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ROLES OF ADIPOCYTES AND FIBROBLASTS IN ACTIVATION OF THE ALTERNATIVE PATHWAY OF COMPLEMENT IN INFLAMMATORY ARTHRITIS IN MICE

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Abstract

The complement system is involved in mediation of joint damage in rheumatoid arthritis, with evidence suggesting activation of both the classical and alternative pathways (AP). The AP is both necessary and sufficient to mediate collagen antibody-induced arthritis (CAIA), an experimental animal model of immune complex (IC)-induced joint disease. The AP in mice is dependent on MASP-1/3 cleavage of pro-factor D (pro-FD) into mature FD. The objectives of the present study were to determine the cells synthesizing MASP-1/3 and pro-FD in synovial tissue. CAIA was studied in wild-type C57BL/6 mice, and the localization of mRNA and protein for FD and MASP-1/3 in synovial adipose tissue (SAT) and fibroblast-like synoviocytes (FLS) was determined using various techniques, including laser capture micro-dissection (LCM). SAT was the sole source of mRNA for pro-FD. Cultured differentiated 3T3 adipocytes, a surrogate for SAT, produced pro-FD but no mature FD. FLS were the main source of MASP-1/3 mRNA and protein. Using cartilage micro-particles (CMP) coated with anti-collagen mAb and serum from *MASP-1/3*$^{-/-}$ mice as a source of factor B, pro-FD in 3T3 supernatants was cleaved into mature FD by MASP-1/3 in FLS supernatants. The mature FD was eluted from the CMP, and was not present in the supernatants from the incubation with CMP, indicating that cleavage of pro-FD into mature FD by MASP-1 occurred on the CMP. These results demonstrate that pathogenic activation of the AP may occur in the joint through IC adherent to cartilage and the local production of necessary AP proteins by adipocytes and FLS.

Keywords

complement; arthritis; immune complex; inflammation

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Disclosures

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INTRODUCTION

Both cellular and humoral immunity appear to be involved in the pathogenesis of rheumatoid arthritis (RA), although the precise role of each arm of the immune system in initiation and/or perpetuation of the autoimmune disease process remains unclear. Recent studies have emphasized the presence of Ab to citrullinated protein antigens (ACPA) prior to the onset of clinically apparent RA and their correlation with worse joint damage during the disease course (1). Activation of the complement system is thought to mediate inflammation and tissue damage in RA (2–4). Immune complexes (IC) containing IgG Ab are found in the cartilage and synovium of rheumatoid joints and have been implicated in induction of local tissue damage primarily through activation of the complement system (5–7). IgG in arthritis-related IC may activate both the classical (CP) and alternative (AP) pathways of the complement system (8–11) and, indeed, ACPA from RA patients activate both pathways (12).

Different proteins have been thought to be involved in the initiation phases of the three pathways of the complement system leading to a final common effector phase following the cleavage of C3 into C3a and C3b (11). The CP is initiated by Ab leading to the formation of the CP C3 convertase, C4b2b. The lectin pathway (LP) is initiated by binding of complexes of mannose-binding lectins (MBL) and MBL-associated serine proteases (MASP-1, -2, and -3) with the eventual formation of the LP C3 convertase, C4b2b. The AP may be initiated by spontaneous turnover of C3 with transient formation of C3(H_2O) and subsequent generation of C3b, followed by binding of factor B (FB) with cleavage by factor D (FD) and generation of the AP C3 convertase, C3bBb. Cleavage of C3 also exposes a short-lived thioester in C3b that covalently attaches to amine and carboxyl groups on target surfaces. The AP may also be initiated by properdin bound to target-containing molecular patterns (13) or by adherent IgG or IgA (10, 14). Recent studies have shown that generation of the AP in mice is dependent on MASP-1/3 cleavage of pro-FD to form mature, but inactive, FD found in the circulation (15). The AP may greatly increase complement initiated by all 3 pathways by generating further C3b on cell surfaces through the amplification loop (11).

We have utilized an animal model of inflammatory joint disease induced by IC to examine the role of the complement system in mediating inflammation and tissue damage. Collagen antibody-induced arthritis (CAIA) is caused by the passive infusion of mAb to type II collagen (CII) into wild-type (WT) mice with the formation of IC in the articular cartilage and synovium. Studies in mice deficient in specific components of the complement system have shown that the AP is both necessary and sufficient to mediate CAIA as neither the LP nor CP appear to be required (16, 17). Moreover, mice deficient in MASP-1/3 do not develop CAIA and sera from these mice exhibit an absence of AP activation in vitro (18).

The mechanism of activation of the AP in inflammatory joint diseases has not been established and may involve complement components derived from the circulation. The possibility also exists that the AP may be initiated locally by IgG deposition in the articular cartilage and synovium with synthesis of the necessary complement components by cells within the joint. Macrophages from the synovial fluid or synovial membrane of patients with RA synthesized C3, FB, FD and properdin (19). C3 and FB were also synthesized by fibroblasts cultured from the rheumatoid synovium (20). In another study, mRNA for all components of the AP, except for properdin, was present in rheumatoid synovium; cultured synovial macrophages synthesized C3 and properdin while fibroblast-like synoviocytes (FLS) secreted FB (21). Using in situ hybridization, immunohistochemistry, and Western blot analysis, the presence of C3, FB, C3aR, and C5aR were demonstrated in rheumatoid synovium (22). FD is primarily synthesized by adipocytes as a precursor molecule (pro-FD); it remains unclear whether mature FD is generated during release from the cell or pro-FD is

secreted with subsequent cleavage to mature FD occurring in the cell microenvironment or circulation (23–27). The production of FD by adipocytes in the joint has not been examined. Lastly, mRNA for MASP-1/3 is found in a variety of cells and tissues, however MASP-1/3 is synthesized primarily in the liver by alternate splicing from a common mRNA (28). The production of MASP-1/3 by cells in the joint has not been examined.

The objectives of these studies were to examine for the presence of mRNA and protein for components of the AP in synovial tissue and synovial adipose tissue (SAT) and to explore the possible mechanisms of local activation of the AP in the joint. Our results indicate that the SAT represents the primary site of synthesis of pro-FD in the joint with FLS in the synovium synthesizing MASP-1. The mRNA for C3, FB, and properdin are also present in joint cells. An in vitro system was devised using cartilage microparticles (CMP) as a biological substrate to examine activation of the AP. The CMP were coated with a mixture of anti-CII mAb and serum from *MASP-1/3*$^{-/-}$ mice was used as a source of FB. In this experimental system pro-FD secreted from 3T3 adipocytes was cleaved into mature FD by MASP-1/3 from FLS, with the FD remaining associated with the CMP. These results establish the possibility that the AP of the complement system can be activated in the joint through the local synthesis of key components by adipocytes and FLS with assembly on IC on the cartilage surface.

MATERIALS AND METHODS

Mice

Ten to twelve week-old WT C57BL/6 male mice (n = 28) were used for this study. We obtained *Df*$^{-/-}$ mice from Drs. Kazue Takahashi and Gregory Stahl and *MASP-1/3*$^{-/-}$ mice from Dr. Minoru Takahashi. Our laboratory has maintained colonies of both *Df*$^{-/-}$ and *MASP-1/3*$^{-/-}$ C57BL/6 homozygous mice with the F10 progeny used for this study; sera from these mice were used for Western blot analyses. All WT C57BL/6 mice were obtained from Jackson Laboratories. The *Df*$^{-/-}$ and *MASP-1/3*$^{-/-}$ mice were genotyped by DNA PCR prior to use. All mice were kept in a barrier animal facility with a climate-controlled environment having 12 h light/dark cycles. Filter top cages were used with three mice in each cage. During the course of this study, all experimental mice were fed breeder's chow provided by the Center for Laboratory Animal Care, University of Colorado School of Medicine.

Induction of collagen antibody-induced arthritis

CAIA was induced in WT mice (n = 12) using a cocktail of 4 mAb to bovine CII (Arthrogen-CIA, Chondrex) suspended in sterile PBS as previously described (17). All WT mice received i.p. injections of 8 mg/mouse of Arthrogen on day 0 and 50 ug/mouse of LPS from E. coli strain 0111B4 on d 3 to synchronize the development of arthritis. All mice started to develop arthritis at d 4 and were sacrificed at d 10. Clinical disease activity (CDA) was examined daily according to our previously published studies (17).

Immunohistochemistry of synovium and synovial adipose tissue

Knee joints from WT mice with CAIA were fixed in 10% neutral-buffered formalin and examined by immunohistochemical staining for macrophages (F4/80), MASP-1, and pro-FD according to our published methods (16). Oil Red O (Sigma-Aldrich) staining was also used to examine for the presence of differentiated adipocytes in the SAT. A counter hemotoxylin stain (VWR) was used to show the presence of FLS.

Cell culture and differentiation

Two different cell types were used for RT-PCR studies: the murine adipocyte cell line 3T3 (American Type Culture Collection) and FLS. Undifferentiated (undiff) and differentiated (diff) 3T3 cells were cultured according to the described protocol (Zin-Bio Inc., Research Triangle Park). Undiff and diff 3T3 cells are also known as immature and mature 3T3 cells and we used the former terminology. The presence of diff 3T3 cells was evaluated using Oil Red O staining. To obtain supernatants for Western blot analyses and RT-PCR, 8×10^5 undiff or diff 3T3 cells were cultured in 3 ml medium without serum.

A primary culture of FLS was obtained from Dr. Gary Firestein (University of California San Diego). These cells were originally derived from the synovium of mice with collagen-induced arthritis. FLS were cultured in T75 flasks in DMEM high glucose medium (Sigma) containing 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine and 0.5% gentamycin. The FLS became confluent at 7–14 days after thawing or splitting. Cultured FLS were used up to 10 passages when a new aliquot of cells was thawed. FLS used for evaluation of protein production were cultured in the absence of serum.

Lectin mRNA in FLS

The presence of mRNA for MBL-A, MBL-C, ficolin (FCN)-A, and FCN-B was examined in cultured FLS by RT-PCR using specific primers. The binding of MASP-1 protein in FLS supernatants to FCN-A was determined by adsorption of FCN-A onto agarose beads coated with N-acetyl-D-glucosamine (NAG) (29). The proteins associated with the NAG beads were eluted under dissociating conditions with subsequent determination by Western blot analysis, as described (29).

Western blot analysis

To examine for the presence of mature FD and Pro-FD, sera were obtained from *Df*$^{-/-}$ and *MASP1/3*$^{-/-}$ mice and supernatants were harvested from both undiff and diff 3T3 cells with Western blot analyses performed as previously described (18). The blots were incubated overnight at 4° C with goat anti-mouse FD Ab (dilution 1:2000) (a gift from Dr. Minoru Takahashi). This Ab reacts with murine pro-FD in serum, with mature murine FD in serum, and with recombinant human FD (Quidel), but not with murine pro-FD in cultured 3T3 cell supernatants. Rabbit anti-goat HRP was used as the secondary Ab (dilution 1:2000) (Hycult Biotech). The blots were washed 3×10 minutes in 1xPBS 0.5% Tween 20 and developed for 3 min by using a 1:1 mixture of SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). To detect pro-FD, a primary Ab (1:2000) was used that reacts only with the 5-residue N-terminal peptide (QPRGR) that is present on murine pro-FD and absent from mature FD (15, 18). The presence of MASP-1 and FCN A in supernatants harvested from both undiff and diff 3T3 cells was analyzed by Western blot analyses as previously described (18, 29).

Laser capture micro-dissection (LCM)

To obtain adequate amounts of SAT, WT mice with maximal CAIA (scores of 10–12) were selected for studies using LCM as previously described (30–32). The knee joints were located at 2X magnification for the synovium and the SAT was viewed at 10X. For LCM studies, we have used an average of 2603.8 ± 338.8 (mean ± SEM) FLS from the synovium of WT mice (n = 7). All cells were counted after cutting the synovium using a UV-laser. The Riboamp HS kit, used to amplify LCM RNA, has a minimum requirement of 10 cells, however, a minimum of 50–500 cells is recommended by the manufacturer. It is imperative to identify cell type because synovium contains not only FLS but also macrophages, neutrophils and other types of cells. Nonetheless FLS are abundantly present in the

synovium. To identify FLS and SAT in the knee joints a Toluidine(T)-blue dye was used. This dye does not affect the quality of the RNA as we examined all samples prior to the amplification by using a bioanalyzer. By using T-blue it was easier to differentiate cobble stone-like SAT from FLS and utmost care was taken to exclude the other cell type while cutting with a UV-laser. In parallel we also separately carried out immunohistochemical staining of the inflamed synovium using specific stains for macrophages and neutrophils. The morphology of the SAT was also confirmed using Oil O Red stain. All immunostains were done separately because of interference with the quality of the RNA. All other areas in the joint, except for FLS and SAT, were excluded, based on immunostaining in parallel sections. The RNA from the synovium and the SAT were obtained using the capture Macro LCM Cap. The RNA was isolated using the PicoPure RNA isolation kit. The isolated RNA was then amplified (1.5 round amplification) using the RiboAmp Plus kit and the RNA concentration was measured using a nanodrop. RT-PCR was performed using specific primers for the mRNA levels of complement components C3, FB, FD, MASP-1 and MASP-3. 18s ribosomal RNA was used as an internal control to quantify the mRNA levels in the synovium and SAT.

RT-PCR

Total RNA was extracted from cultured cells or from LCM samples using a RNAeasy Mini kit (Qiagen). For some RT-PCR studies, mesenteric adipose tissue and liver were obtained from WT mice without disease. All samples for RT-PCR were analyzed for C3, FB, MASP-1, MASP-3, and pro-FD according to Methods published in Nature Protocols (33). Forty thermal cycles (CT) was used to measure mRNA. According to this method all samples should be amplified until 40 cycles to determine positivity. A CT value of 40 or more indicates no amplification and this value was not included in the calculations. All RT-PCR data were analyzed by using a cDNA based standard curve. For example, the standard curves for pro-FD and MASP-1/3 were constructed by using mRNA from mouse adipose tissue and liver, respectively. In case of low mRNA expression, the RT-PCR product was confirmed by a 2 % agarose gel. The primers used for these studies are available upon request from the corresponding author.

In vitro activation of the AP on cartilage micro-particles

For these studies articular cartilage (AC), anti-CII mAb, serum from *MASP-1/3*$^{-/-}$ mice, diff 3T3 cell culture supernatant, and culture supernatant from FLS were used. AC from 16-week old C57BL/6 WT mice (n = 5) without CAIA was obtained by surgically removing the knee joints. AC fragments were obtained and CMP were prepared as recently described (34).

To examine cleavage of pro-FD into mature FD on the surface of CMP by MASP-1, Western blot analysis was performed. Supernatants collected from the diff 3T3 cells and FLS at 120 h of incubation in serum free media were used as a source of pro-FD and MASP-1, respectively. Sera from *MASP-1/3*$^{-/-}$ mice were used as a source of FB required to form the AP C3 convertase on the CMP. For this experiment, 500 μl of CMP (as a source of collagen) were washed three times with 1xPBS without Ca^{++}or Mg^{++}. To make solid phase IC, CMP were incubated overnight at 4°C with a mixture of anti-CII mAb (2 μg/ml) suspended in 2 ml of 0.1 M sodium carbonate-bicarbonate buffer (pH 9.5). After washing (3x) with 1xPBS without Ca^{++} or Mg^{++}, 125 μl of pooled sera from *MASP-1/3*$^{-/-}$ mice was added (1:1 ratio v/v) followed by the supernatant from diff 3T3 cells (25 μl per sample, 1:1 ratio v/v) containing pro-FD, and incubated for 1 h at 4°C. The supernatant from the FLS (1:1 ratio v/v) containing MASP-1was then added. This above mixture of IC (CMP/anti-CII mAb), sera from *MASP-1/3*$^{-/-}$ mice, diff 3T3 supernatant, and FLS supernatant was incubated overnight on a shaker at 4°C. After centrifugation of the CMP, supernatants were collected and eluates of the material adherent to the CMP were prepared. The pellet was

washed with cold 1xPBS and 50 μl of RIPA (Radio immunoprecipitation assay) buffer (Sigma) was added followed by incubation for 20 min at 4°C; these samples were then boiled for 10 min. After centrifugation of the pellet, the eluate of the CPM was collected.

All supernatant and eluate samples were electrophroresed using a 10% Bis-tris gel under reducing conditions. After transfer to the polyvinylidene difluoride membranes, detection of FD was performed by Western blot analysis, as described earlier (18). To show the specificity of cleavage of the pro-FD into mature FD on CMP, all relevant positive and negative controls were analyzed. These included CMP treated with FLS and 3T3 supernatants without *MASP-1/3*$^{-/-}$serum, CMP with *MASP-1/3*$^{-/-}$ serum, CMP with *MASP-1/3*$^{-/-}$ serum and 3T3 supernatant without FLS supernatant, and CMP with *MASP-1/3*$^{-/-}$ serum and FLS supernatant without 3T3 supernatant. *FD*$^{-/-}$ serum and purified human recombinant FD (hu rFD, 10 ng/ml) were used as controls to identify the FD band on Western blot analysis.

Statistical analyses

Student's t test was used to calculate *p*-values using the GraphPad Prism® 4 statistical program. The data in all graphs, histograms and tables are shown as the mean ± SEM with $p < 0.05$ considered significant. Preliminary analyses using a null hypothesis for w-statistics indicated that the data were normally distributed.

RESULTS

Immunohistochemistry of the inflamed joint

Knee joints were obtained from WT mice with CAIA at d 10 after the injection of mAb to CII, i.e. at d 7 after the injection of LPS. Studies were performed on the inflamed synovium and on the SAT. H&E staining showed the histological characteristics of each of these areas in the inflamed joint (Figs. 1A, B, G and H). Although the SAT was present as a distinct layer of cells adjacent to the synovium (Fig. 1H), adipocytes were often locally intermingled with synovial cells (Figs. 1G and H). Staining for macrophages, as F4/80 positive cells, revealed their presence scattered throughout the synovium and between adipose cells in the SAT (Figs. 1C and 1D). Cells staining weakly for MASP-1 were also localized throughout the synovium and within the SAT (Figs. 1E and 1F). Staining for MASP-3 could not be performed because of the unavailability of specific Ab. Lastly, Pro-FD was abundantly seen throughout the SAT (Fig. S1, middle panel).

These results indicate that adipocytes were present in a distinct layer adjacent to the inflamed synovium in mice with CAIA as well as intermingled with nearby synovial cells. Macrophages were also present in both the SAT and synovial tissue. Whereas weak staining for MASP-1 was also present in both areas, strong staining for pro-FD was observed only in the SAT.

mRNA in synovium and SAT obtained by Laser capture micro-dissection

LCM has been used to examine the localization within the synovium of differentially expressed genes (31, 32). Samples of synovium and SAT were obtained by LCM, the RNA was extracted, and mRNA for components of the AP were measured by RT-PCR. The SAT contained high levels of mRNA for C3 (Fig. 2A), FB (Fig. 2B), and Pro-FD (Fig. 2C). The synovium exhibited substantial levels of mRNA for C3 (Fig. 2A) and FB (Fig. 2B), as well as for MASP-1 (Fig. 2D) and MASP-3 (Fig. 2E). No mRNA for pro-FD was found in the synovium (Fig. 2C).

These results establish that the synovium contained mRNA for MASP-1 and MASP-3. In contrast, the SAT was the sole source of mRNA for pro-FD. Murine mesenteric adipose tissue and liver served as positive controls for mRNA for pro-FD and MASP-1, respectively, and as negative controls for the opposite protein (Fig. S2A).

mRNA and protein levels in cultured FLS

In order to examine for the levels of mRNA for components of the AP over time and in response to stimulation, FLS were cultured for 144 h in the absence of stimulation or in the presence of 50 μg/ml LPS, 100 ng/ml TNF-α, or 50 ng/ml IL-1α. Cell pellets were harvested for determination of mRNA levels of AP components by RT-PCR and supernatants were collected for determination of presence of MASP-1 protein by Western blot analysis. Cultured FLS showed low levels of mRNA for FB and C3, with slight induction by LPS and IL-1α (Figs. 3A and 3B). MASP-1 and MASP-3 mRNA were present in FLS without any exogenous stimulation (Figs. 3C and 3D). FLS exhibited virtually no mRNA for pro-FD (Fig. 3E). To calculate the amount of mRNA in each sample, 18s rRNA was also amplified as an internal control (Fig. 3F).

Supernatants of FLS showed the presence of MASP-1 protein when examined at 120 and 144 h, either without any exogenous stimulation or in the presence of all 3 stimulants (Fig. 4A). Because MASPs are largely bound to lectins for delivery to tissues, experiments were carried out to examine for the presence of the lectin(s) binding MASP-1. FLS contained only mRNA for the lectin FCN-A (Fig. 4B); mRNA for MBL-A, MBL-C, or FCN-B were absent in FLS (data not shown). FCN-A mRNA was also expressed in diff 3T3 cells with no increase after stimulation with LPS, TNF-α, or IL-1α (data not shown). Western blot analysis showed the presence of FCN-A protein in FLS supernatants and in diff 3T3 supernatants (Fig. 4C). The presence of double bands may indicate the presence of FCN A variants as have been reported in mouse serum (35). Positive and negative controls include sera from WT and *MBL A/C/FCN A*$^{-/-}$ mice, respectively (Fig 4C lane 1, 2). Attempts were unsuccessful to co-localize MASP-1 and FCN-A in FLS supernatants by adsorption on NAG-coated beads, although FCN-A in serum was readily bound by NAG-coated beads (data not shown).

mRNA levels in cultured 3T3 cells

To further explore the production of mRNA for components of the AP in adipose tissue cells, experiments were carried out over 144 hours with cultured undiff and diff murine 3T3 adipocytes in the absence or presence of 50 μg/ml LPS, 100 ng/ml TNF-α, or 50 ng/ml IL-1α. The mRNA extracted from these cells was examined by RT-PCR. The undiff 3T3 cells demonstrated increasing and high levels of mRNA for FB over time in response to TNF-α (Fig. 5A) and for C3 in response to all 3 stimuli (Fig. 5B). Low levels of mRNA for MASP-1 (Fig. 5C), and MASP-3 (Fig. 5D), were observed in the undifferentiated 3T3 cells with little response to any stimulus. However, these cells produced no detectable pro-FD mRNA in response to culture with any stimulant (Fig. 5E). To calculate the amount of mRNA in each sample, 18s rRNA was also amplified as an internal control (Fig. 5F).

Confirmation of differentiation of the 3T3 cells was obtained by staining for oil red O (Fig. S3B). The diff 3T3 cells exhibited different responses producing high levels of mRNA for C3 (Fig. 6B) and low levels of mRNA for FB (Fig. 6A), both in response primarily to TNF-a. Low levels of mRNA for pro-FD were found at 33 CT in diff 3T3 cells at all time periods, primarily in response to culture with IL-1α (Fig. 6E). Since the levels of mRNA for pro-FD in diff 3T3 cells were low, we confirmed the RT-PCR product by electrophoresis on a 2% agarose gel (Fig. S2B). A band of 104 base pairs of RT-PCR product for pro-FD was detected in most of the samples confirming the presence of pro-FD mRNA (Fig. S2B). Low

levels of mRNA for MASP-1 (Fig. 6C), and MASP-3 (Fig. 6D) were observed in the diff 3T3 cells with little response to any stimulus. Although diff 3T3 cells contained low levels of MASP-1 mRNA, small amounts of MASP-1 protein were found only in cell lysates but not supernatants (data not shown). To calculate the amount of mRNA in each sample, 18s rRNA was also amplified as an internal control (Fig. 6F).

These results indicate that diff 3T3 murine adipocytes, but not undiff 3T3 cells, synthesized pro-FD mRNA over time in culture.

Production of factor D proteins in stimulated 3T3 cells

Experiments were then performed to examine for the effects of stimulation of undiff and diff 3T3 cells with IL-1α, TNF-α, and LPS on the secretion of pro-FD or mature FD proteins. Western blot analyses were performed on the cell supernatants collected from the cultured cells in the mRNA experiment. Specific Ab were utilized that recognized only pro-FD or both pro-FD and mature FD. The undiff 3T3 cells failed to produce any detectable pro-FD or mature FD proteins at any time point in response to any stimulant (Fig. 7A, upper two panels). However, the diff 3T3 cells produced pro-FD, but not mature FD, at 96 and 120 h of culture in the absence of exogenous stimulation (Fig. 7A, Panel 2 and 4). Although the antibody to FD detected pro-FD in murine serum, it failed to do so when the protein was present in 3T3 supernatants (Fig. 7A, panels 2 and 4).

In vitro initiation of the AP on cartilage microparticles

We devised an experimental system to examine whether the AP could be activated by solid phase IC on a biological substrate. CMP were coated with anti-CII mAb and cultured with sera from *MASP-1/3*$^{-/-}$ mice as a source of FB. Sera from *MASP-1/3*$^{-/-}$ mice contained FB protein (Fig. 7B), as previously reported (18). Supernatants from FLS and diff 3T3 cells, both cultured for 120 h in the absence of any exogenous stimuli, were used as sources of MASP-1 and pro-FD, respectively (Fig. 4A and 8A). The results indicated that no mature FD was detected in the supernatants of cultures using any combination of reagents (Fig. 8B).

However, mature FD with a MW of 29 kDa was clearly present in the CMP eluates only when supernatants from both 3T3 cells and FLS were present during the culture (Fig. 8C, lane 7). Although sera from *MASP-1/3*$^{-/-}$ mice contain pro-FD as well as FB, no cleavage into mature FD occurred when this serum was incubated with CMP and supernatants from FLS, in the absence of supernatants from 3T3 cells (Fig. 8C, lane 6). To explain this complex figure more completely, lane 1 shows no mature FD in the serum of a *FD*$^{-/-}$ mouse and lane 2 shows the migration of recombinant 29 kDa human FD. Lane 3 shows no mature FD when CMP were incubated with supernatants from 3T3 cells and lane 4 shows no mature FD after incubation of CMP with sera from *MASP-1/3*$^{-/-}$ mice. No pro-FD was detected in lane3 because the anti- FD Ab used does detect pro-FD in 3T3 cell supernatants. Lane 5 indicates an absence of mature 29 kDa FD with the incubation of CMP with *MASP-1/3*$^{-/-}$ serum and 3T3 supernatant, but in the absence of FLS supernatant. Lane 6 also shows an absence of mature FD with the incubation of CMP with *MASP-1/3*$^{-/-}$ serum and FLS supernatant, but in the absence of 3T3 supernatant. Lane 7 shows the results of complete conditions where a clear band of 29 kDa mature FD was present when CMP were incubated with *MASP-1/3*$^{-/-}$ serum and supernatants from both 3T3 cells and FLS. These data strongly suggested that MASP-1 present in FLS supernatant cleaved pro-FD present in the supernatant from 3T3 cells. The other necessary control conditions examined in the experiment examining eluates from the CMP outlined in Fig. 8, failed to show any conversion of pro-FD to mature 29 kDa mature FD.

One of the unique features of these ex vivo CMP Western blots analyses, in contrast to purified proteins or serum proteins, is that the 35 – 50 kDa bands in the blots from supernatants or lysates of the CMP were elongated when using *MASP-1/3*$^{-/-}$ mouse serum. We do not know the reason for this observation. However, it was not due to over-loading as the blots were highly reproducible even when using lesser amounts of the *MASP-1/3*$^{-/-}$ mouse serum.

DISCUSSION

The AP has been found to be both necessary and sufficient for mediation of CAIA, an experimental model of IC disease in mice, and MASP-1/3 cleavage of pro-FD into mature FD is required for activation of the AP and induction of CAIA in mice. Our results indicate that solid phase IC of CII and anti-CII mAb on the biological substrate CMP can activate the AP in vitro, in the presence of a source of FB, using FD formed by MASP-1 cleavage of pro-FD. Moreover, our results suggest that MASP-1 released from FLS may cleave pro-FD secreted by synovial adipocytes in vivo, generating mature FD locally in the joint.

We found that mRNAs for the essential components of the AP were found in synovial tissue samples from mice with CAIA obtained by LCM. SAT contained mRNA for C3, FB, and pro-FD with synovium containing mRNA for C3, FB, MASP-1, and MASP-3. Synovial macrophages may also contribute complement components as mRNA for all of the AP proteins, except for properdin, were previously described in the rheumatoid synovium (21). We observed weak staining for MASP-1 in cells scattered throughout the SAT and synovium but mRNA for MASP-1 and MASP-3 were found only in the synovium obtained by LCM and not in the SAT (Fig. 2E and F). Thus, synovium can produce MASP-1/3 locally in joints, in addition to the liver, which is the major source of circulating MASP-1/3 in vivo.

In initial experiments designed to examine whether pro-FD from adipocytes could be cleaved by MASP-1 from FLS, we mixed these supernatants or co-cultured the cells. However, in both cases we failed to observe any cleavage of pro-FD to FD by Western blot analysis of the supernatants (data not shown). Since the events of activation of the AP in vivo in disease states occur not in the fluid phase but after deposition of C3b on cell or tissue surfaces, we developed an in vitro system using a biological substrate, CMP, to simulate events occurring in the joint. The CMP consist of both proteoglycans and CII; mAb to CII bind and provide a substrate for subsequent binding of C3, FB, and FD. Proteoglycans may play an important role in this in vitro experimental system through providing secondary glycosoaminoglycan binding sites for complement components. Proteoglycans were lacking in our previously published in vitro system using solid phase mAb to CII where FH did not bind (34).

The majority of pro-FD is synthesized by adipocytes throughout the body and is present in small amounts in the circulation (27, 36). Most of the FD found in human circulation is the mature form of the protein. The generation of mature FD has been hypothesized to occur by cleavage from pro-FD by the action of a trypsin-like enzyme during the process of secretion from adipocytes (27). FD in circulation is in an inactive form with subsequent activation due to conformational changes induced by binding to its natural substrate C3b-FB (27, 37). The possibility also exists that pro-FD is released from adipocytes and mature FD is generated locally in tissues through release of a critical protease, presumably MASP-1/3. This possibility is supported by the observation that diff 3T3 adipocytes released only pro-FD in vitro and that only pro-FD, and not mature FD, was present in the circulation of mice deficient in MASP-1/3 (15, 18).

In our studies, pro-FD secreted by synovial adipocytes may bind to the C3b-FB complex present on the cartilage coated with mAb to CII. MASP-1 produced locally by FLS could then bind to the pro-FD in the cartilage complex, producing active FD that then cleaves FB into fragments Ba and Bb with generation of the AP convertase C3bBb. In previous studies to reconstitute the AP of complement in vitro, cobra venom factor (CVF), recombinant FB, and recombinant pro-FD were used (15). CVF acted as a source of C3b because it is a structural and functional analogue of C3. Both CVF and C3b can bind to FB and form a stable complex with FB. rMASP-1K did not cleave endogenous pro-FD present in the serum of *MASP-1/3*$^{-/-}$ mouse (15). These authors proposed that rMASP-1K may already been activated during expression and purification. Thus rMASP-1K can cleave recombinant pro-FD in vitro but it cannot cleave pro-FD present in *MASP-1/3*$^{-/-}$ mouse serum. This observation suggests that some other conditions are required in vivo for MASP-1 to cleave pro-FD, possibly the presence of a biological substrate or surface to mediate binding of native MASP-1/3 to native pro-FD. We have not used CVF, instead an endogenous source of C3 was utilized. The AP convertase formed in our experiments presumably was C3b-FB adherent to the CMP with the capacity to bind pro-FD. The design of our experiments was unique due to the use of ex vivo CMP biological substrate. A model diagram of this proposed mechanism for activation of the AP by solid phase IC in the joint is presented in Fig. S4.

Pro-FD in the circulation of *MASP-1/3*$^{-/-}$ mice and FD in both human and murine blood are present by Western blot analysis as broad bands (15, 18, 24). These are thought to represent variably glycosylated species; the possibility of binding to other serum proteins is rendered unlikely since the addition of recombinant human FD to WT mouse serum did not yield any higher MW species of FD (data not shown). An unexplained observation in our studies is that the goat anti-mouse FD Ab recognized the 37–50 kDa broad band of pro-FD in serum of *MASP-1/3*$^{-/-}$ mice but not the 29 kDa pro-FD present in 3T3 cell supernatants (Fig. 7B). The conformational determinants recognized by this Ab may be present on pro-FD in serum but may be absent on the newly synthesized molecule present in the supernatants of diff 3T3 cells cultured in the absence of serum. Another unexplained and unanticipated observation is that the MASP-1 in FLS supernatants appeared not to cleave the 37–50 kDa pro-FD in serum but only the 29 kDa species of pro-FD secreted by 3T3 cells (Fig. 8C). It should be noted that a broad band of pro-FD was present in the CMP eluates where *MASP-1/3*$^{-/-}$ serumwas present (Fig. 8C), suggesting that the variably glycosylated species of pro-FD bound to the CMP. Thus, generation of active FD may primarily occur locally in tissues in the microenvironment at sites of synthesis. The absence of FD in the supernatants of the CMP experiment (Fig. 8B) suggests either the protein was present in too low an amount to be detected by the Western blot analysis or the FD was not released from the C3b-FB complex. Further experiments will be necessary to examine these possibilities.

MASPs are thought to bind to lectins such as MBLs, ficolins, or collectins in the circulation or body fluids with the enzymes delivered to tissues through specific local binding of the lectin. The tissue specificities of binding of lectins, with associated MASPs, remain incompletely understood. We observed that FLS contain only mRNA for ficolin (FCN)-A and not for FCN-B, MBL-A or MBL-C. Although both MASP-1 and FCN-A were present in FLS supernatants by Western blot analysis (Fig. 4A & C), neither protein was adsorbed from FLS by NAG-coated beads. Whether some other carrier lectin was required to transport the MASP-1 from the FLS to the cartilage surface, or if free MASP-1 was responsible, was not determined by our studies (15).

In addition to adipocytes, FD synthesis and secretion has also been described in cultured normal human blood monocytes, rheumatoid synovial fluid and tissue macrophages, and by the human monocyte cell line U937 (19, 38, 39). Synovial tissue macrophages were not

cultured in the current studies and their contribution to CAIA remains unknown. Macrophages are also present between adipocytes in adipose tissue and may play a role in local inflammation and activation of the AP.

The role of circulating C3 versus locally synthesized C3 was examined in passively transferred arthritis in K/BxN mice, an IC model dependent on the AP, using bone marrow chimeras and parabiotic mice (40). The results showed that circulating C3 was necessary and sufficient to induce this experimental animal model of arthritis, and that locally produced C3 was not required. Similar experiments will be necessary in CAIA to examine the role of circulating versus local synthesis of FD and MASP-1 since pro-FD and MASP-1 in the circulation are present in inactive forms and may not be readily delivered to inflamed tissues. Although we did not examine a role for properdin in CAIA, studies in K/BxN (41) and C57BL/6 (42) mice showed markedly reduced disease severity in mice lacking properdin.

Adipose tissue is hypothesized to be part of the innate immune system and to play a role in human immune and inflammatory diseases including RA (43, 44). Adipokines such as resistin and adiponectin may exert proinflammatory and destructive effects in the joint (45–47). Leptin may lead to joint destruction through inducing metalloproteinase production in chondrocytes (48) and enhancing a Th17 response in collagen-induced arthritis (49). However, white adipose tissue, particularly from the joint, may play a local anti-inflammatory role through the production of IL-1 receptor antagonist (50, 51). The effects of adipokines in CAIA have not yet been examined.

Cultured 3T3 cells, used as a surrogate for adipocytes in these studies, may not possess properties completely equivalent to synovial adipocytes in vivo. This possibility is suggested by the observation that cultured undiff and diff 3T3 cells possessed some mRNA for MASP-1 and MASP-3 (Figs. 5 and 6) whereas SAT obtained by LCM contained no mRNA for MASP-1/3 (Fig. 2). Sera from *MASP-1/3*$^{-/-}$ mice demonstrated a broad band of pro-FD by Western blot analysis, presumably representing variably glycosylated species of this molecule (Fig. 8B & C). However, the supernatants of cultured diff 3T3 cells showed a narrow band of secreted pro-FD, suggesting an absence or a minimum of glycosylation (Fig. 7). The cleavage of pro-FD by MASP-1 in FLS led to a small band of mature FD of the expected MW for recombinant FD, leaving behind a large and broad band of pro-FD that was not cleaved (Fig. 8C, lane 7). This result suggested that the amount of MASP-1 in the FLS supernatant was much less than the amount of potential substrate present in the experiment, or that the FLS cleaved only the pro-FD in the 3T3 supernatant and not the pro-FD in the serum from *MASP-1/3*$^{-/-}$ mice. Indeed, in the absence of the 3T3 supernatant, MASP-1 in the FLS appeared not to cleave any of the pro-FD in the serum from *MASP-1/3*$^{-/-}$ mice (Fig. 8C, lane 6).

There are other possible explanations why the entire broad band of pro-FD in serum was not cleaved into mature FD by the MASP-1 in FLS supernatants. The enzymatic sites for cleavage of pro-FD into mature FD may have been obscured by glycosylation. In addition, the catalytic activities of MASP-1 are influenced by rapid intracellular autoactivation and autodegradation (52). Recombinant MASP-1 was only expressed in a mammalian expression system when co-expressed with the protease inhibitor, C1 inhibitor; the expressed MASP-1 in this system bound MBL and was catalytically active (52). In a complex biological system, pro-FD might be bound to unknown inhibitory proteins thereby preventing its binding to MASP-1 present in FLS supernatants leading to an absence of cleavage of pro-FD present in *MASP-1/3*$^{-/-}$serum (Fig. 8 lane 6). The possibility also exists that pro-FD present in the 3T3 supernatant is in a different conformation compared to pro-FD in *MASP-1/3*$^{-/-}$ serum and more avidly binds to MASP-1 present in FLS supernatant.

Lastly, MASP-1 may cleave pro-FD bound to solid phase IC more efficiently than pro-FD present in sera due to conformational differences. These are important questions and the focus of future investigations in our laboratory.

Our data incriminate MASP-1 in the FLS supernatant in cleavage of pro-FD bound to CMP. However, the possibility exists that some other protease in the FLS supernatant may contribute to this activity, such as thrombin (27). However, no conversion of pro-FD to mature FD was observed in the serum of *MASP-1/3* $^{-/-}$ mice with CAIA, suggesting that other proteases potentially able to cleave pro-FD were not operative in vivo (18).

The role of MASP-1 cleavage of pro-FD into FD in activation of the AP in human diseases is unknown. A patient genetically lacking both MASP-1 and MASP-3 exhibited normal AP function in peripheral blood, although this blood lacking both MASP-1 and MASP-3 demonstrated a nonfunctional lectin system (53). Thus, MASP-1 is essential to the lectin pathway but cleavage of pro-FD by MASP-1/3 appears not to be necessary for activation of the AP in human blood. Although systemic activation of the AP in humans may not require MASP-1 cleavage of pro-FD, local activation of the AP in tissues through this mechanism remains possible. Evidence for this possibility has been described recently where human recombinant MASP-1 cleaved the pro-enzyme of human FD in vitro (54). The results of our experiments further emphasize the possible role of adipose tissue in forms of inflammatory arthritis and point to the potential role of these cells in regulating complement-dependent injury. Further studies will be necessary to examine the role of MASP-1 cleavage of pro-FD into FD in activation of the AP in tissues in RA and other human inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACPA	Ab to citrullinated protein antigens
AP	alternative pathway
CII	bovine type II collagen
CMP	cartilage micro-particles
CP	classical pathway
CDA	clinical disease activity
CAIA	collagen Ab-induced arthritis
diff	differentiated
FB	factor B
pro-FD	pro-factor D

FD	factor D
FCN	ficolin
FLS	fibroblast-like synoviocytes
AT	adipocyte
SYN	synovium
M	macrophage
IC	immune complexes
LCM	laser capture micro-dissection
LP	lectin pathway
MBL	mannose-binding lectin
MASP	MBL-associated protein
NAG	N-acetyl-D-glucosamine
rhu FD	recombinant human FD
SAT	synovial adipose tissue
RA	rheumatoid arthritis
T-blue	toluidine blue
undiff	undifferentiated
WT	wild type

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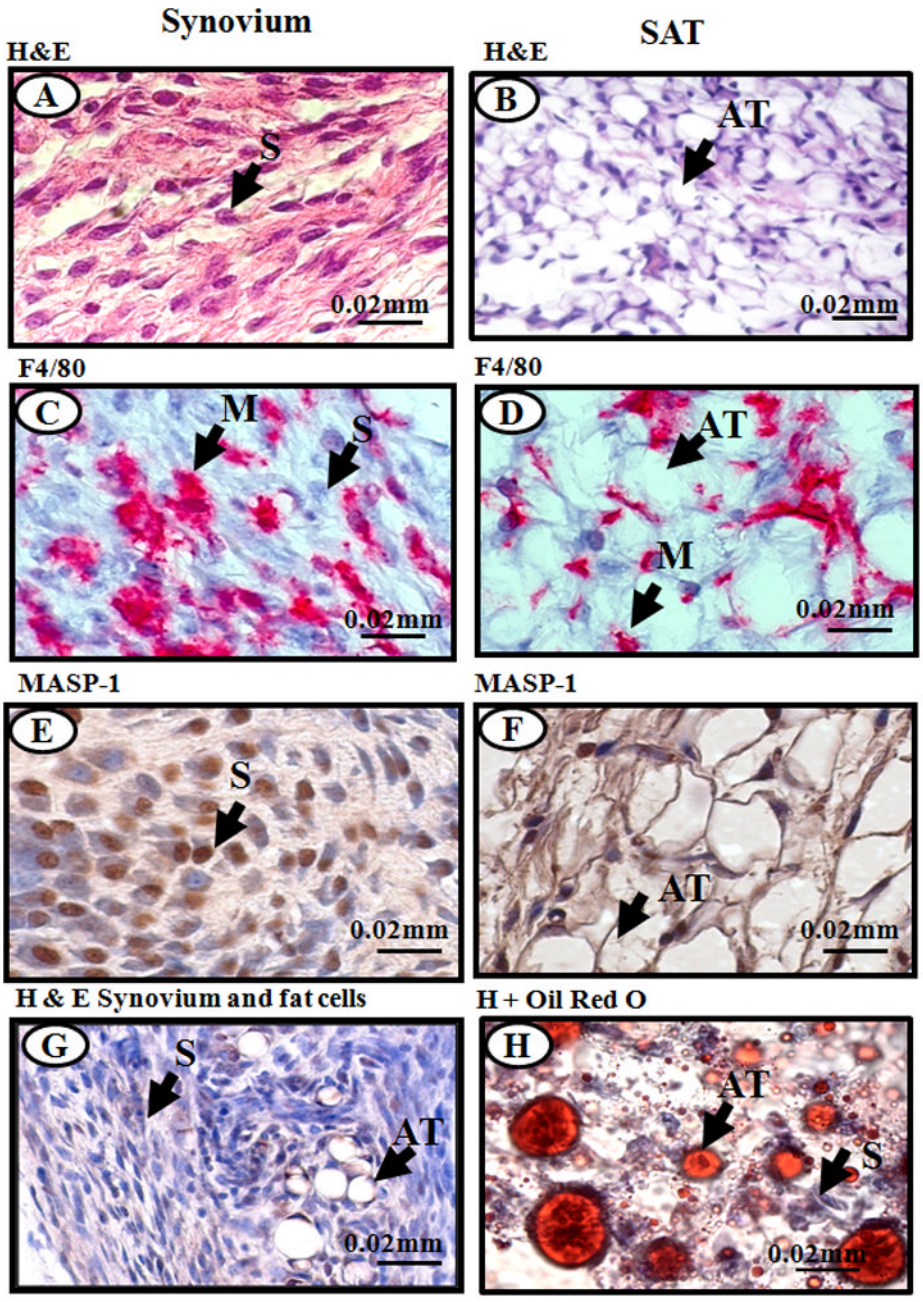

Figure 1.

Representative immunohistochemical analysis of the synovium (S) and synovial adipose tissue (SAT) of joints from WT mice with CAIA. At day 10 following Arthrogen injection, mice were sacrificed and immunohistochemical staining was performed using joints from the left hind limb following tissue processing. The integrity of the histology sections was confirmed by using Toluidine-blue staining. Paraffin embedded sections were used for *panels* ***A, B, C, D, E, F & G.*** Paraffin embedded fat cells in sections appear empty because xylene dissolves the lipid contents present inside the cells and the nucleus is deviated towards one side. Fresh frozen sections were used for *panel* ***H. A.*** Hematoxylin and Eosin (H & E) staining of the synovium. ***B***. Hematoxylin (blue color) staining of the AT (black arrow pointing to an adipocyte). Adipocytes appear as hollow sacs where the nucleus is

pushed towards one side (black arrow). ***C***. F4/80 staining identifies abundant macrophages (M) in the synovium (black arrow pointing to red stain). ***D.*** Frequent macrophages are also present in the SAT between the adipocytes (black arrow pointing to a red staining macrophage). ***E.*** MASP-1 staining of the synovium with large numbers of positive cells present (black arrow pointing to brown stain). ***F.*** MASP-1 positive cells are also present between adipocytes in the SAT (black arrow) . None of the adipocytes were positive for MASP-1. ***G.*** Hematoxylin and Eosin staining showing the synovium embedded with white hollow adipocytes (pointed by black arrow). ***H.*** Adipocyte islands (pointed by black arrow) are also embedded sparsely throughout the synovium. Red droplets are fat contents of adipocytes (pointed by black arrow). The synovium is multi-layered and filled with infiltrating fibroblasts (blue). Magnification of all panels was 100x. A magnification scale bar at 100x of 20 μm (0.02 mm) is included in the lower right hand corner of each panel.

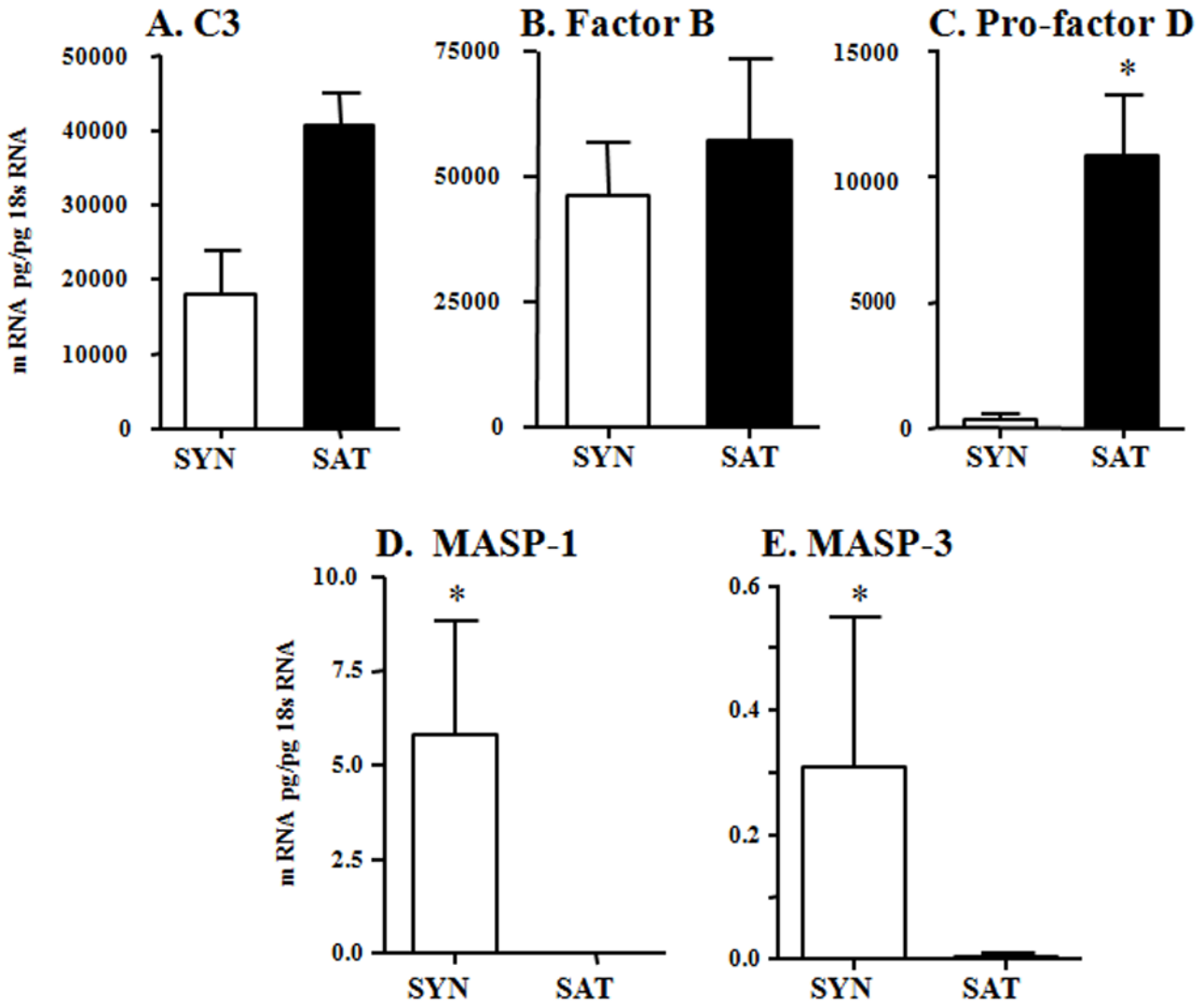

Figure 2.

mRNA levels of complement components in the synovium (SYN) and in synovial adipose tissue (SAT) obtained by laser capture micro-dissection (LCM) from the knee joints of WT mice with CAIA. Several sections (~10–15) were precisely cut using UV lasers from the knee joints of each mouse and SYN and SAT from each mouse were pooled prior to the RNA extraction. The mRNA levels for synovial (empty white bar) and SAT (solid black bar) were measured by quantitative RT-PCR using cDNA made from aRNA, as described in Methods, with specific primers and probes for C3 (Fig. 3A), FB (Fig. 3B), pro-FD (Fig. 3C), MASP-1 (Fig 3D), and MASP-3 (Fig. 3E). The mRNA levels for each cytokine were expressed as specific mRNA (pg)/18s rRNA (pg). All data represent the mean ± SEM based on n = 5. *$p < 0.05$: significantly different between synovium and SAT.

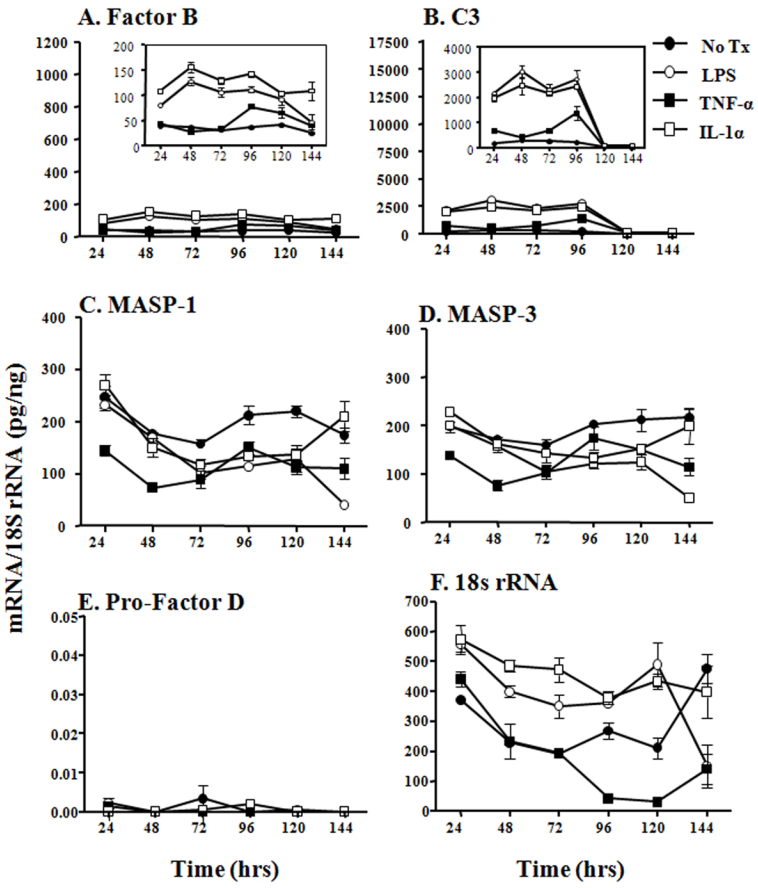

Figure 3.

mRNA levels in FLS cultured for up to 144 h in the presence of no stimulant, 5 μg/ml LPS, 100 ng/ml TNF-α, or 50 ng/ml IL-1α. The data were obtained from 3 separate experiments with each sample determined in duplicate and expressed as mean ± SEM based on n = 6. The insets in Fig. A and B show data on a full scale so that the differences can be compared with other figures (Fig 3A and Fig 4A).

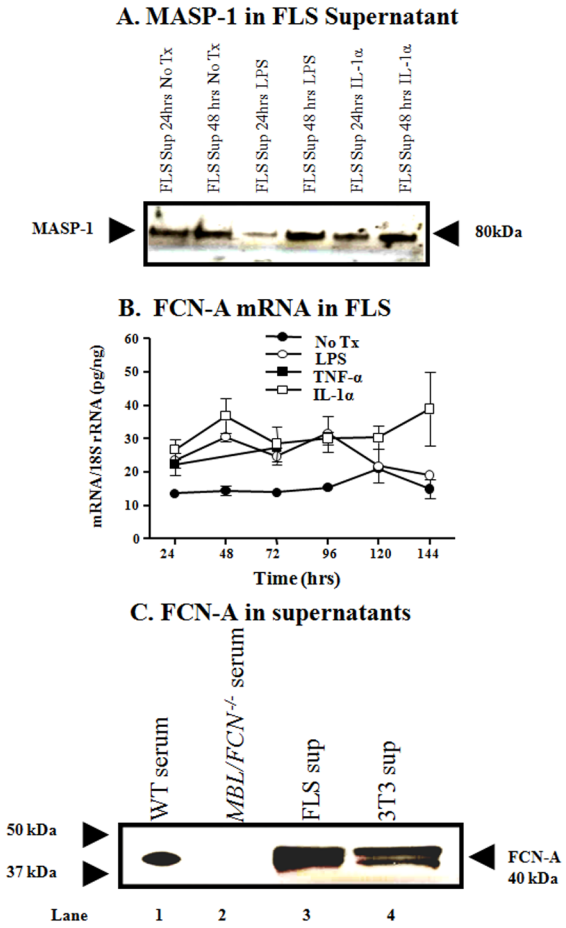

Figure 4.

MASP-1 and FCN A in FLS. *Panel A*. Western blot analysis of FLS supernatants without and with treatments with LPS and IL-1α using an Ab for MASP-1. *Panel B*. RT-PCR of mRNA extracted from FLS using primers specific for FCN A. Panel C. FCN-A in the supernatants of FLS and diff 3T3 adipocytes by Western blot analysis. Sera from WT and *MBL A/C*$^{-/-}$*/FCN A*$^{-/-}$ were used as positive and negative controls, respectively.

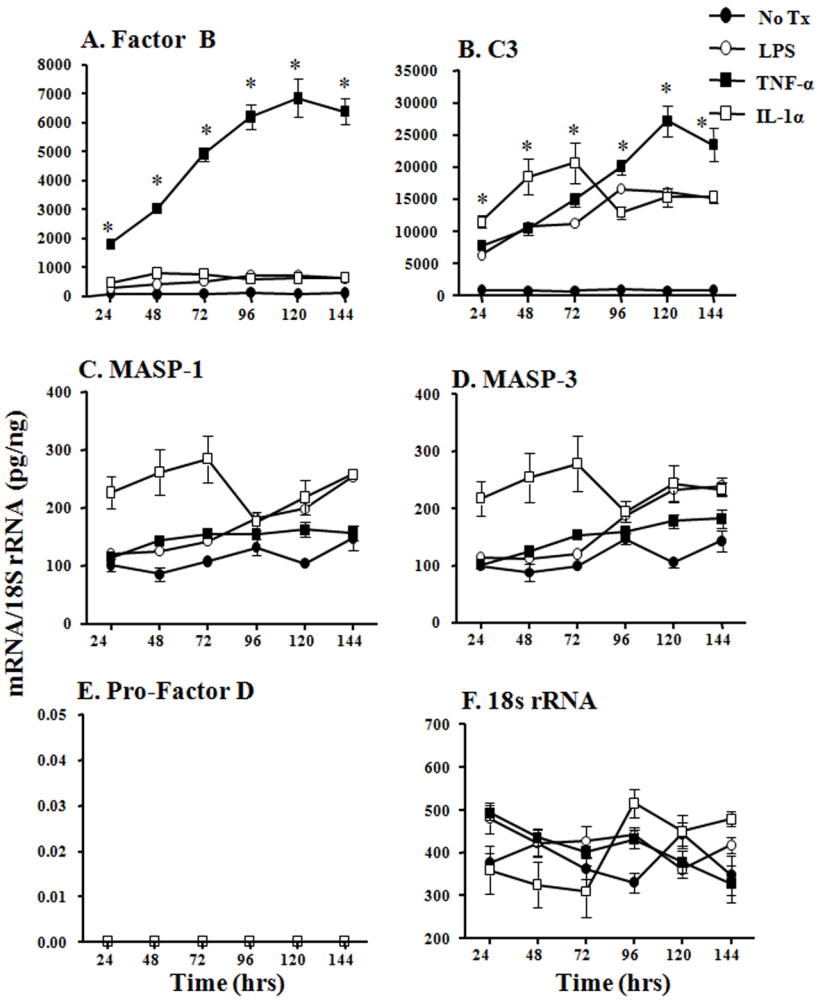

Figure 5.

mRNA levels in undiff 3T3 adipocytes cultured for up to 144 h in the presence of no stimulant, 5 μg/ml LPS, 100 ng/ml TNF-α, or 50 ng/ml IL-1α. The data were obtained from 3 separate experiments with each sample determined in triplicate and are expressed as mean ± SEM based on n = 9. *$p < 0.05$: significantly different between TNF-α or IL-1α stimulation and no stimulation.

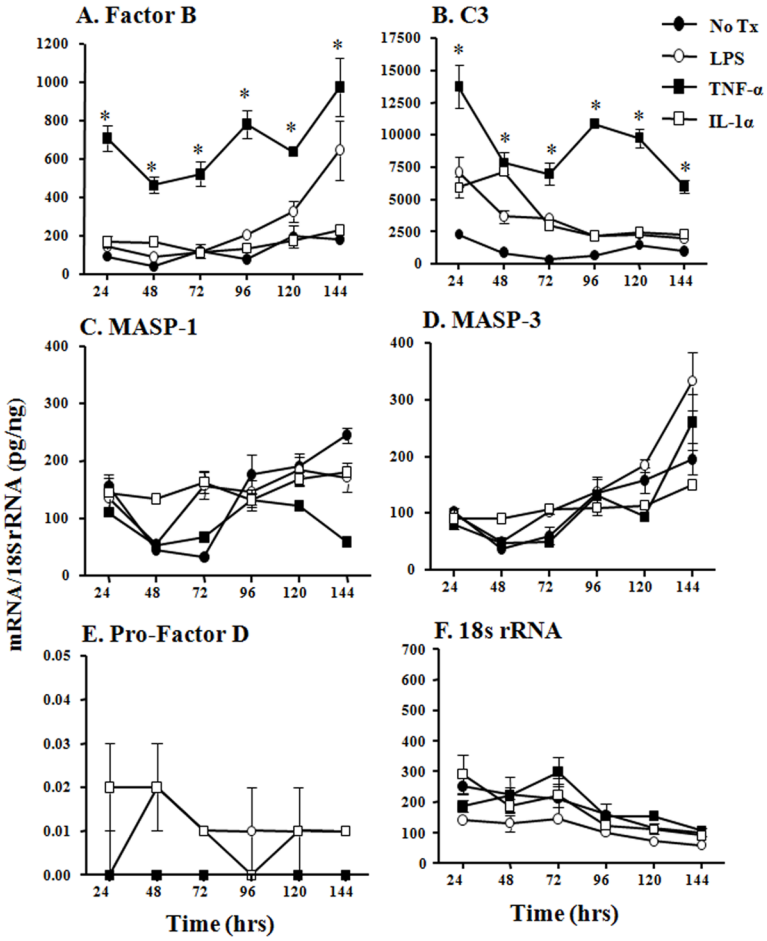

Figure 6.

mRNA levels in diff 3T3 adipocytes cultured for up to 144 h in the presence of no stimulant, 5 μg/ml LPS, 100 ng/ml TNF-α, or 50 ng/ml IL-1α. The data were obtained from 3 separate experiments with each sample determined in triplicate and are expressed as mean ± SEM based on n = 9. *$p < 0.05$: significantly different between TNF-α stimulation and no stimulation.

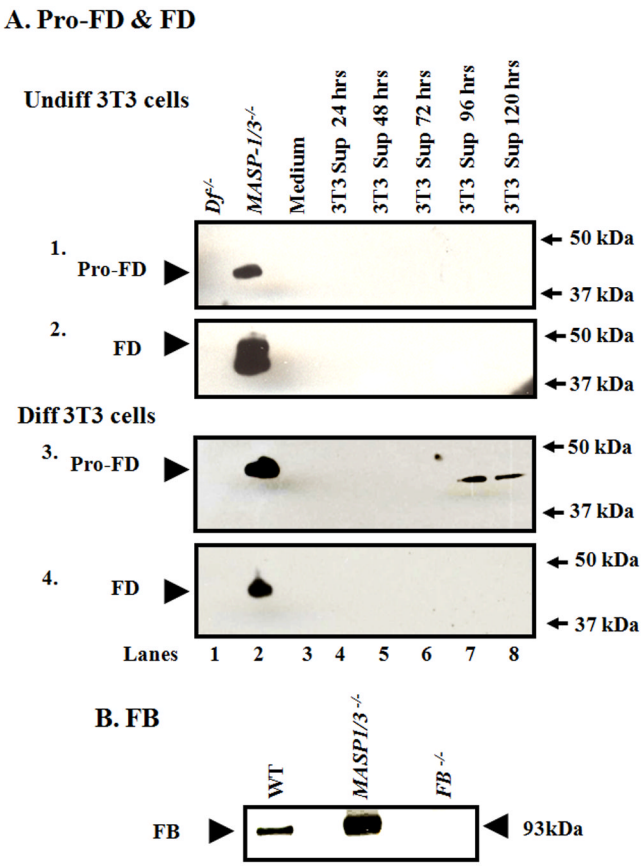

Figure 7.

Diff 3T3 cells secrete only pro-FD and FB is present in sera from *MASP-1/3*$^{-/-}$ mice. *Panel A.* Western blot analysis of supernatants from undiff (upper two blots) or diff (lower two blots). The blots with supernatants from 3T3 cells were probed with a specific Ab to pro-FD or with an Ab to mature FD. The Ab to mature FD recognized pro-FD in serum but not in supernatants of 3T3 cells. *Panel B.* Western blot analysis of sera from WT, *MASP-1/3*$^{-/-}$, and *FB*$^{-/-}$ mice using an Ab to FB.

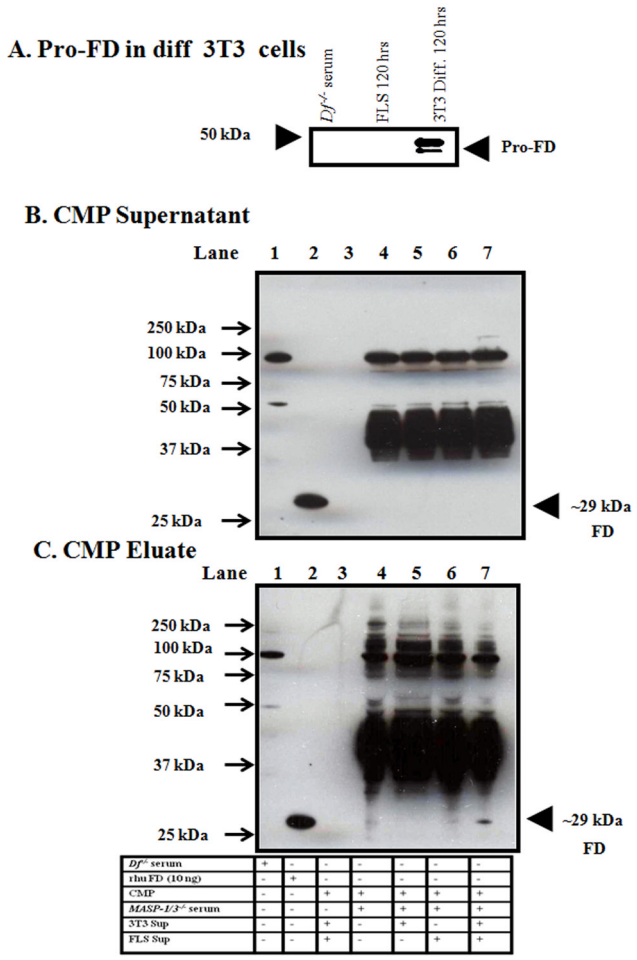

Figure 8.

Conversion of pro-FD protein in the supernatant of diff 3T3 adipocytes by MASP-1/3 secreted by cultured FLS. The identities of the components present in each lane are indicated in the table below the figure and include: sera from WT, *FD*$^{-/-}$, and *MASP-1/3*$^{-/-}$ mice; CMP; and supernatants from diff 3T3 cells or FLS. *Panel A*. Pro-FD in the supernatants of diff 3T3 cells with this supernatant used for the ex vivo CMP experiment shown below. The pro-FD in this Western blot was detected using an Ab specific for this protein. *Panel B*. Supernatants from each treatment lane shown in table below. The Western blot was probed with an Ab to mature FD that detects this protein in sera and cell supernatants but detects pro-FD only in sera. No FD band (29 kDa) was detected in any lane except in lane 2, which contains a positive control (rhu FD). *Panel C*. CMP eluates from a mixutre of components shown in each lane in table below. The Ab used was directed against mature FD, described under panel B. A distinct band of FD was present only in lane 7 containing CMP, *MASP-1/3*$^{-/-}$ serum, diff 3T3 cell supernatant, and FLS supernatant. As expected, a band of FD was also present in lane 2, which contains a positive control (rhu FD). This experiment was repeated 5 times with identical results