# Gottron's Papules Exhibit Dermal Accumulation of CD44 Variant 7 (CD44v7) and Its Binding Partner Osteopontin: A Unique Molecular Signature

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The accumulated mucin in non-Gottron's dermatomyositis (DM) lesions is primarily chondroitin-4-sulfate (C4S), which is immunomodulatory *in vitro*. Gottron's papules are a particularly resistant manifestation of DM that often persist after other lesions have resolved with therapy. We examined non-Gottron's DM lesions and Gottron's papule skin biopsies for C4S, CD44 variant 7 (CD44v7), a chondroitin sulfate-binding isoform causally implicated in autoimmunity, and osteopontin (OPN), a CD44v7 ligand implicated in chronic inflammation. Gottron's papule dermis contained more C4S and CD44v7 than non-Gottron's lesions. Normal skin showed less CD44v7 over joints relative to Gottron's lesions. All DM dermis had increased OPN compared with healthy skin. Mechanically stretching cultured fibroblasts for 6 hours induced CD44v7 mRNA and protein, whereas IFN-γ treatment induced OPN mRNA and protein. OPN alone did not induce CD44v7, but stretching dermal fibroblasts in the presence of OPN increased human acute monocytic leukemia cell line (THP-1) monocyte binding, which is blunted by anti-CD44v7 blocking antibody. C4S, CD44v7, and OPN are three molecules uniquely present in Gottron's papules that contribute to inflammation individually and in association with one another. We propose that stretch-induced CD44v7 over joints, in concert with dysregulated OPN levels in the skin of DM patients, increases local inflammatory cell recruitment and contributes to the pathogenesis and resistance of Gottron's papules.

*Journal of Investigative Dermatology* (2012) **132,** 1825–1832; doi:10.1038/jid.2012.54; published online 29 March 2012

## INTRODUCTION

Dermatomyositis (DM) is an autoimmune disorder with characteristic skin findings, including Gottron's papules over the extensor surfaces of large and small joints, most frequently of the hands (Callen, 2010). The pathogenesis of these skin findings remains unexplained.

Histology of DM skin lesions includes upper dermal deposition of mucin, which is strongest where it co-localizes with the mononuclear infiltrate (Janis and Winkelmann, 1968). Recently, our group molecularly characterized the accumulated mucin in non-Gottron's DM lesions as primarily chondroitin-4-sulfate (C4S; Kim and Werth, 2011). Published reports indicate that C4S has immunomodulatory effects *in vitro*, which may contribute to inflammation via monocyte activation or counteract it by blocking the effects of tumor necrosis factor-α and IL-1β (Rachmilewitz and Tykocinski, 1998; Fioravanti and Collodel, 2006; Xu *et al.*, 2008).

In the current study, we focused on Gottron's papules, a particularly resistant manifestation of DM that often persists after other lesions have resolved with therapy. Two reports to date describe histologically similar findings in Gottron's papules as in other DM lesions (Hanno and Callen, 1985; Mendese and Mahalingam, 2007), but these similarities cannot explain the divergent clinical courses. Because C4S has not previously been reported in Gottron's papules, we now hypothesized that this molecule and its associated binding partners might differ between Gottron's papules and photodistributed non-Gottron's DM skin lesions.

## RESULTS

### Gottron's papules contain increased content of C4S and CD44 variant 7 (CD44v7) compared with other active DM lesions or location-matched healthy controls

Immunohistochemical analysis of Gottron's biopsies of DM patients demonstrated a similar distribution of papillary dermal C4S as in all other DM skin lesions, but with a striking density exceeding that of samples from other locations. Compared with healthy control biopsies (Figure 1a), samples taken from non-Gottron's DM lesions (Figure 1b) exhibited a mean 2.9-fold increased density of C4S.

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*Abbreviations: CD44s, CD44 standard; CD44v7, CD44 variant isoform 7; C4S, chondroitin-4-sulfate; CS, chondroitin sulfate; DM, dermatomyositis; IP, interphalangeal; OPN, osteopontin; rOPN, recombinant human OPN*

*Received 17 July 2011; revised 29 December 2011; accepted 24 January 2012; published online 29 March 2012*

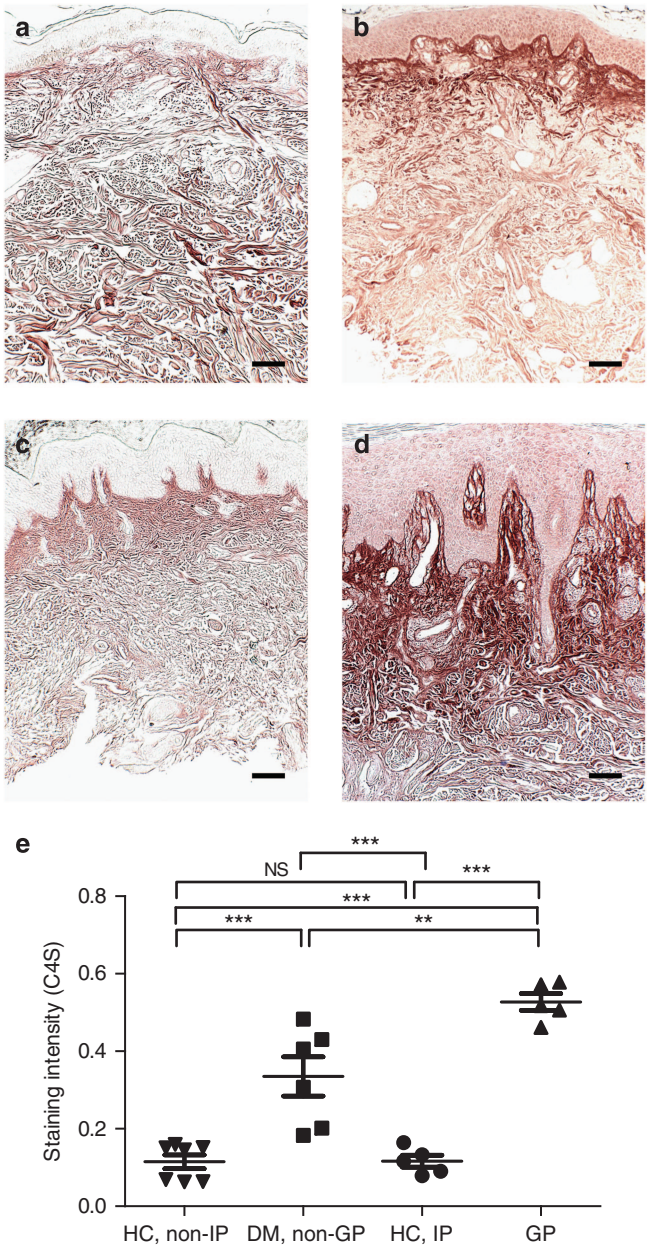


**Figure 1. Gottron's papules (GP) exhibit unusually dense deposits of chondroitin-4-sulfate (C4S) in the papillary dermis.** Biopsies are stained with an antibody against C4S. (**a**) Healthy control skin (HC, non-interphalangeal (non-IP)). (**b**) Dermatomyositis (DM) lesions (DM, non-GP). (**c**) The proximal IP of healthy volunteers (HC, IP). (**d**) GP. Representative photomicrographs are shown, original magnification ×5, bar = 100 μm. (**e**) Systematic quantification of C4S staining intensity within the papillary dermal matrix. Every data point is displayed; wide and short horizontal bars represent mean and SEM, respectively. $P<0.0001$ by analysis of variance (***$P<0.001$; **$P<0.01$). NS, not significant.

Compared with healthy control interphalangeal (IP) biopsies (Figure 1c), samples from Gottron's lesions (Figure 1d) exhibited a 4.6-fold increased density of C4S (both $P<0.001$). Gottron's lesions had a mean 1.6-fold C4S density compared with biopsies from other non-Gottron's DM lesions ($P<0.01$). C4S was not present in the epidermis of any samples.

We next investigated the Gottron's biopsies for dermal expression of proteins that interact with chondroitin sulfate (CS). Many proteins covalently or non-covalently attach to CS, but of these proteins only the variant 7 (v7) isoform of CD44 has also been causally implicated in autoimmunity (Wittig *et al.*, 2000, 2002; Marhaba *et al.*, 2003; Farkas *et al.*, 2005; Hoffmann *et al.*, 2007). The v7 domain, as well as one region within the ''standard'' CD44 (CD44s) that lacks any of the variant domains, acts as a covalent attachment site for CS, whereas CD44v6-7 has also been shown to non-covalently bind CS (Sleeman *et al.*, 1997; Keller *et al.*, 2007). Thus, we examined our samples for expression of CD44s and variant isoforms CD44v3, v6, and v7 (Kim and Werth, 2011). Consistent with previous reports, we observed strong epidermal expression of CD44s, CD44v3, and CD44v6 in healthy control skin, particularly within the basal layer and stratum spinosum, but scant dermal matrix expression beyond staining of infiltrating leukocytes (Leigh *et al.*, 1996; Seiter *et al.*, 1996, 1998). Non-Gottron's and Gottron's DM lesions exhibited qualitatively stronger CD44s and CD44v3 staining within vessels compared with healthy controls; however, dermal staining patterns of CD44s, CD44v3, and CD44v6 in healthy controls, non-Gottron's, and Gottron's lesions did not show significant differences (data not shown). We found no detectable CD44v6 in any dermal sample, despite abundant pericellular keratinocyte staining in the epidermis in the same slides, which served as an internal positive control.

By contrast, Gottron's biopsies demonstrated a mean 4.5-fold denser accumulation of CD44v7 within the papillary dermal matrix compared with healthy control non-IP biopsies ($P<0.001$), and a mean 2.5-fold CD44v7 density compared with the dermis of non-Gottron's lesions ($P<0.01$; Figure 2). Non-Gottron's lesions did not differ from controls, and this differential expression was specific to CD44v7.

To study the effect of location versus disease in our patient samples, we acquired biopsies from the skin overlying the fourth proximal IP joint of five healthy volunteers with no history of autoimmune disease. Immunohistochemistry of healthy IP skin was compared with Gottron's samples to reveal a 4.5-fold greater density of C4S ($P<0.001$) and a 2.8-fold greater density of CD44v7 ($P<0.01$) in the IP dermis of patients with disease. For within-person comparisons, we also obtained a second biopsy from either the shoulder or the arm of two healthy volunteers. C4S density was not significantly different in the skin overlying the IP joint compared with non-IP skin of either volunteer. CD44v7 density in IP skin, however, demonstrated a modest but significant 1.6-fold (*t*-test $P=0.0149$) and 1.4-fold (*t*-test $P=0.0307$) increase compared with that in non-IP skin in the two volunteers, respectively.

### DM dermis contains increased osteopontin (OPN) protein

OPN is a known binding ligand for CD44v7, and this specific interaction has been implicated in chronic inflammation (Denhardt *et al.*, 2001). OPN is present in the spinous and granular layers of normal epidermis, and around sweat glands, hair follicles, and sebaceous glands of the dermis (Chang *et al.*, 2008). Our examination of healthy control skin

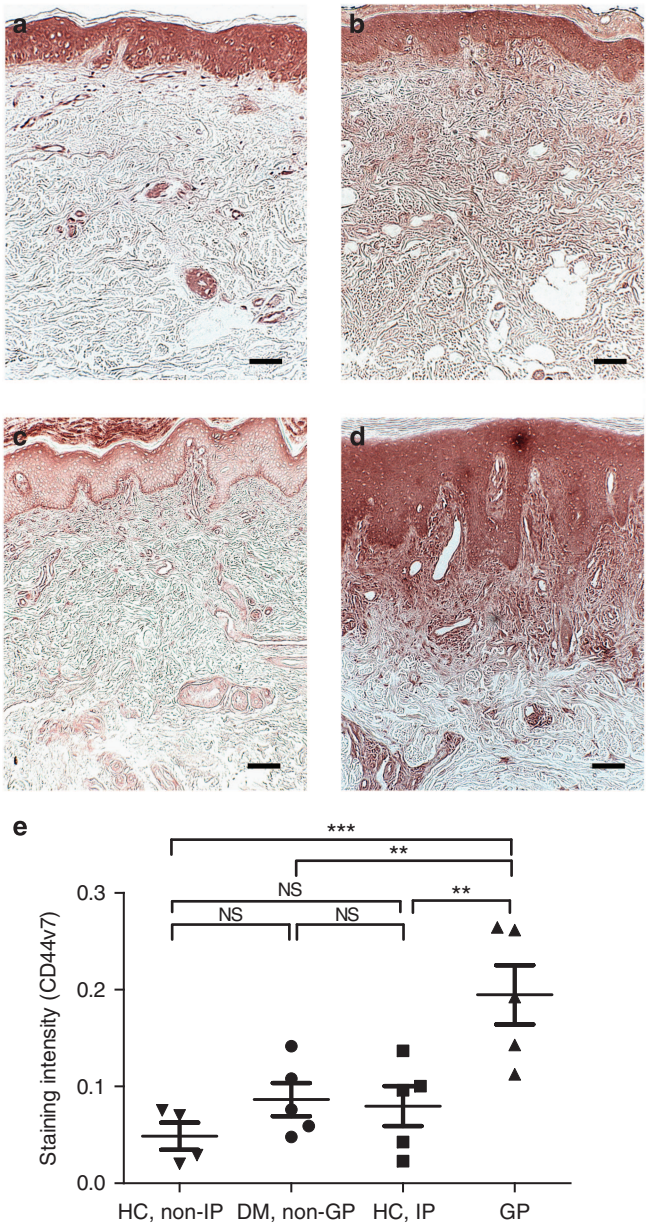


**Figure 2. Gottron's papules (GP), but not non-Gottron's dermatomyositis (DM) lesions, exhibit abundant accumulation of CD44 variant 7 (CD44v7) in the papillary dermis.** Sequential sections taken from the same biopsies shown in Figure 1 are stained with an antibody specific for CD44v7. (**a**) Healthy control skin (HC, non-interphalangeal (non-IP)). (**b**) Non-Gottron's DM lesions (DM, non-GP). (**c**) The proximal IP of healthy volunteers (HC, IP). (**d**) GP. Representative photomicrographs are shown, original magnification ×5, bar = 100 μm. (**e**) Systematic quantification of CD44v7 staining intensity within the papillary dermal matrix. Every data point is displayed; wide and short horizontal bars represent mean and SEM, respectively. $P = 0.0003$ by analysis of variance (***$P < 0.001$; **$P < 0.01$). NS, not significant.

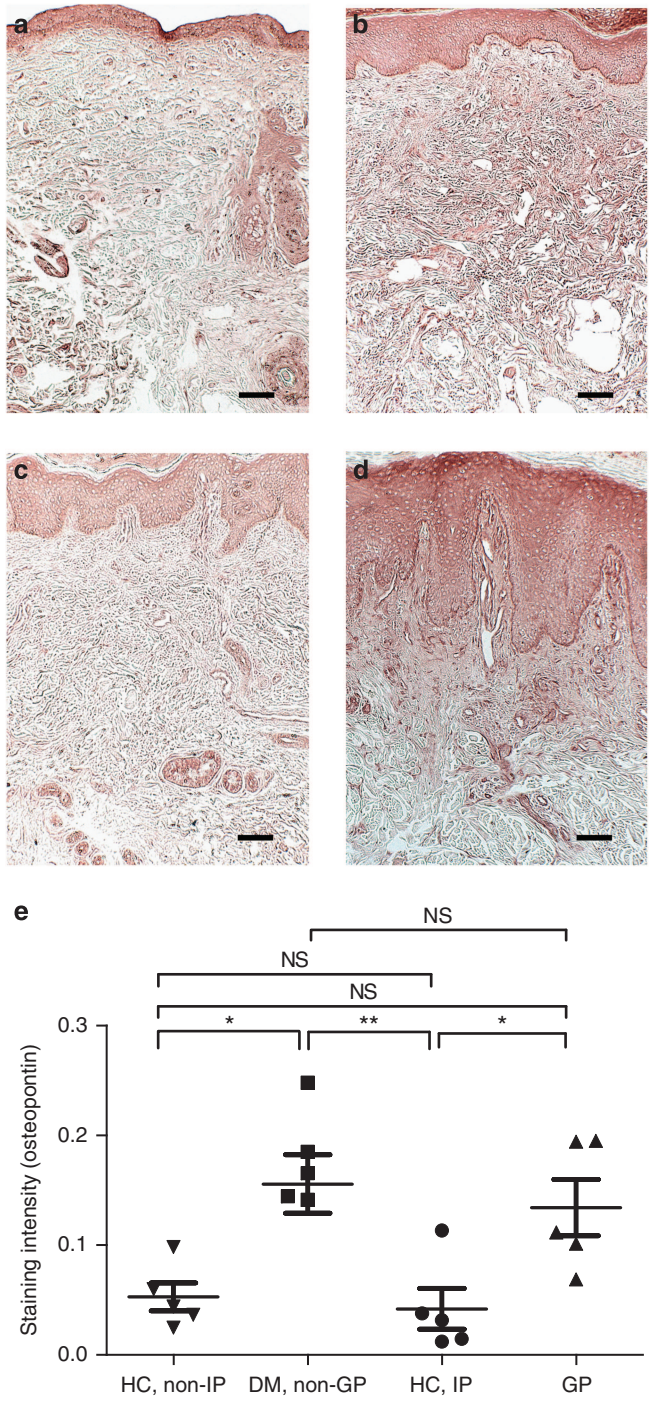


**Figure 3. Both Gottron's papules (GP) and non-Gottron's dermatomyositis (DM) lesions exhibit abundant accumulation of osteopontin, a CD44 variant 7-binding partner, in the papillary dermis.** Sequential sections taken from the same biopsies shown in Figures 1 and 2 are stained with an antibody specific for osteopontin. (**a**) Healthy control skin (HC, non-interphalangeal (non-IP)). (**b**) Non-Gottron's DM lesions (DM, non-GP). (**c**) The proximal IP of healthy volunteers (HC, IP). (**d**) GP. Representative photomicrographs are shown, original magnification ×5, bar = 100 μm. (**e**) Systematic quantification of osteopontin staining intensity within the papillary dermal matrix. Every data point is displayed; wide and short horizontal bars represent mean and SEM, respectively. $P = 0.0036$ by analysis of variance (**$P < 0.01$; *$P < 0.05$). NS, not significant.

revealed similar patterns (Figure 3a). However, compared with location-matched healthy controls, biopsies taken from non-Gottron's (Figure 3b) and Gottron's lesions (Figure 3c) exhibited increased accumulation of OPN diffusely distributed throughout the dermis. Quantitative analysis of dermal

OPN revealed a mean 3.3-fold greater density in non-Gottron's samples compared with healthy control samples ($P<0.01$) and a 2.5-fold OPN density in Gottron's samples compared with healthy controls ($P<0.05$; Figure 3d). OPN in Gottron's lesions was not found to be significantly different from that in non-Gottron's DM lesional skin, nor did OPN in healthy control IP skin differ from healthy control non-IP skin.

### Mechanical stretching of dermal fibroblasts *in vitro* induces CD44v7 mRNA expression

Because mechanical stretching has been reported to induce expression of CD44v7 and v8 in porcine ocular trabecular meshwork cells (Keller *et al.*, 2007), we hypothesized that the increased CD44v7 seen in the IP skin of DM patients and healthy volunteers is induced by chronic stretching of the skin over joints. Thus, we stretched primary dermal fibroblasts on cell culture membrane inserts and then determined CD44v7 mRNA expression by quantitative real-time PCR (reverse-transcriptase–PCR). We found that mechanical stretching of dermal fibroblasts induced a significant 1.6-fold increase in CD44v7 mRNA expression at 6 hours ($P<0.01$), which returned to a baseline expression by 12 hours (Figure 4a). CD44v7 protein in the supernatant of stretched fibroblasts at 6 hours increased $3.3 \pm 1.3$ times relative to control fibroblasts ($P<0.05$; Figure 4b and c). OPN alone did not induce CD44v7 mRNA (data not shown). These results were reproducible in three repeat experiments. By contrast, mechanical stretching did not have a significant effect on fibroblastic OPN mRNA expression.

### IFN-γ induces OPN mRNA expression in cultured dermal fibroblasts

IFN-γ has been reported to stimulate OPN expression in monocytes (Li *et al.*, 2003), but this induction has not been studied in dermal fibroblasts. Because IFN-γ is present in the skin of DM patients (Caproni *et al.*, 2005; Wenzel *et al.*, 2005), and mutations in the IFN-γ pathway have been associated with DM (Chinoy *et al.*, 2007), we hypothesized a role for IFN-γ in the dysregulation of OPN expression in the skin. Treatment of human primary dermal fibroblasts with IFN-γ ($10\,ng\,ml^{-1}$) induced a 4.4-fold mean OPN mRNA expression compared with untreated control cells *in vitro* ($P<0.001$; Figure 5a). IFN-γ ($10\,ng\,ml^{-1}$) induced an 8.1-fold increase in OPN protein in fibroblast supernatant ($P<0.001$; Figure 5b). OPN mRNA and protein in the fibroblast supernatant were not induced by the pro-inflammatory cytokines IL-1α or IFN-α, indicating specificity (Figure 5a and b). Neither IFN-γ nor OPN treatment had a significant effect on fibroblast CD44v7 mRNA expression (data not shown).

### Mechanical stretching of dermal fibroblasts treated with OPN increases human acute monocytic leukemia cell line (THP-1) monocyte adhesion

On the basis of these findings, we hypothesized that the increased CD44v7 in association with its ligand OPN could have a crucial role in the localized inflammation of Gottron's papules. To study the functional effect of increased CD44v7 ligation with OPN on immune cell recruitment, we incubated dermal fibroblast monolayers overnight, without or with stretching, without or with recombinant human OPN (rOPN), followed by rinsing. Fluorescently labeled THP-1 monocytes were then cocultured with the prepared fibroblast monolayers, followed by rinsing to remove non-adherent

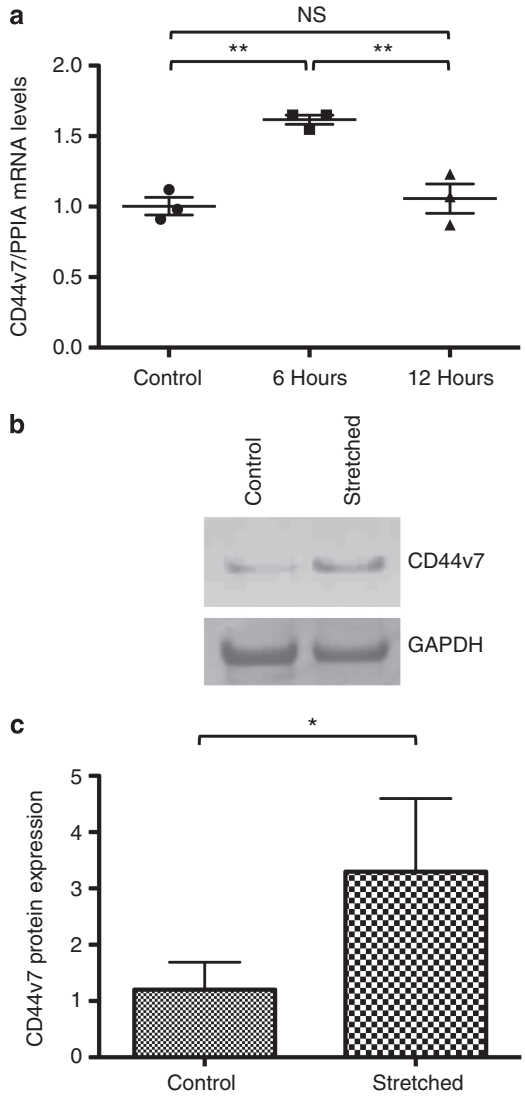


**Figure 4. Mechanical stretching induces CD44 variant 7 (CD44v7) in cultured human dermal fibroblasts.** Confluent cultured human dermal fibroblasts grown on flexible membranes were subjected to tonic mechanical stretching. All cells were plated simultaneously and stretched for the final 6 or 12 hours of incubation, after which RNA was harvested simultaneously. (**a**) CD44v7 mRNA levels were assayed by quantitative real-time reverse-transcriptase–PCR, normalized to peptidylprolyl isomerase A (PPIA) mRNA levels, and then expressed relative to unstretched controls. Every data point from a representative experiment is displayed; wide and short horizontal bars represent mean and SEM, respectively. $P=0.0017$ by analysis of variance (ANOVA; $^{**}P<0.01$). (**b**) CD44v7 protein in the supernatant of stretched fibroblasts at 6 hours was assayed by western blotting and expressed relative to unstretched controls. (**c**) Mean CD44v7 protein detected in one representative experiment; horizontal bar represents SEM. $P=0.0321$ by ANOVA ($^{*}P<0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant.

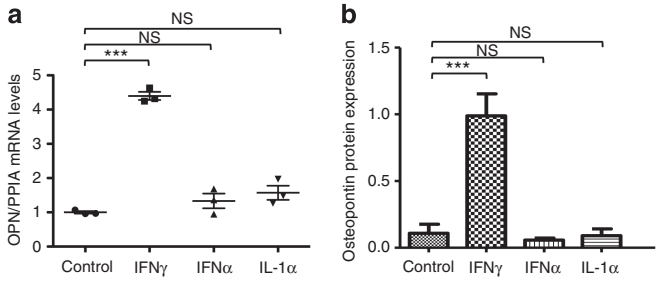


**Figure 5. IFN-γ, but not IFN-α or IL-1α, induces osteopontin (OPN) in cultured dermal fibroblasts.** Confluent cultured human dermal fibroblasts were incubated for 6 hours in control medium or in medium supplemented with IFNγ (10 ng ml$^{-1}$), IFNα (100 U ml$^{-1}$), or IL-1α (20 ng ml$^{-1}$), after which RNA or conditioned media for ELISA was harvested; data from representative experiments are displayed. (**a**) OPN mRNA levels were assayed by quantitative real-time reverse-transcriptase–PCR, normalized to peptidylprolyl isomerase A (PPIA) mRNA levels, and expressed relative to control; points denote mean OPN/PPIA mRNA for each well (sampled in triplicate); wide and short horizontal bars represent mean and SEM of triplicate conditions, respectively. $P<0.0001$ by analysis of variance (ANOVA; ***$P<0.001$). (**b**) OPN protein in control and conditioned media was quantified by ELISA; mean OPN protein detected (ng ml$^{-1}$) is indicated; horizontal bar represents SEM of triplicate conditions. $P=0.0003$ by ANOVA (***$P<0.001$). NS, not significant.

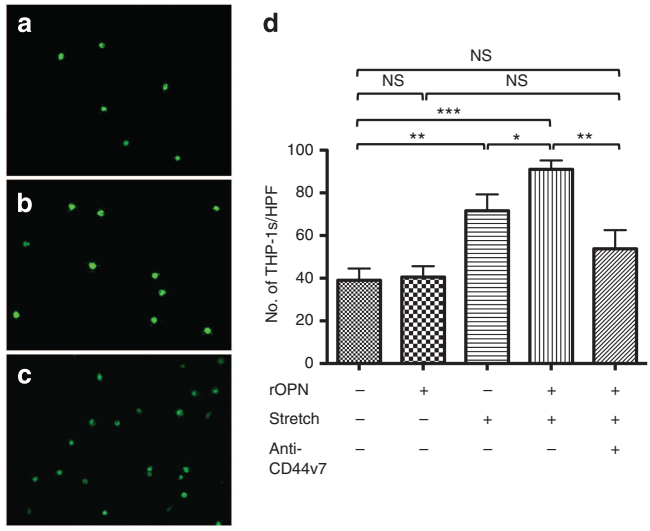


**Figure 6. Mechanical stretching of fibroblasts, particularly in combination with osteopontin (OPN) treatment, augments the adhesion of human acute monocytic leukemia cell line (THP-1) monocytes, in a process dependent on CD44 variant 7 (CD44v7).** Confluent monolayers of cultured human dermal fibroblasts grown on flexible membranes were incubated overnight without or with OPN, without or with stretch, as indicated. Unbound OPN was rinsed away, and then adherence of fluorescently labeled THP-1 monocytes onto these monolayers was assessed. Wells were incubated for 1 hour following treatment with anti-CD44v7 blocking antibody or isotype control Ig, and then washed just before addition of the labeled monocytes. Representative fluorescent micrographs of adherent monocytes (green dots) are shown. (**a**) Control fibroblasts. (**b**) Mechanically stretched fibroblasts. (**c**) Mechanically stretched fibroblasts pretreated with recombinant OPN (rOPN). (**d**) Counts of adherent, fluorescent monocytes per high-power field (HPF; means ± SEM, $n=6$), $P<0.0001$ by analysis of variance (***$P<0.001$; **$P<0.01$; *$P<0.05$). NS, not significant.

monocytes, and the adherent monocytes were visualized with an inverted fluorescent microscope. We found that overnight incubation of fibroblast monolayers in medium supplemented with rOPN had no effect on THP-1 adhesion in the absence of stretch (Figure 6). Stretching the fibroblasts in the absence of rOPN supplementation resulted in an increase in the number of adherent THP-1 cells by 1.8 times to that of unstretched, no-rOPN controls (Figure 6; $P<0.01$). Stretching fibroblasts overnight in medium supplemented with rOPN resulted in an increase in THP-1 adhesion by 2.2 times to that of stretched fibroblasts without rOPN ($P<0.001$) and by 2.3 times to that of unstretched, no-OPN controls ($P<0.001$).

To demonstrate the specific effect of fibroblast CD44v7 on THP-1 adhesion, we incubated prepared fibroblast monolayers with anti-CD44v7 blocking antibody or isotype control IgGκ for 1 hour immediately before THP-1 coculture. Stretched, rOPN-treated monolayers blocked with anti-CD44v7 antibody demonstrated a 41% reduction in THP-1 adhesion compared with identically prepared fibroblast monolayers incubated with control isotype IgGκ (Figure 6; $P<0.001$).

## DISCUSSION

In the current study, we found a unique molecular signature of Gottron's papules that consists of a dense dermal deposition of C4S, abundant amounts of a C4S-binding molecule, CD44v7, which has been causally linked to autoimmunity, and co-localized deposits of OPN, a cytokine-like factor that cooperates with CD44v7 to recruit inflammatory cells. By contrast, non-Gottron's DM lesions had far less C4S and exhibited levels of CD44v7 that were indistinguishable from the low amounts in skin from healthy controls. OPN was present in non-Gottron's DM lesions, but the ability of this molecule to recruit immune cells remains unproven in the near absence of CD44v7, its major binding partner. The combination of mechanical stretch to induce CD44v7 over the extensor surfaces of joints with IFN-γ, a factor that induces OPN expression and occurs more generally in the skin of DM (Caproni *et al.*, 2005; Wenzel *et al.*, 2005), may explain the co-localization of these molecules in Gottron's papules.

The CD44v7 chondroitin sulfate proteoglycan is of particular interest in autoimmunity as a demonstrated mediator of cell homing, adhesion, and survival. The soluble pattern of CD44v7 seen in our samples is probably the result of CD44 shedding and sequestration through its association with the cell-associated matrix; shed CD44 also competes with cell surface CD44 for ligand binding, modulation, or disruption of CD44-dependent cell–cell and cell–matrix adhesion (Cichy and Pure, 2003). In a mouse model of experimental colitis, anti-CD44v7 antibody treatment blocks T-cell extravasation and recruitment to the intestinal mucosa, thus curing colitis (Wittig *et al.*, 2002; Farkas *et al.*, 2005). Blockade of CD44v7 also induces apoptosis of mononuclear cells that had been extracted from the inflamed mucosa of patients with Crohn's disease (Wittig *et al.*, 2002).

The role of OPN in autoimmunity is an area of active investigation. OPN gene polymorphisms associate with

autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis, and plasma OPN concentration is correlated with systemic lupus erythematosus susceptibility, serum cytokine profiles, and disease activity (Forton *et al.*, 2002; Wong *et al.*, 2005; Han *et al.*, 2008; Kariuki *et al.*, 2009). A polymorphism for OPN in a cohort of newly diagnosed juvenile DM patients was associated with increased serum IFN-α level (Niewold *et al.*, 2010).

Demonstrated immunological functioning of OPN includes polyclonal B cell and macrophage activation in autoimmune murine models and activation-induced T-cell death (Singh *et al.*, 1990; Lampe *et al.*, 1991; Weber and Cantor, 2001; Chiocchetti *et al.*, 2004). In the skin, OPN secreted by activated T cells is thought to enhance T helper type 1-mediated immunity through interaction with immune cells: OPN enhances production of IFN-α in plasmacytoid dendritic cells, has chemotactic properties for macrophages, dendritic cells, and T cells, and OPN encountered by dendritic cells in the dermal matrix activates and polarizes Langerhans cells and myeloid-type dendritic cells toward a T helper type 1 phenotype, directing them to draining lymph nodes (Giachelli *et al.*, 1998; O'Regan *et al.*, 2000; Weiss *et al.*, 2001; Kawamura *et al.*, 2005; Renkl *et al.*, 2005). The specific dose- and integrin-dependent binding of OPN to CD44 is implicated with roles in adhesion, migration, modulation of cytokine secretion, anti-apoptotic signaling, and tumor invasion (Anborgh *et al.*, 2010). For example, OPN induces CD44-dependent chemotaxis and down-modulates anti-inflammatory IL-10 secretion through interactions with CD44, and is hypothesized as an activator of the CD44 variant survival signal in lymphocytes by the extracellular matrix of the gut mucosa (Weber *et al.*, 1996; Katagiri *et al.*, 1999; Ashkar *et al.*, 2000; Denhardt *et al.*, 2001).

Intriguingly, CD44v7, a principal CD44 isoform sufficient for OPN binding (Katagiri *et al.*, 1999), is the isoform present in our Gottron's papules. We have previously shown CD44v7 expression in discoid lupus erythematosus skin (Kim and Werth, 2011). However, OPN is not found in abundance within discoid lupus erythematosus lesions, and accordingly the OPN/CD44v7 complex is not seen.

C4S and CD44 are known to contribute to cellular adhesion (Murai *et al.*, 2004; Charbonneau *et al.*, 2007). Our THP-1 cell adhesion assay mimics the co-expression of CD44v7 and OPN in active areas of active inflammation in Gottron's papules of DM, and its results demonstrate the adhesive interactions regulated by stretch-induced CD44v7 on dermal fibroblasts complexed with OPN. Stretching of dermal fibroblasts may induce upregulation of a variety of molecules, but the inhibitory effect of anti-CD44v7 monoclonal antibody implicates a functional role specific to this molecule in adhesion.

Thus, the present study identifies C4S, CD44v7, and OPN as three molecules that contribute to inflammation individually and in association with one another and are uniquely present together in Gottron's papules. We propose that stretch-induced expression of CD44v7 overlying joints, in concert with dysregulated levels of OPN generally in the skin of DM patients, increases local recruitment of leukocytes and contributes to the pathogenesis and resistance of Gottron's papules. A greater understanding of these molecules and the consequence of their accumulation in DM skin lesions may ultimately provide new therapeutic targets.

## MATERIALS AND METHODS

### Skin specimen collection

Lesional skin biopsies from patients with DM ($n=10$) were obtained in the autoimmune skin disease clinic at Penn. Five biopsies were taken from Gottron's papules over the proximal IP or metacarpophalangeal joints, and five unmatched biopsies were taken from active lesions on the arm, shoulder, or chest. Healthy control skin was provided by volunteers ($n=10$) without a history of autoimmune skin disease; five biopsies were from skin overlying the proximal IP of the third digit, and five biopsies were from sun-protected arm. Two volunteers provided both a proximal IP and arm or shoulder sample. All biopsies were obtained with written, informed patient consent and adherence to the Helsinki Guidelines according to our approved protocol under the UPenn Institutional Review Board. Biopsies were fixed in 10% neutral-buffered formalin overnight, embedded in paraffin, and cut into 4-μm sections.

### Immunostaining

Immunostaining for C4S was performed on human skin as described previously (Chang *et al.*, 2011a) by predigestion with chondroitinase AC II (Sigma, St Louis, MO), followed by incubation with an anti-deltaDi-4S monoclonal antibody that recognizes a terminal glucuronic acid residue adjacent to N-acetylgalactosamine-4-sulfate at the nonreducing end of chondroitin sulfate chains in proteoglycans produced after chondroitinase digestion of chondroitin sulfate chains (clone 2-B-6, Seikagaku Biobusiness, Tokyo, Japan). We also performed immunostaining using monoclonal antibodies against a fusion protein of glutathione S-transferase with individual variant domains (v3, v6, and v7) of human CD44 (AbD Serotec, Raleigh, NC). Staining for CD44 v6 required heat-induced antigen retrieval, whereby slides were incubated for 10 minutes at 70 °C in Target Retrieval Solution (Dako, Carpinteria, CA). OPN was detected using a rabbit polyclonal antibody recognizing full-length, as well as thrombin- and matrix metalloproteinase-cleaved, OPN (AbCam, Cambridge, MA). Sections were then incubated with biotinylated secondary antibody and streptavidin-horseradish peroxidase (Dako, Carpinteria, CA), followed by Vector NovaRED substrate (Vector, Burlingame, CA). Negative controls included mouse IgGκ isotype antibody (Sigma, St Louis, MO) and phosphate-buffered saline instead of chondroitinase AC II enzyme digestion.

### Systematic quantification of immunostaining

All high-power fields in three sections of each 3- to 5-mm skin biopsy were examined at original magnification ×10. Among all fields examined, one high-power field appearing most representative of all sections was selected from each biopsy to measure matrix intensity, and this field was photographed at original magnification ×10. An intensity channel was applied to photograph files using commercial software to isolate staining intensity (Image-Pro Plus v5.0, Cybernetics, Bethesda, MD). One-hundred 5-μm circular manual tags were selected by systematic uniform random sampling of the area of interest (e.g., within the upper dermis for CD44v7; Mayhew, 2008). The mean intensity of staining within the total area

selected by the manual tags is reported as the representative staining intensity value for each specimen.

### Cell culture, mechanical stretch, and cytokine treatment

Normal adult, human fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Mechanical stretching experiments were conducted by seeding primary human dermal fibroblasts onto membrane inserts (six-well format, 23.1-mm diameter, 3.0-μm pore size, BD Falcon no. 353091, BD Biosciences, Franklin Lakes, NJ) and culturing the cells to confluence (Keller *et al.*, 2007). Next, a smooth 5-mm glass bead was placed in the dish beneath the center of the insert membrane, and constant weight was applied to the lid of the insert to force the edge of the insert down onto the bottom of the dish. This produced distortion of the membrane, increasing its surface area and thereby mechanically stretching the cells and their extracellular matrix. Stretching was continued for 6 or 12 hours, and then each stretched membrane insert was cut out, vortexed in RNAeasy lysate buffer, and RNA purified, followed by quantitative PCR.

For cytokine stimulation, fibroblasts were plated in 100-mm-diameter dishes and grown to confluence. Fibroblasts were treated for 6 hours with 10 ng IFN-γ $ml^{-1}$ (R&D Systems, Minneapolis, MN), 100 U IFN-α per 2 ml (PBL Interferon Source, Piscataway, NJ), or 20 ng recombinant human IL-1α $ml^{-1}$, before lysis with the RNAeasy mini kit lysate buffer and processing RNA (Qiagen, Valencia, CA). Experiments were performed in triplicate and repeated three times with reproducible results.

### Quantitative real-time reverse-transcriptase–PCR

Quantification of specific mRNAs was performed as previously described (Chang *et al.*, 2011a). Total RNA was reverse transcribed using the SuperScript First-Strand Synthesis System for reverse-transcriptase–PCR (Invitrogen, Carlsbad, CA) with random hexamer primers. Real-time PCR was performed using Taqman Gene Expression Assays for human OPN (Taqman Assay ID Hs00960942_m1; Applied Biosystems, Foster City, CA) and peptidylprolyl isomerase A (cyclophilin A), the housekeeping control (Taqman Assay ID Hs99999904_m1; Applied Biosystems). Custom-made sequences for detection of human CD44 exon 11 transcripts, which encode exclusively the v7 motif, were 5′-TGCAAGGAAGGACAACACCAA-3′ (sense primer) and 5′-GGGTGTGAGATTGGGTTGAAGAAA t-3′ (antisense primer). All reactions were performed in triplicate. The ΔΔCt method was used to analyze the differential gene expression in cytokine-treated fibroblast cultures compared with controls, as described previously (Chang *et al.*, 2011b).

### Western blot

Serum-free supernatant from control and stretched fibroblasts were collected after 6 hours. The supernatants were centrifuged at 10,000*g* for 1 minute. Equal amounts of protein (10 μg) were separated on a 4–12% bis–tris gel (Invitrogen, Grand Island, NY), transferred to a membrane (Millipore, Billerica, MA), and stained with 1:2,000 dilution of mouse anti-human CD44v7 (AbD Serotec, Oxford, UK). For loading control, the washed membrane was stained with 0.1% Coomassie R-350 (GE Healthcare, Piscataway, NJ). Western blot results were recorded by X-ray films (Kodak Scientific Imaging, Truesense Imaging, Rochester, NY) and quantified by ImageJ (Collins, 2007).

### OPN protein determination by ELISA

Conditioned media from IFN-γ, IL-1α, or IFN-α-treated cultured fibroblasts were collected and concentrated 2-fold. The protein was quantified by the CB-Protein-Assay-Kit (G-Biosciences, St Louis, MO) and volume was adjusted to 100 uL, so that equal protein was used for each sample. OPN protein was quantified by commercial ELISA (R&D Systems, Minneapolis, MN).

### Adhesion assay

Primary dermal fibroblasts were cultured to confluence on 23.1-mm polyethylene terephthalate membrane inserts and then stretched, or not, with 5-mm glass beads. Here, stretching lasted overnight and was combined with treatment with 0 or 5 μg of recombinant OPN $ml^{-1}$. Unbound OPN was rinsed with warmed phosphate-buffered saline before the assay of monocyte adherence. Fibroblasts were maintained in serum-free, phenol red–free DMEM for the remainder of the assay. For inhibition studies, 25 μg of mouse anti-human CD44v7 (AbD Serotec, Raleigh, NC) per milliliter or mouse anti-human IgGk isotype antibody control (Sigma, St Louis, MO) per milliliter was added to stretched monolayers after rinsing of unbound OPN for 1 hour, and then washed. Living THP-1 monocytes labeled with 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA, Invitrogen, Eugene, OR) were added ($5 \times 10^5$/insert), followed by a 30-min incubation at 37 °C. Unbound monocytes were then removed by rising four times, and the number of monocytes firmly bound to the fibroblast monolayers was counted under fluorescent microscopy in six random high-power fields near the centers of the stretched membranes, where the stretching was the greatest. Experiments were repeated four times with reproducible results.

### Statistical analysis

Comparison of several groups simultaneously was performed by initially using analysis of variance (GraphPad Prism v5.01). When analysis of variance indicated differences among the groups, pairwise comparisons between experimental groups were made using the Student–Newman–Keuls *q* statistic and are indicated in Figures 1–6. Unless otherwise indicated, summary statistics are reported as mean ± SEM.

## CONFLICT OF INTEREST

This material is based on the work supported by the Lupus Foundation of America and in part by a Merit Review Grant from the Department of Veterans Affairs (Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development) and by the National Institutes of Health (NIH K24-AR 02207) to VPW. The authors state no conflict of interest.

## ACKNOWLEDGMENTS

We thank Dr Ming-Lin Liu for expert technical assistance. We also thank Dr Kevin Jon Williams for guidance with the refining and editing of the final manuscript. A preliminary report of this work was presented at the Society for Investigative Dermatology (May 2010, Atlanta, GA; Kim and Werth, 2010).

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