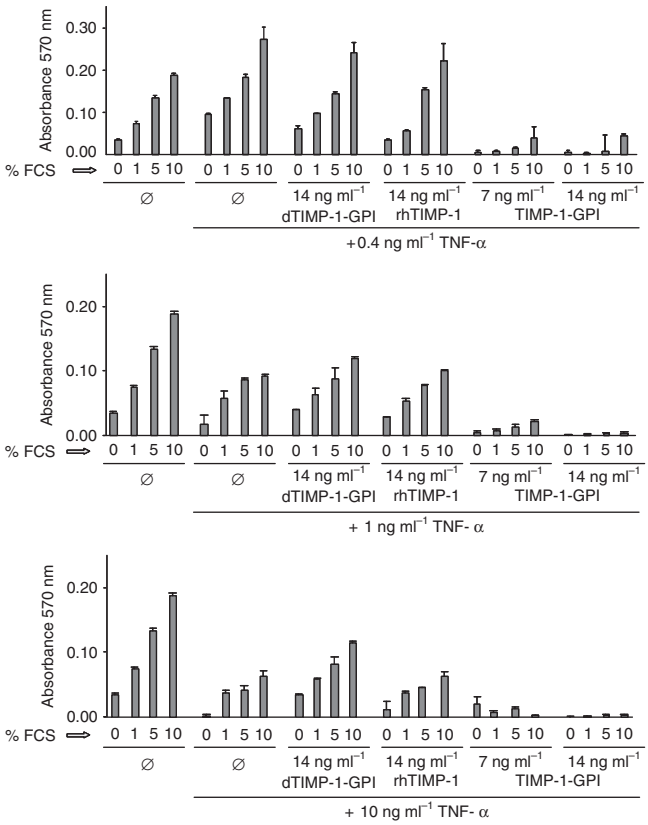


Figure 3. Tissue inhibitor of metalloproteinases-1-glycosylphosphatidyl-inositol (TIMP-1-GPI) treatment suppresses serum and tumor necrosis factor-α (TNF-α)-induced fibroblast proliferation. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assays were performed to assess the effect of TIMP-1 surface engineering on the proliferation of fibroblasts. Serum and/or TNF-α were used to stimulate proliferation. Fibroblasts treated with 7 or 14 ng ml^{-1} TIMP-1-GPI showed a reduction to serum (0, 1, 5, or 10%) and/or TNF-α (0.4, 1, or 10 ng ml^{-1}) induced proliferation after 48 hours of treatment. Control recombinant human (rh) TIMP-1 or heat-denatured TIMP-1-GPI at 14 ng ml^{-1} did not strongly influence proliferation of the cells. dTIMP, denatured tissue inhibitor of metalloprotein-1; FCS, fetal calf serum; rhTIMP-1, recombinant human TIMP-1.

To determine whether the effects of TIMP-1-GPI on steady-state mRNA expression were reversible, in a parallel set of experiments, 24 hours after the fibroblasts had been treated with 14 ng ml^{-1} TIMP-1-GPI, 14 ng ml^{-1} of heat-treated TIMP-1-GPI, or 14 ng ml^{-1} of rhTIMP-1 as described above, the cells were subjected to PLC digestion (120 ng ml^{-1}) and placed back into culture for an additional 24 hours; RNA was then isolated and tested for the level of expression of the various genes. The results demonstrated that the TIMP-1-GPI effects are reversible and that the overall process does not appear to be overly toxic to primary dermal fibroblast cells (Figure 5a–f).

DISCUSSION

By linking a GPI anchor to TIMP-1, an agent was generated that moderates the response of primary dermal fibroblasts to stimulation by TNF-α or serum. Treated cells showed a shift toward apoptosis, reduced proliferation, and suppressed the expression of matrix components.

TIMPs and MMPs are key components in the formation, remodeling, and degradation of matrix proteins (Gabbiani, 2003; Hetzel *et al.*, 2005; Mirastschijski *et al.*, 2010). A balance between ECM deposition and protease activity needs to be optimally regulated during wound healing. In skin, tissue repair involves the proliferation of fibroblasts, which migrate into the wound, differentiate into myofibroblasts, produce, and contract the ECM, leading to wound closure and restoration of tissue function (Singer and Clark, 1999; Gabbiani, 2003; Denton and Abraham, 2004; Leask and Abraham, 2004). This is accompanied by apoptosis of fibroblasts/myofibroblasts within the wound (Gabbiani, 2003). Where the balance between the breakdown and deposition of ECM is disturbed, or when apoptosis is inefficient, abnormal wound healing may result in excessive scarring or keloid scarring (Gabbiani, 2003). The present study suggests that GPI-anchored TIMP-1 may effectively control, alter, inhibit, or prevent undesirable processes associated with excessive scaring.

The fusion of a GPI anchor to human TIMP-1 protein yields a protein with pronounced effects on fibroblasts. TIMP-1-GPI treatment resulted in a sequestering of MMPs on the surface of cells and reduced expression of matrix-associated protein genes. The effects of treatment were reversible, as cleavage of the GPI anchor using PLC, resulting in the release of TIMP-1 from the surface, led to re-secretion of the gelatinase MMPs and the re-expression of profibrotic genes.

The increased expression of fibronectin, collagen 1A1, collagen 4A2, and 16A1 is linked to fibroproliferative scarring (Zhu *et al.*, 2008). Serum- and TNF-α-induced mRNA expression of these genes by dermal fibroblasts was reduced in a dose-dependent manner in response to TIMP-1-GPI treatment. The cytokines connective tissue growth factor and TGF-β1 also have important roles in wound healing (Singer and Clark, 1999; Leask and Abraham, 2004; Margadant and Sonnenberg, 2010). TIMP-1-GPI treatment reduced the mRNA expression of both cytokines. In addition, TIMP-1-GPI treatment also inhibited the steady-state level of MMP-2 and MMP-9, suggesting that, in addition to sequestering the proteins on the cell surface, TIMP-1-GPI also reduced transcription of the genes (Supplementary Figure S1 online).

The observation that increased concentrations of TIMP-1 could lead to a reduction in fibrosis seems at first glance counter-intuitive. A reduction in MMP activity by increased levels of TIMP-1 at the cell surface should result in a more pronounced accumulation of matrix components because of a blockade in their catabolism. The effects seen may be linked to the observed reduced expression of tgf-β1 mRNA and protein resulting from treatment (Djafarzadeh *et al.*, 2012). TGF-β1 is known to stimulate the proliferation of fibroblasts (Moses *et al.*, 1990; Verrecchia *et al.*, 2001; Leask and Abraham, 2004). Recent studies by our group have shown that TIMP-1-GPI treatment can block the processing of latent TGF-β1 to its active form in other cell types (Djafarzadeh *et al.*, 2012; Djafarzadeh *et al.*, unpublished results). This effect may underlie, in part, the generally positive effects of TIMP-1-GPI treatment seen in *in vitro* and *in vivo* models of

wound healing. As many autocrine and paracrine growth factors also undergo proteolytic processing (Leask and Abraham, 2004; Broughton *et al.*, 2006), TIMP-1-GPI may have additional effects on fibroblast biology. Finally, treatment brought about a shift in the intracellular levels of Bcl-2 proteins toward a more proapoptotic posture in the dermal fibroblasts. Thus, TIMP-1-GPI treatment may positively influence a series of events linked to pathologic scar formation, including the dysregulation of apoptosis associated in wound-healing abnormalities. Topical application of TIMP-1-GPI may find efficacy in specific wound healing situations.

MATERIALS AND METHODS

Cellular reagents and tissue culture

Primary human dermal fibroblast cells NHDF-c (PromoCell, Heidelberg, Germany, No. C-12300) were cultured in Dulbecco's MEM with Glutamax-1 (GIBCO BRL, Life Technologies GmbH, Eggenstein, Germany, No. 21885-025) supplemented with 10% heat-inactivated fetal calf serum. All experiments adhered to the Declaration of Helsinki Principles.

Fluorescence-activated cell scanning analysis

Human TIMP-1, MMP-1, MMP-2, MMP-9, MMP-13, and MMP-14 were identified on the cell surface of primary fibroblasts by FACS

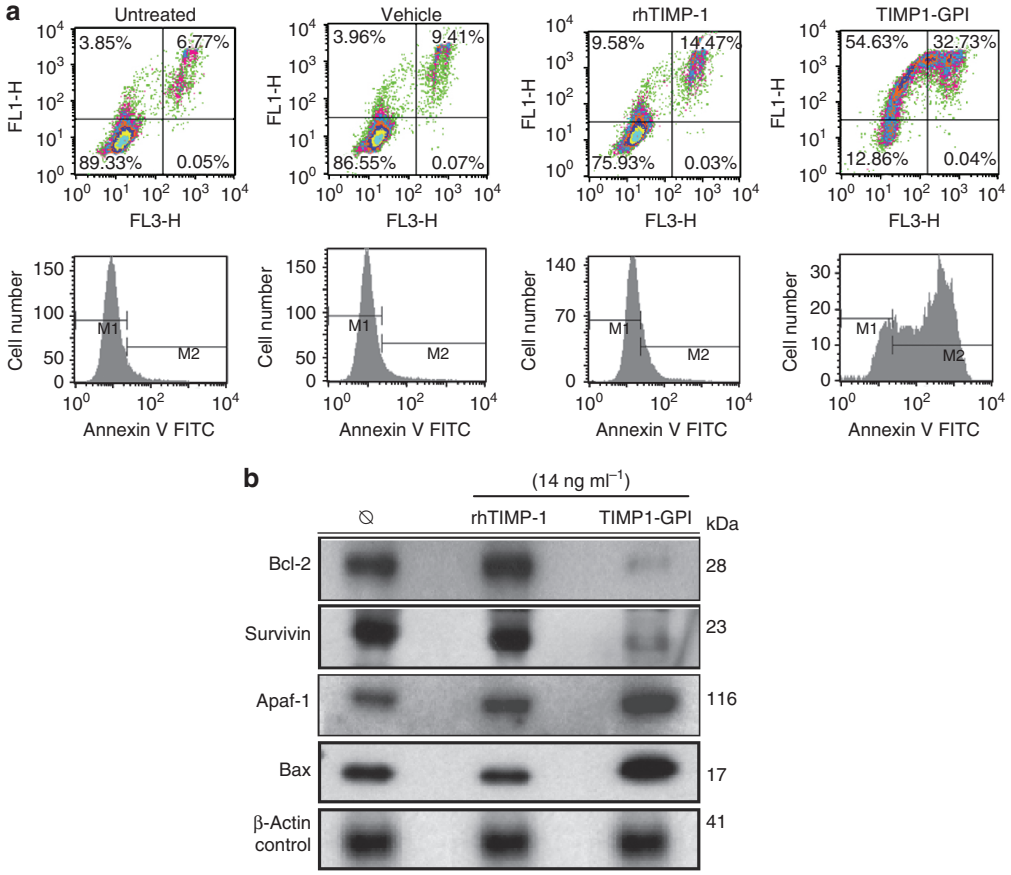
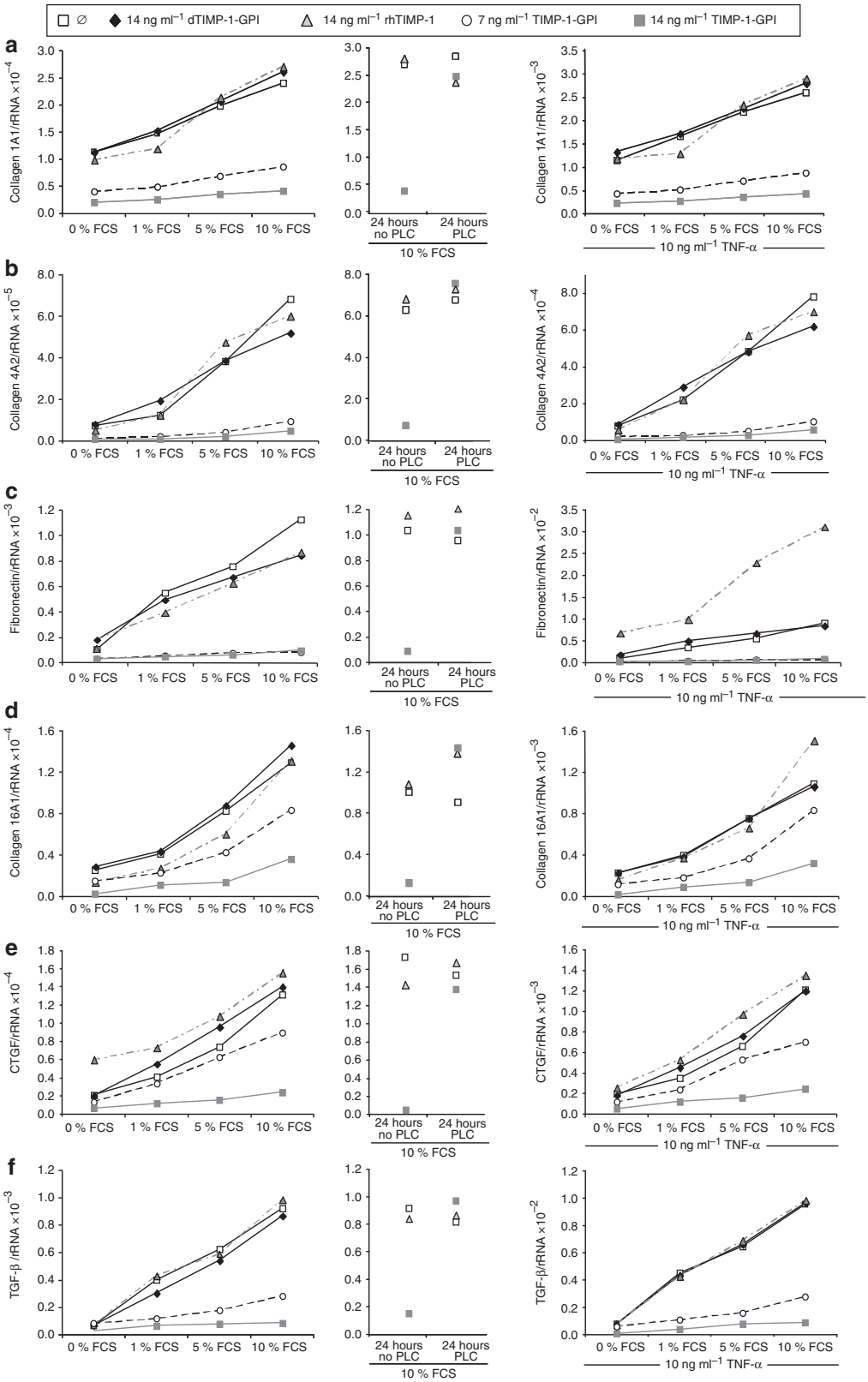


Figure 4. Treatment of fibroblasts with tissue inhibitor of metalloproteinases-1-glycosylphosphatidylinositol (TIMP-1-GPI) renders the cells more sensitive to apoptosis. Fibroblast cells were untreated or treated with vehicle, 12 ng ml^{-1} TIMP-1-GPI, or recombinant human (rh) TIMP-1. (**a**) Binding of annexin V-FITC (FL1) and 7-aminoactiomycin D (FL3) was used to detect viable, early, and late apoptosis by flow cytometry. (**b**) Regulation of apoptotic proteins (Bcl-2, Bax, Survivin, and AFAP-1) with TIMP-1-GPI in fibroblast cells. Bcl-2, Bax, Survivin, and Apaf-1 expression in fibroblast cells following treatment with TIMP-1-GPI or rhTIMP-1 was analyzed by western blot analysis. FL, fluorescence channel; rhTIMP-1, recombinant human TIMP-1.

Figure 5. Tissue inhibitor of metalloproteinases-1-glycosylphosphatidylinositol (TIMP-1-GPI) treatment transiently moderates the steady-state expression of fibrosis-associated genes. Primary dermal fibroblasts were treated with 7 ng ml^{-1} TIMP-1-GPI, 14 ng ml^{-1} TIMP-1-GPI, 14 ng ml^{-1} of heat-treated TIMP-1-GPI, or 14 ng ml^{-1} of recombinant human (rh) TIMP-1 and then stimulated with serum (0, 1, 5 and 10% FCS), with or without the presence of 10 ng ml^{-1} tumor necrosis factor-α (TNF-α). After 48 hours, messenger RNA was isolated and subjected to analysis using TaqMan reverse transcriptase PCR (RT-PCR). In parallel, fibroblasts treated for 24 hours were subjected to phospholipase C (PLC) digestion (120 ng ml^{-1}) and placed back into culture for an additional 24 hours. The genes analyzed were (**a**) collagen 1A1, (**b**) collagen 4A2, (**c**) fibronectin, (**d**) collagen 16A1, (**e**) connective tissue growth factor, and (**f**) transforming growth factor beta 1 (TGF-β). FCS, fetal calf serum; rhTIMP-1, recombinant human TIMP-1; rRNA, ribosomal RNA.

analysis. Cells at 80% confluency were washed with serum-free medium and incubated with 14 ng ml^{-1} TIMP-1-GPI or rhTIMP-1 for 2 hours at 37 °C. The cells were washed again and incubated in serum-free medium overnight. Cells were detached using Biotase (Biochrom A, Berlin, Germany, No. L2193) and incubated for 60 minutes on ice with TIMP-1 antibody (No. IM32L), MMP-1



antibody (No. IM35L-100 UG), MMP-13 antibody (No. IM44L) (all from Calbiochem, Merck Darmstadt, Germany), MMP-2 antibody (No. IM 51L), MMP-9 antibody (No. IM 61-100 UG) (from Oncogene, Bad Soden, Germany), anti-hFibronectin (DakoCytomation A/S, Glostrup, Denmark, No. A0245), or isotype control $IgG_{1\kappa}$ (Sigma-Aldrich, Taufkirchen, Germany, No. M9269). The cells were washed with 1 × phosphate buffered saline (PBS) and incubated with an FITC-conjugated human anti-mouse (Dako A/S, Glostrup, Denmark, No. F0313) IgG for 45 minutes on ice. The cells were washed with PBS and analyzed via FACS (FACS Calibur, Becton, Dickinson and Company, San Jose, CA) using the CellQuest analysis software (FACS).

Purification of TIMP-1-GPI protein

The TIMP-1-GPI protein was produced and purified as previously described (Djafarzadeh *et al.*, 2004).

Incorporation of TIMP-1-GPI into cell membranes and enzymatic cleavage of the GPI anchor

Fibroblasts (5×10^5 cells ml^{-1}) were incubated for 1 hour with 14 ng ml^{-1} of purified hTIMP-1-GPI at 37 °C/5% CO_2. The cells were then harvested and washed once with cold PBS and analyzed by FACS using human TIMP-1-specific monoclonal antibodies (see above). For GPI-anchor digestion, TIMP-1-GPI-anchored fibroblast cells were treated with 120 ng ml^{-1} phosphatidylinositol-specific PLC (Sigma-Aldrich, No. 661-9) in serum-free medium for 30 minutes at 37 °C. The cells were washed three times with cold PBS and analyzed by FACS.

Zymography

Dermal fibroblast cells were cultured in 24-well plate (5×10^4 cells in each well). The medium was changed after 24 hours with serum-free medium containing the TIMP-1-GPI and rhTIMP-1 and incubated for 1 hour. The cells were then washed once with medium, and incubated for 24, 48, and 72 hours in 1 ml of serum-free medium. Cell supernatants were analyzed by gelatinase zymography using 10% SDS-polyacrylamide gels (Invitrogen, Groningen, The Netherlands, No. EC61755BOX) containing 0.1% gelatin, and cell supernatants were loaded in a 1:2 dilution with SDS sample buffer (Invitrogen, No. LC2676). Recombinant MMP-9 enzyme (Amersham Biosciences, Uppsala, Sweden, No. RPN2634) was used as positive control. After electrophoresis at 130 Volts for 1.5 hours, the gels were renatured for 30 minutes and subsequently developed for 96 hours at 37 °C. Gels were stained with Coomassie blue and destained with deionized water.

Proliferation

Primary dermal fibroblasts at a density of 5×10^3 were cultured in 96-well microtiter plates for 24 hours to yield firmly attached and stably growing cells. After discarding supernatants, 50 µl of medium containing native TIMP-1-GPI, denatured TIMP-1-GPI (1 hour at 90 °C), vehicle, or rhTIMP-1 were added and the cells were incubated from 24 to 72 hours. Next, 50 µl of 1 mg ml^{-1} solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added. After 3 hours of incubation at 37 °C, formazan crystals were dissolved by the addition of 100 µl isopropanol and 0.04 N HCl. Absorbance was measured at 570 nm using GENios plus TECAN ELISA reader (Männedorf, Switzerland). For each experiment, at least six wells were analyzed per experimental condition and time point.

Annexin V detection of apoptosis

Detection and quantification of apoptotic versus necrotic cells at the single-cell level was performed using Annexin-V-FLUOS staining kit (Becton, Dickinson and Company, Heidelberg, Germany, No. 556547). Normal human dermal fibroblast cells from an adult donor (PromoCell, No. C-12302) were seeded at a density of 1×10^6 cells per flask into 25-cm^2 flasks and allowed to attach overnight. The flasks were then rinsed once with serum-free medium and 1 ml of serum-fibroblast growth medium (PromoCell, No. C-23010) was added, followed by 12 ng ml^{-1} of TIMP-1-GPI or rhTIMP-1. Cells were incubated overnight at 37 °C/5% CO_2. The cells were washed with PBS, pelleted, and resuspended in staining solution (Annexin-V-fluorescein labeling reagent and 50 µg ml^{-1} 7-Aminoactiomycin D (Sigma-Aldrich, No. A9400)) for 15 minutes at room temperature. The cells were then analyzed by flow cytometry.

Western blot analysis

The fibroblasts were washed three times with cold PBS and detached with 1.5 mM EDTA. The cells were rotated for 1 hour at 4 °C with hypertonic lysis buffer (5 mM Tris, 2 mM MgCl, 0.1 mM EDTA, 1 mM PMSF (Roche, Basel, Switzerland, No. 236608), 2 µg ml^{-1} Aprotinin (Roche, No. 1583794), 2 µg ml^{-1} leupeptin (Sigma, Taufkirchen, Germany, No. L2884), 1 µg ml^{-1} pepstatin A (Roche, Basel, Switzerland, No.1524488), pH 7.4). Cell membranes were isolated with extraction buffer (100 mM NaCl, 1% Triton X-100, 10 mM Tris, 5 mM EDTA, 1 mM PMSF, 2 µg ml^{-1} aprotinin, 2 µg ml^{-1} leupeptin, 1 µg ml^{-1} pepstatin A, pH 7.4) and were rotated for 1 hour at 4 °C, followed by centrifugation at 11,000*g* for 20 minutes at 4 °C. The supernatant was used for further western blot tests.

Whole-cell lysates containing 40 µg of protein were denatured by boiling for 5 minutes in SDS sample buffer and then loaded and separated by 4–20% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Invitrogen, No. LC2002) and tested. Western blot analysis was used for the detection of BCL-2 (ALX-804-225, Axxora, LLC Farmingdale, NY), BAX (ANC-357-040, Axxora), APAF-1 (AAP-300, Assay Designs (Ann Arbor, MI)), Survivin, (MAB886, R&D Systems, Minneapolis, MN), and β-Actin (Acris, Hiddenhausen, Germany, No. ab8227) using a commercial western blot analysis kit, Chemiluminescent Immunodetection System (Invitrogen).

RT-PCR analyses

Total fibroblast RNA was isolated for standardized quantitative RT-PCR by using the RNeasy MiniKit 50 (Quiagen, Hilden, Germany, No. 74104). In brief, 2 µg of total RNA from cultured fibroblast cells was used for random primed reverse transcription using a modified MMLV reverse transcriptase (Superscript II, Life Technologies, Karlsruhe, Germany) for 1 hour at 40 °C. Real-time RT-PCR was performed on a TaqMan 7000 Sequence Detection System (Applied Biosystems, Warrington, UK) using heat-activated TaqDNA polymerase (Amplitaq Gold, PE Biosystems, Weiterstadt, Germany). After 2 minutes at 50 °C and 10 minutes at 95 °C, the samples were cycled 45 times at 95 °C for 15 seconds and 60 °C for 60 seconds. PCR products were labeled by internal fluorescence probes (MMP-9 and β-Actin) or with intercalating DNA dyes (MMP-2, Applied Biosystems). mRNA expression for each signal was calculated following the ΔCt procedure (Cohen *et al.*, 2001). The amplification efficiency was defined as 1, as all compared analyses were performed during the

same run including the control dilution series. Glyceraldehyde-3-phosphate dehydrogenase and β-Actin were used as reference genes. Commercial TaqMan reagents were used for collagen 16A1 (No. HS00156876-ml), collagen 1A1 (No. HS00156876-m1), tgf-β (No. HS99999918-m1), connective tissue growth factor (No. HS00170014-m1), fibronectin (No. HS00277509-m1), and β-actin (No. 4333762F). MMP-2 and MMP-9 primers were obtained from Applied Biosystems.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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