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Shedding of Large Functionally Active CD11/CD18 Integrin Complexes from Leukocyte Membranes during Synovial Inflammation Distinguishes Three Types of Arthritis through Differential Epitope Exposure

Louise Carstensen Gjelstrup,*‡ Thomas Boesen,§ Tue Wenzel Kragstrup,*‡ Annette Jørgensen,* Nigel J. Klein,‖ Steffen Thiel,† Bent Winding Deleuran,‡,§ and Thomas Vorup-Jensen*†,‡

CD18 integrins are adhesion molecules expressed on the cell surface of leukocytes and play a central role in the molecular mechanisms supporting leukocyte migration to zones of inflammation. Recently, it was discovered that CD11a/CD18 is shed from the leukocyte surface in models of inflammation. In this study, we show that shedding of human CD11/CD18 complexes is a part of synovial inflammation in rheumatoid arthritis and spondyloarthritis but not in osteoarthritis. In vivo and in vitro data suggest that the shedding is driven by TNF-α, which links the process to central events in the inflammatory response. The shed complexes contain multiple heterodimers of CD11/CD18, are variable in size, and differ according to the type of synovial inflammation. Furthermore, the differential structures determine the avidity of binding of the complexes to the ICAM-1. With the estimated concentrations of CD11/CD18 in plasma and synovial fluid a significant coverage of binding sites in ICAM-1 for CD18 integrins is expected. Based on cell adhesion experiments in vitro, we hypothesize that the large soluble complexes of CD11/CD18 act in vivo to buffer leukocyte adhesion by competing with the membrane-bound receptors for ICAM-1 binding sites. As reported here for synovial inflammation changes in the concentration or structure of these complexes should be considered as likely contributors to disease activity. The Journal of Immunology, 2010, 185: 4154–4168.

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Abbreviations used in this paper: 7-AAD, 7-amino-actinomycin D; AU, arbitrary units; BT, β tail; CRP, C-reactive protein; DMARD, disease-modifying antirheumatic drug; GPC, gel permeation chromatography; HV, human volunteer; I-EFG, integrin-epidermal growth factor; LAD, leukocyte adhesion deficiency; Max, maximum; Min, minimum; MMP, matrix metalloproteinase; MNC, mononuclear cell; NS, nonsignificant; OA, osteoarthritis; P, plasma; PSI, plein/segaphorin/integrin; RA, rheumatoid arthritis; Rq, hydrodynamic radius; rs, recombinant soluble; RT, room temperature; s, soluble; SF, synovial fluid; SpA, spondyloarthritis; TRIFMA, time-resolve immunofluorometric assay.

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their work that the platelet receptor (integrin αIIbβ3) forms stable complexes comprising two or more heterodimers (17), apparently in a process influenced by conformational changes in the ecto domain (18). However, no previous studies have addressed the contribution of integrin oligomerization to the function of solubilized receptors and the question of whether integrins other than αIIbβ3 form such complexes.

Inflammatory diseases have often provided valuable material for studying the biological role of solubilized adhesion molecules (19). Synovial inflammation is a feature of a highly diverse range of diseases of which rheumatoid arthritis (RA) is one of the most common (20). The inflammatory response in RA involves all major leukocyte subsets and a vast array of cytokines and adhesion molecules (21–23). Spondyloarthritis (SpA) encompasses a heterogeneous group of inflammatory diseases, many with manifestations affecting spinal and peripheral joints. These diseases encompass ankylosing spondylitis, reactive arthritis, and psoriatic arthritis (24). Both in the case of RA and SpA elevated plasma concentrations of inflammatory markers, for example, C-reactive protein (CRP), are associated with increased disease activity. Migration of leukocytes into the synovium likewise characterizes all of these diseases. By contrast, markers of the inflammatory process that distinguish RA and SpA have not been identified although several lines of clinical evidence suggest that the progression of RA and SpA follows different routes, notably with a relatively poor response to disease-modifying antirheumatic drugs (DMARD) among the SpA patients. The spectacular success of treatment of these diseases with function-blocking Ab to TNF-α may, at least in part, rely on the influence of this cytokine on both the endothelial expression of ICAM-1 and integrin ligand-binding activity (21, 22). In osteoarthritis (OA), inflammation is less dominant compared with RA and SpA (25). Based on the involvement of inflammation in the pathogenesis of RA and SpA and the site of tissue damage shared with OA, these three disorders of the synovium are particularly well-suited for a study of soluble (s) CD18 integrins in inflammatory diseases.

In this study, we show that ICAM-1–binding complexes of CD11/CD18 are shed from leukocytes, both under the conditions of normal physiology and, at an enhanced level, in the pathophysiology of synovitis. The shedding was found to be driven by TNF-α as evidenced by clinical and experimental data. Surprisingly, we report that the epitope exposure of sCD18 distinguishes the type of synovial inflammation. Guided by experiments with soluble recombinant integrin and recently gained insight into the structure of CD18 integrin receptors (26), we interpret these findings as a result of oligomerization of the sCD11/CD18 complexes. These findings open up a new perspective on the molecular biology of CD18 integrins where the soluble receptor may play a role in “buffering” leukocyte adhesion and hence in regulating inflammation.

Materials and Methods

**Patients and samples**

Synovial fluid (SF) and plasma samples were collected from patients with RA, SpA, and OA admitted to Department of Rheumatology at Aarhus University Hospital, Aarhus, Denmark, in the period 2006–2007 (Table I). The category SpA subgroups includes the subgroups psoriatic arthritis, reactive arthritis, entheopathic arthropathies associated with inflammatory bowel disease, and undifferentiated SpA. Patients with RA and OA were diagnosed in accordance with the criteria established by the American College of Rheumatology (27). Patients with SpA were classified according to the European spondyloarthropathy criteria (28), and psoriatic arthritis (29). Blood samples and SF was collected during therapeutic arthrocentesis, transferred to tubes containing heparin, centrifuged, and stored as frozen at −80°C. All samples were obtained after informed consent according to guidelines set forth by the Danish Data Protection Agency, the local ethical committee (project nos. 20050046 and 20060012) and the Declaration of Helsinki. A sample of heparin plasma from a 26-y-old female patient diagnosed with LAD type I with clinical characteristics published earlier (30) was collected at the Department of Infectious Diseases and Microbiology, University College London, London, U.K. Citrate plasma samples were collected from human volunteers (HVs) through collaboration with the Blood Bank at Department of Clinical Immunology, Aarhus University Hospital, Skejby, Denmark.

**Hybridoma cell culture, Ab purification, and biotinylation**

The three hybridoma cell lines KIM127 (CRL-2838), KIM185 (CRL-2839), and TS 1/18 (HB-203) producing murine IgGl mAbs specific to human CD11c were purchased from the American Type Culture Collection (LGC Promochem, Boras, Sweden) and cultivated in medium containing 1% (v/v) FCS with a low concentration (≤5 μg/ml) of IgG (16250078, Invitrogen, Carlsbad, CA) (31–33). Abs from culture supernatants were purified on a protein G Sepharose column and biotinylated essentially as described earlier (34).

**Quantification of sCD18 and CD11 by time-resolved immunofluorometry**

Detection of sCD18 in plasma samples was carried out by time-resolved immunofluorometric assays (TRIFMs) using a “sandwich” technique with pairs of polystyrene-coated and biotinylated mAbs applied for the detection of the CD18 chain. Microtiter wells (FluoroNunc Maxisorp; 437958, Nunc, Roskilde, Denmark) were coated for 16 h at 4°C in a volume of 100 μl PBS with 10 μg/ml Ab to CD18 (KIM127, KIM185, or TS1/18) or, as control, murine IgGl. The wells were washed in 10 mM Tris-HCl, 140 mM NaCl, 0.1% (w/v) Na2SO4 (TBS) with 0.05% (v/v) polyoxyethyleneorbitan monolaureate (Twee-20), and residual binding sites blocked by incubation with 200 μl TBS with 1 mg/ml HSA for 1 h at room temperature (RT). Following three washes of the wells in TBS/Tween, samples of 100 μl plasma or heparinized SF diluted in TBS/Tween with 1 mM CaCl2, 1 mM MgCl2, and 100 μg/ml aggregated IgG were added to the wells, and the plates incubated for 1 h at RT. The heat-aggregated human IgG was prepared by incubating IgG (Beriglobin, catalog no. 070815, ZLB Behring, Hattersheim am Main, Germany) at 56°C for 30 min, followed by centrifugation at 13,000 × g. Inclusion of heat-aggregated IgG was made to quench contributions to the assay signal from anti-IgG Abs (rheumatoid factors), which are particularly abundant in plasma and SF from RA patients. After incubation of the diluted samples in the wells for 1 h at RT, the wells were washed and subsequently incubated for 1 h at RT with 100 μl biotinylated mAbs to CD18 in the wells, and the plates incubated for 1 h at RT. The heat-aggregated human IgG was prepared by incubating IgG (Beriglobin, catalog no. 070815, ZLB Behring, Hattersheim am Main, Germany) at 56°C for 30 min, followed by centrifugation at 13,000 × g. Inclusion of heat-aggregated IgG was made to quench contributions to the assay signal from anti-IgG Ab (rheumatoid factors), which are particularly abundant in plasma and SF from RA patients. After incubation of the diluted samples in the wells for 1 h at RT, the wells were washed and subsequently incubated for 1 h at RT with 100 μl biotinylated mAbs to CD18 (KIM127 or KIM185) diluted to a concentration of 1 μg/ml in TBS/Tween with 100 μg/ml bovine IgG. Bovine IgG was added to the buffers to quench any contribution to the assay signal from human Abs to bovine IgG; such Abs may cross-link bovine IgG Abs (derived from calf serum used in the hybridoma culture medium) possibly contaminating the preparations of Abs (31). After washing the wells, 1 μl biotinylated streptavidin was applied and the signals read by time-resolved fluorometry as described (34). Signals from patient samples were compared against a standard curve made from titrations of an HV plasma defined to contain 1000 mU/ml. Calibration of the standard plasma against a source with a known absolute concentration of CD18 was made by comparison with dilutions of recombinant soluble (rs) CD11a/CD18 (17661-36R, lot no. L8080557 C9072217, U.S. Biologicals, Swampscoct, MA), expressed in Chinese hamster ovary cells. The proteins were the human CD33 signal peptide (Met 1–Ala 16) fused with the CD11a chain (Tyr 26–Met 1089), followed by an acidic tail with a calculated Mr of 121,700. The CD33 signal peptide was also fused with the CD18 chain (Gln 23–Asn 700), followed by a basic tail with a calculated Mr of 79,000. Due to glycopolisiation the CD11a and CD18 chains migrated in SDS-PAGE as proteins bands with Mr’s of ~160,000 and 100,000 bands under reducing conditions according to the manufacturer’s information (U.S. Biologicals). The protein sample, provided as a lyophilized powder, was dissolved in TBS to give a final concentration 0.56 mg/ml.

Detection of sCD11 was carried out as described for the CD18 chains, but using commercially available biotinylated Abs to CD11a (MC148/6B, AbD Biotech, Oxford, U.K.), CD11b (555387, BD Pharmingen, San Diego, CA), or CD11c (P01146B, Biodesign International, SacO, ME) for the development.

**Quantification of ICAM-1 binding CD18 integrins in plasma**

Recombinant human ICAM-1 (ADP, lot no. WV1507082, R&D Systems, Oxon, U.K.) was coated onto polystyrene wells similar to the coating with Abs described previously. HV plasma was diluted in buffer with heat-aggregated IgG and Mg2+ and Ca2+ as described for sCD18 assays.
Following incubation for 1 h at RT, the procedure described previously for developing TRIFMAs signals with KIM127 Ab was followed. As a control, biotinylated murine IgG1k, produced as described previously, was also applied to wells coated with ICAM-1 and incubated with HV plasma.

**Experimental induction of CD18 shedding**

Peripheral mononuclear cells (MNCs) were harvested from HV peripheral blood as described elsewhere (35) and used either immediately or following storage at −134˚C in 70% (v/v) RPMI 1640, 20% (v/v) FCS, and 10% (v/v) DMSO. For studies on the time course of CD18 shedding freshly isolated granulocytes were separated from MNCs and erythrocytes by centrifugation on a Histopaque 1077 and 1019 gradient (Sigma-Aldrich) as described (36). The cells were resuspended in RPMI 1640 with 10% (v/v) heat-inactivated bovine serum and penicillin and streptomycin at a final concentration of 1 × 10³ cells/ml. Samples of 400 μl culture medium, including cells were harvested after 24 h, 48 h, 72 h, and 120 h of incubation at 37˚C in a humidified atmosphere with 5% (v/v) CO₂, followed by separation of cells and medium by centrifugation. The cells were analyzed by flow cytometry (35). MNCs were stained for CD45 and CD14 expression as well as with 7-AAD and an antibody to CD18 with well-characterized binding epitopes. These included the human plasma samples. To study the influence of recombinant TNF-α (210TA, R&D Systems, Minneapolis, MN) on CD18 shedding, MNCs were thawed in cold AIM-V medium (12055, Life Technologies–Invitrogen). The cultures of granulocytes were stained with 7-AAD as well as with 5.8 nm), aldolase (RH = 6.1 nm), IgG (RH = 5.8 nm), albumin (RH = 4.8 nm), and HSA (RH = 3.5 nm); a standard curve was made from the relationship between RH and the elution volume (Ve):

\[
\log V_e = \log \left(\frac{V_o}{V_e} - \frac{V_o}{V_o} - V_t - V_0 \right) = \alpha R_H
\]

where \(V_o\) and \(V_e\) are the void and total volumes, respectively, for the GPC column and \(\alpha\) a numerical constant. Fitting of \(\alpha\) from \(R_H\) and \(K_{av}\) calculated from the Ve of the calibration markers as well as the estimation of \(R_H\) and the estimate errors for sCD18 complexes from the Ve,s were made in SigmaPlot 11 (Systat Software, Chicago, IL).

The rsCD11a/CD18 was studied as for the sCD18 assays. The diluted supernatants were applied to V-shaped wells (Precision Plus Protein, 161-0363, Bio-Rad). Following electro-elution, the samples were analyzed by GPC on the Superdex 200 column. The rsCD11a/CD18-expressing cells were applied (Precision Plus Protein, 161-0363, Bio-Rad) at a flow rate of 0.5 ml/min using TBS/Tween as running buffer.

**Gel permeation chromatography and SDS-PAGE analysis**

The HV standard plasma was analyzed by gel permeation chromatography (GPC) on a HiLoad 16/60 Superdex 200 column operated in the AKTA FPLC chromatography system (Pharmacia AB, Uppsala, Sweden). Samples were injected in a volume of 2 ml with a flow rate of 1 ml/min in TBS/Tween, and fractions of 2 ml were collected. The fractions were tested in the TRIFMAs described previously. For analysis of patient samples, named paired samples of SF and plasma, GPC was carried out on Superose 6 10/30 column (Pharmacia), followed by analysis of the fractions in the KIM185–KIM27 assay. Each run was performed with 200 μl sample at a flow rate of 0.5 ml/min using TBS/Tween as running buffer.

To estimate the hydrodynamic radii (\(R_H\)) of complexes, the GPC columns were calibrated with a set of markers (17-0441-01, Pharmacia) with known \(R_H\), including thyroglobulin (\(R_H = 8.5\) nm), ferritin (\(R_H = 6.1\) nm), IgG (\(R_H = 5.8\) nm), albumin (\(R_H = 4.8\) nm), and HSA (\(R_H = 3.5\) nm); a standard curve was made from the relationship between \(R_H\) and the elution volume (\(V_e\)):

\[
\log V_e = \log \left(\frac{V_o}{V_e} - \frac{V_o}{V_o} - V_t - V_0 \right) = \alpha R_H
\]

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domain (I-EGF) 2 and KIM185 (33), which binds the I-EGF 4 domain (40, 41) (Fig. 1). Both Abs are able to activate CD18 integrins for ligand binding (32, 33) and KIM127 has been shown to stabilize the unbound conformation of the integrin receptors (42). KIM127 binding is, furthermore, routinely used for investigating the level of integrin activation (41, 43–45). TS1/18 is a function-blocking Ab (31) that works through an allosteric mechanism (46) with an epitope either at or in close proximity to the Ca²⁺ chelation site in the CD18 I-like domain (46, 47) (Fig. 1). The I-like domain forms a contact between the CD18 and CD11 chains and is important in the allosteric regulation of integrin ligand binding (48, 49). By combining these Abs in three “sandwich”-type assays we probed the level of sCD18 Ag and the epitope exposure in heparinized HV and patient plasma. As reported below, both the concentration and epitope exposure contained information specific for the inflammatory disease.

In assays with KIM185 and KIM127 as capture and developing Abs, respectively, a signal at 27,000 counts/s was obtained with an isotypic control IgG and development with KIM127 produced a signal nearly 20-fold lower (Fig. 2) while coating of polystyrene wells with an isotypic control IgG and development with KIM127 produced a signal nearly 20-fold lower (Fig. 2B). The plasma used in these experiments was chosen as a standard defined to contain 1000 mU/ml sCD18. Comparisons with the standard enabled the expression of the sCD18 concentration in other HV and patient plasma samples in these units. Ultracentrifugation of the plasma sample at 100,000 × g did not change the TRIFMA signal (data not shown) suggesting that sCD18 species detected were kept in suspension independent of lipid vesicles such as ectosomes or lipid microparticles (50).

As a test of the selectivity of the assays, we analyzed plasma from a patient (GF) with diagnosed LAD-I (30), namely, CD18 deficiency caused by mutations in the ITGB2 locus. GF was reported to present an unusual case carrying two nonsynonymous mutations (A270V and C590R) that lower the expression of CD18 to ~5% of the wild-type level as monitored by flow cytometry (30). However, as LAD-I patients show at least a 5-fold-higher-than-normal neutrophil count (4), the total level of expressed CD18 is consequently expected to be ~20% of that found in ITGB2 wild-type individuals. Although the C590R mutation is located in the segment known to contain the KIM185 epitope the binding of KIM185 to CD18 is not affected (30). Consistently, side-by-side titrations of the standard and patient GF plasma showed that the LAD patient plasma was ~5-fold less potent in generating TRIFMA signals (Fig. 2A) corresponding to a sCD18 concentration of 200 mU/ml.

We tested additional plasma samples in this assay as well as in two additional assays with KIM185 as capture Ab and TS1/18 as developing Ab (KIM185-TS1/18) or TS1/18 as capture Ab and KIM127 as developing Ab (TS1/18-KIM127). To test the inter-assay variation, plasma samples from three HVs were included in the KIM127 as developing Ab (TS1/18-KIM127). To test the inter-assay variation, plasma samples from three HVs were included in the KIM127 as developing Ab (TS1/18-KIM127). The half-maximum TRIFMA signal for sCD18 binding and the corresponding plasma concentration is indicated with a hatched line.

As a test of the selectivity of the assays, we analyzed plasma from a patient (GF) with diagnosed LAD-I (30), namely, CD18 deficiency caused by mutations in the ITGB2 locus. GF was reported to present an unusual case carrying two nonsynonymous mutations (A270V and C590R) that lower the expression of CD18 to ~5% of the wild-type level as monitored by flow cytometry (30). However, as LAD-I patients show at least a 5-fold-higher-than-normal neutrophil count (4), the total level of expressed CD18 is consequently expected to be ~20% of that found in ITGB2 wild-type individuals. Although the C590R mutation is located in the segment known to contain the KIM185 epitope the binding of KIM185 to CD18 is not affected (30). Consistently, side-by-side titrations of the standard and patient GF plasma showed that the LAD patient plasma was ~5-fold less potent in generating TRIFMA signals (Fig. 2A) corresponding to a sCD18 concentration of 200 mU/ml.

We tested additional plasma samples in this assay as well as in two additional assays with KIM185 as capture Ab and TS1/18 as developing Ab (KIM185-TS1/18) or TS1/18 as capture Ab and KIM127 as developing Ab (TS1/18-KIM127). To test the inter-assay variation, plasma samples from three HVs were included in 11–12 independent measurements over a period of 4 mo. The coefficients of variation ranged from (on average) 0.28 for the KIM127–KIM185 and KIM185–TS1/18 assays to 0.5 for the TS1/18–KIM127 assay (data not shown).

The TRIFMA signal from standard plasma was calibrated by comparison with the signal of a sample of rsCD11a/CD18 with a known concentration (Fig. 2A). We found that 1 pg CD18 corre-
sCD18 levels in plasma and SF samples from RA, SpA, and OA patients

Paired samples of plasma and SF from RA, SpA, and OA patients as well as plasma samples from HV donors (Table I) were analyzed in the KIM185–KIM127 (Fig. 3A, Table II), KIM185–TS1/18 (Fig. 3C, Table III), and TS1/18–KIM127 (Fig. 3E, Table IV) assays. The material included samples from patients with a various degree of inflammation as a consequence of disease activity as well as ongoing treatment with DMARD.

The median sCD18 concentration for the HVs in all three assays responded to ~877 mU sCD18, taking into account that sCD18 accounts for 38% of the mass of rsCD11a/CD18. The standard plasma thus contained 1.14 pg/ml sCD18 or, with the M₄ of CD18 at ~95,000 (31), ~10 fm.

Considering earlier reports on soluble complexes of CD11a/CD18 (6), we investigated the presence of such complexes as well as complexes with CD11b and CD11c. The polystyrene wells were coated with KIM185, KIM127, or TS1/18 Abs, or a control IgG, followed by incubation with plasma dilutions or buffer. Development with the Ab “38” (51, 52) that binds CD11a gave signals higher than development with a control IgG (Fig. 2C). Application of an Ab to CD11b gave a signal 5-fold higher than background (data not shown), namely, considerably weaker than the reactivity found with Ab to CD11a. Ab to CD11c did not produce a signal higher than background (data not shown).

The integrins CD11a/CD18 and CD11b/CD18 expressed on the cell surface bind to ICAM-1 (53, 54). The presence of these integrins in plasma prompted us to probe the functional activity of these complexes by testing their ability to bind ICAM-1. Dilutions of plasma were applied to wells coated with ICAM-1, followed by development via KIM127 Ab. sCD18 was easily detected in wells coated with ICAM-1 (Fig. 2D), whereas the absence of ICAM-1 coating (Fig. 2D) or the application of a control Ab did not produce a signal higher than background (data not shown). The half-maximum signal was obtained consistently for all coatings with ICAM-1 at a plasma concentration of 12% (v/v) corresponding to a sCD11/CD18 molarity of 1.2 fM assuming each binding complex to contain equimolar amounts of CD11 and CD18.

Table I. Patient samples

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Samples from Patients with RA (N = 33)</th>
<th>Samples from Patients with SpA (N = 31)</th>
<th>Samples from Patients with OA (N = 10)</th>
<th>Samples from HVs (N = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (y)Δ</td>
<td>57</td>
<td>41</td>
<td>62</td>
<td>18–60</td>
</tr>
<tr>
<td>Sex, (number of females)Δ</td>
<td>28</td>
<td>24</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>CRP, mean concentration (nM)Δ</td>
<td>337</td>
<td>217</td>
<td>All &lt;75</td>
<td>—</td>
</tr>
<tr>
<td>Number of repetitive samples</td>
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<td>9</td>
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<td>0</td>
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<tr>
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<td>11</td>
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<tr>
<td>DMARDΔ</td>
<td>15</td>
<td>19</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>13</td>
<td>12</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Salazopyrine</td>
<td>7</td>
<td>6</td>
<td>—</td>
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<tr>
<td>Chloroquine</td>
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<td>—</td>
<td>—</td>
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<td>1</td>
<td>—</td>
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<td>Penicillamine</td>
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<td>—</td>
<td>—</td>
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<td>Anti-TNF-α</td>
<td>1</td>
<td>7</td>
<td>—</td>
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</tr>
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</table>

ΔSpecific information on the mean age and sex of HVs was not released from the Blood Bank. Danish blood donors are aged between 18 and 60 y.

ΔΔCRP levels were not measured in plasma samples from national HV donors.

ΔΔΔNeither OA patients nor HVs received DMARD treatment.
Median levels and distribution of sCD18 measurements with the KIM185-KIM127 assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>N</th>
<th>Min (mU/ml)</th>
<th>25% (mU/ml)</th>
<th>Median (mU/ml)</th>
<th>75% (mU/ml)</th>
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<td>878</td>
<td>1.348</td>
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<td>RA SF</td>
<td>33</td>
<td>915</td>
<td>2.520</td>
<td>3.519</td>
<td>7.587</td>
<td>28.967</td>
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<td>866</td>
<td>1.388</td>
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<td>SpA SF</td>
<td>31</td>
<td>665</td>
<td>1.811</td>
<td>2.675</td>
<td>3.245</td>
<td>14.159</td>
</tr>
<tr>
<td>P</td>
<td>31</td>
<td>447</td>
<td>791</td>
<td>1.179</td>
<td>1.623</td>
<td>2.629</td>
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<tr>
<td>OA SF</td>
<td>10</td>
<td>771</td>
<td>834</td>
<td>990</td>
<td>1.474</td>
<td>2.872</td>
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<tr>
<td>P</td>
<td>10</td>
<td>698</td>
<td>908</td>
<td>1,100</td>
<td>1,351</td>
<td>1,478</td>
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</table>

Compilations for each of the three assays of the number of samples tested (N), the minimum (Min), and maximum (Max) sCD18 levels, the 25% and 75% percentiles, and median sCD18 levels in SF and plasma (P) (in bold) from the three patient groups and from HV plasma samples.

Median levels and distribution of sCD18 measurements with the KIM185-TS1/18 assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>N</th>
<th>Min (mU/ml)</th>
<th>25% (mU/ml)</th>
<th>Median (mU/ml)</th>
<th>75% (mU/ml)</th>
<th>Max (mU/ml)</th>
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</thead>
<tbody>
<tr>
<td>HV</td>
<td>15</td>
<td>1,080</td>
<td>1,209</td>
<td>1,310</td>
<td>1,419</td>
<td>1,675</td>
</tr>
<tr>
<td>RA SF</td>
<td>33</td>
<td>405</td>
<td>1,809</td>
<td>3,125</td>
<td>7,162</td>
<td>28,869</td>
</tr>
<tr>
<td>P</td>
<td>31</td>
<td>135</td>
<td>1,261</td>
<td>1,759</td>
<td>2,631</td>
<td>13,259</td>
</tr>
<tr>
<td>SpA SF</td>
<td>31</td>
<td>143</td>
<td>1,185</td>
<td>2,112</td>
<td>3,363</td>
<td>19,475</td>
</tr>
<tr>
<td>P</td>
<td>31</td>
<td>528</td>
<td>1,049</td>
<td>1,307</td>
<td>2,360</td>
<td>5,224</td>
</tr>
<tr>
<td>OA SF</td>
<td>10</td>
<td>617</td>
<td>660</td>
<td>737</td>
<td>975</td>
<td>2,127</td>
</tr>
<tr>
<td>P</td>
<td>10</td>
<td>1,130</td>
<td>1,236</td>
<td>1,460</td>
<td>1,785</td>
<td>1,895</td>
</tr>
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</table>

Compilations for each of the three assays of the number of samples tested (N), the minimum (Min), and maximum (Max) sCD18 levels, the 25% and 75% percentiles, and median sCD18 levels in SF and plasma (P) (in bold) from the three patient groups and from HV plasma samples.

Association between sCD18 levels and inflammation markers in arthritis

Significant correlations were found between the concentration of MNCs and the sCD18 SF levels measured by the KIM185–KIM127 and KIM185–TS1/18 assays for the SpA patients (Fig. 4A, 4C). By contrast, the sCD18 SF levels for SpA patients measured by the TS1/18–KIM127 assay and for the RA patients measured by any of the assays did not correlate with the MNC concentration (Fig. 4E). The sCD18 levels in SF for RA patients measured by all assays correlated excellently with granulocyte concentration for sCD18 (Fig. 4B, 4D, 4F). However, only in the case of the KIM185–TS1/18 assay did the sCD18 SF level for SpA patients correlate with the granulocyte concentration (Fig. 4D).

The correlation of the plasma CRP concentration with the levels of sCD18 was measured in three assays with either KIM185 as a capture Ab and biotinylated KIM127 as a signal-developing Ab (A, B), KIM185 as capture Ab and TS1/18 as developing Ab (C, D), or TS1/18 as capture Ab and KIM127 as developing Ab (E, F). TRIFMA signals were converted to units of mU/ml from comparison with the HV standard plasma. In A, C, and E, the median level is indicated with a gray bar. For the paired patients samples of SF and P the levels were compared in a Wilcoxon signed rank test with the p values indicated in brackets for each of the assays. In B, D, and F, the correlation between the sCD18 level in SF and in P was compared by calculating Spearman’s rank order correlation coefficient (r) and the level of significance (p) for the three assays.

We also considered the correlation between plasma CRP and sCD18 levels in SF with the patient samples stratified according to diagnosis. In samples from SpA patients, significant correlations were found between the sCD18 levels in SF measured in the KIM185–TS1/18 and TS1/18–KIM127 assay and the plasma CRP concentration, whereas the same correlations for RA patients were not significant and with lower r values (Fig. 5B, 5C). In the case of RA patients, a significant correlation was found between sCD18 levels measured in the KIM185–KIM127 assay and the plasma CRP concentration, whereas this was not observed for SpA patients (Fig. 5A). No correlation was found between the plasma CRP concentrations and plasma sCD18 levels (data not shown).

Fifteen samples from one RA and six SpA patients were collected longitudinally, allowing for a pilot study on the correlation between the plasma CRP concentration and the sCD18 levels in either SF or plasma over time (data from four patients are shown in Fig. 5D–G). The sCD18 level in SF measured by the KIM185–...
KIM127 assay followed the CRP concentration in four of the seven patients. In plasma samples from five patients, the TS1/18–KIM127 assay, there was a different trend where a lowering in the plasma CRP concentration was associated with a raised sCD18 level in plasma according to this assay. Comparisons between data from the KIM185–KIM127 assay applied to SF samples and the TS1/18–KIM127 assay applied to plasma samples showed that in six of the seven patients the two assays correlated inversely. This was particularly evident from the two patients in therapy blocking the function of TNF-α (Fig. 5F, 5G) where the sCD18 levels were lowered 5- to 10-fold.

**Experimental induction of CD18 shedding**

The ability of MNCs or granulocytes to shed CD18 was followed by incubation of these cells in culture medium for 24–120 h. The number of dead leukocytes increased over 72–120 h (Fig. 6A, 6B) with a concomitant increase in sCD18 levels (Fig. 6C). It was recently suggested that the mechanisms of CD18 shedding involves MMPs, in particular MMP-9 (7). Based on our observation that blocking of the function of TNF-α apparently lowered the sCD18 concentration in SF (Fig. 5F, 5G), together with an emerging literature linking TNF-α with MMP-9 activation (55, 56), we investigated the influence of this cytokine on CD18 shedding. A K562 cell line expressing recombinant CD11a/CD18 (38) was used together with the parent K562 cell line, as well as HL-60 cells (57). Incubation of CD11a/CD18–K562 cell line with TNF-α induced shedding of the CD11a/CD18 (Fig. 6D), whereas no sCD18 was found in supernatants from similarly incubated HL-60 or nontransfected K562 cells (data not shown). Shedding of CD18 from MNCs was markedly higher than for CD11a/CD18–HL-60 or nontransfected K562 cells (data not shown). Shedding of CD18 was analyzed the functional activity of the sCD11/CD18 complexes in a static adhesion assay with CD11a/CD18-expressing K562 cell as described earlier (13, 14, 37). Supernatants containing sCD11/CD18 complexes (~360 mU/ml sCD18) were produced by cultivation of MNCs for 120 h as described previously. Following the cultivation, the supernatants were centrifuged at 300 × g or 100,000 × g (Fig. 6I). These data indicated that the majority of sCD18 complexes were retained in solution following depletion of lipid vesicles by centrifugation clearly suggesting that these complexes were not associated with vesicles.

**Influence of sCD11/CD18-containing supernatants on CD11a/CD18-mediated adhesion to ICAM-1**

The sCD11/CD18 complexes are able to bind surface-immobilized ICAM-1, which suggest a potential influence of these complexes on cell binding to this ligand through CD11a/CD18 (Fig. 2D). We analyzed the functional activity of the sCD11/CD18 complexes in a static adhesion assay with CD11a/CD18-expressing K562 cell as described earlier (13, 14, 37). Supernatants containing sCD11/CD18 complexes (~360 mU/ml sCD18) were produced by cultivation of MNCs for 120 h as described previously. Following the cultivation, the supernatants were centrifuged at 100,000 × g for 1 h to remove lipid vesicles and thereby excluding this as a source of influence on the cell adhesion. The supernatants were added to ICAM-1–coated wells as well as the HSA-blocked reference wells and incubated under conditions similar to the experiments for detecting sCD11/CD18 binding to ICAM-1. After incubation, the

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Table IV. Median levels and distribution of sCD18 measurements with the TS1/18-KIM127 assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>Min (mU/ml)</th>
<th>25% (mU/ml)</th>
<th>Median (mU/ml)</th>
<th>75% (mU/ml)</th>
<th>Max (mU/ml)</th>
</tr>
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<tbody>
<tr>
<td>HV P</td>
<td>15</td>
<td>491</td>
<td>802</td>
<td>995</td>
<td>1436</td>
</tr>
<tr>
<td>RA SF</td>
<td>32</td>
<td>121</td>
<td>350</td>
<td>935</td>
<td>12718</td>
</tr>
<tr>
<td>P</td>
<td>32</td>
<td>7</td>
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<td>461</td>
<td>2589</td>
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<tr>
<td>SpA SF</td>
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<td>351</td>
<td>522</td>
<td>2749</td>
</tr>
<tr>
<td>P</td>
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<tr>
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<td>526</td>
<td>575</td>
<td>1061</td>
<td>2787</td>
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</table>

Compilations for each of the three assays of the number of samples tested (N), the minimum (Min) and maximum (Max) sCD18 levels, the 25% and 75% percentiles, and median sCD18 levels in SF and plasma (P) (in bold) from the three patient groups and from HV plasma samples.

Table V. Differences between groups of samples with the KIM185-KIM127 assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>RA</th>
<th>SpA</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SF</td>
<td>P</td>
<td>SF</td>
</tr>
<tr>
<td>HV P</td>
<td>p &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>OA P</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>SF</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>SpA P</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>RA P</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

Kruskal-Wallis analyses with Dunn’s correction for testing equality of sCD18 medians in the multiple groups of samples measured. These included plasma (P) and SF samples from OA, SpA, and RA patients, as well as plasma samples from HVs. For significant differences, the maximum p value is indicated.

NS, nonsignificant difference (i.e., p > 0.05).

---

Table VI. Differences between groups of samples with the TS1/18-TSI18 assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>RA</th>
<th>SpA</th>
<th>OA</th>
</tr>
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<tr>
<td></td>
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<td>P</td>
<td>SF</td>
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<tr>
<td>HV P</td>
<td>p &lt; 0.01</td>
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<tr>
<td>OA P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>SF</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>SpA P</td>
<td>p &lt; 0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>RA P</td>
<td>p &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Kruskal-Wallis analyses with Dunn’s correction for testing equality of sCD18 medians in the multiple groups of samples measured. These included plasma (P) and SF samples from OA, SpA, and RA patients, as well as plasma samples from HV assays. For significant differences, the maximum p value is indicated.

NS, nonsignificant difference (i.e., p > 0.05).
wells were carefully washed and the cells were added to the wells. Following centrifugation, the percentage of bound cells was determined according to Equation 2. A robust binding was observed for the CD11a/CD18-expressing K562 cells, whereas the control cell line showed little binding to the ICAM-1–coated wells (Fig. 7A). Treatment of the wells with sCD11/CD18-containing supernatant clearly reduced the cell adhesion to ICAM-1 in a concentration-dependent fashion (Fig. 7A). We also compared the inhibition by sCD11/CD18-containing supernatant with plain culture medium, namely, culture medium not incubated with cells (Fig. 7B). The binding in wells preincubated with supernatant or culture medium was normalized to the binding in wells not incubated with any medium. The cell

FIGURE 4. Correlation between leukocyte concentration in SF and the concentration of sCD18 in SF samples from RA and SpA patients. The correlation between the numbers of either MNC (A, C, E) or granulocytes (B, D, F) in SF and the sCD18 concentration was compared by calculating Spearman’s rank order correlation coefficient (r) and the level of significance (p) for the three assays with either KIM185 Ab-coated wells developed with biotinylated KIM127 Ab (A, B), KIM185 Ab-coated wells developed with biotinylated TS1/18 (C, D), or TS1/18 Ab-coated wells developed with biotinylated KIM127 Ab (E, F). The calculated r and p values are indicated in the keys to each panel together with the number of comparisons included (N).

FIGURE 5. Correlation between SF sCD18 levels and CRP concentrations in RA and SpA patients. A–C, The correlations between the plasma CRP concentration and the sCD18 levels in SF were compared by calculating Spearman’s r and the level of significance (p) for the three assays with either KIM185 Ab-coated wells developed with biotinylated KIM127 Ab (A), KIM185 Ab-coated wells developed with biotinylated TS1/18 (B), or TS1/18 Ab coated into wells developed with biotinylated KIM127 Ab (C). The calculated r and p values are indicated in the keys to each panel together with the number of comparisons included (N). D–G, The sCD18 levels in SF measured by the KIM185–KIM127 assay and the sCD18 levels in plasma measured by the TS1/18–KIM127 assay were compared with the plasma CRP level in consecutive samples from four patients. Arrow heads in F and G indicate that the sample was collected during TNF-α function-blocking therapy.
binding was reduced with >50% in wells treated with sCD11/CD18-containing supernatant at a concentration of 12.5% (v/v) compared with the untreated wells (p < 10^{-5}). By contrast, the binding in wells treated with plain culture medium was only insignificantly reduced (p < 0.18) compared with untreated wells (Fig. 7B). This point was strengthened by comparisons of the inhibition by supernatant and culture medium applied to the wells at a concentration of at 12.5% (v/v), where the cell binding was significantly lower (p < 0.03) in wells preincubated with supernatant than in well preincubated with plain culture medium (Fig. 7B).

Characterization of sCD18 complexes by GPC

To gain insight into the integrity and size of sCD18 complexes, we subjected the standard plasma to high-resolution GPC (Fig. 8A). The sCD18 concentration in the fractions from the GPC were analyzed by the KIM185–KIM127, KIM185–TS1/18, and TS1/18–KIM127 assays (Fig. 8B). The V_r that gave rise to peaks in the TRIFMA signal were compared with calibration markers to establish the R_H of the sCD18-containing species. One peak corresponded to an R_H at 6.4 nm. A second peak in the TRIFMA signals corresponding to integrin complexes with a much larger hydrodynamic radius at R_H ~8.8 nm was also found. Although this value was obtained from extrapolation using Equation 3, the V_r was within 2 ml volume of the largest marker (thyroglobulin) with a known R_H at 8.5 nm (58) supporting the accuracy of the estimate. The precision of the R_H estimates was analyzed by considering the theoretical error introduced from linear regression. The CI for the value estimated to be 6.4 nm ranged from 4.2 to 8.6 nm, whereas the value estimated to be ~8.8 nm had a CI from 7.5 to 10.1 nm. The error introduced from the technical reproducibility of the V_r was also considered. The variation in V_r between GPC runs was estimated from the V_r of HSA, easily identified from the peak in the continuous elution profile monitored by UV absorption (Fig. 8A). In four independent experiments, this volume was 88.0 ± 0.4 ml (mean ± SD) with the variation in V_r contributing an error in the estimation of the R_H of 1.4% (mm/m).

We wanted to test whether any of the sCD18 complexes were associated with integrin CD11 chains. As shown in Fig. 2C, a strong signal for CD11 was produced by Ab to CD11a, whereas an almost 20-fold lower signal was obtained with Ab to CD11b (data not shown). From this comparison, we chose either KIM127 or KIM185 Ab as capture Ab and “38” Ab to CD11a for analysis of the complex constituents in the GPC fractions. Peaks in the signal for detection of CD11a-containing complexes occurred at the same elution volumes as for the analysis of the CD18 Ag (Fig. 8C).

The estimated dimensions of the eluted sCD11/CD18 complexes raised the question of whether sCD11/CD18 formed oligomers larger than one CD11 chain under these conditions. To test for the possibility of integrin CD11/CD18 oligomerization, we probed the GPC fractions for the presence of complexes with more than one identical epitope. Complexes with an R_H at 6.2 nm, namely, with dimensions very similar to the small complexes detected by the KIM185–KIM127, KIM185–TS1/18, and TS1/18–KIM127 assays, produced...
a strong TRIFMA signal (Fig. 8D). The TRIFMA signals were not influenced by nonspecific binding because signals from wells coated with isotypic control Ab and developed with KIM127 Ab gave only a signal corresponding to the background level (Fig. 8E).

As a further test on the KIM127–KIM127 assay, we made a side-by-side comparison of unfraccionated HV and LAD patient plasma. The signal from the HV plasma was easily detectable, whereas no sCD18-specific signal was produced by the LAD patient plasma (Fig. 8F).

**Characterization of sCD18 complexes in SF and plasma from RA and SpA patients**

We analyzed the distribution sCD18 complexes in plasma and SF from arthritis patients by GPC (Fig. 9A, 9B, 9D, 9E, 9G, 9H) on a column with matrix pore sizes of 25–29 nm (59). For comparison the HV standard plasma also analyzed in Fig. 8 was included (Fig. 9C, 9F). The eluates were either analyzed in terms of the total protein concentration according to the absorbance at 280 nm (Fig. 9A–C) or with the KIM185–KIM127 assay (Fig. 9D–H).

In terms of the sCD18 complex formation, both samples of SF from RA patients showed distinct peaks corresponding to species with $R_H$ of ~6.5 nm and ~23 nm (Fig. 9D, 9G). The determination of $R_H$ via Equation 3 for the larger complexes was only possible by extrapolation making the estimates less accurate. However, although some of these complexes eluted at the $V_0$ for the column, other complexes were clearly capable of penetrating the matrix. Plasma samples from the same patients identified a single peak corresponding to an $R_H$ of ~7.2 nm (Fig. 9D, 9G) similar to the HV plasma (Fig. 9F). By contrast, the elution profiles of sCD18 complexes in SF from SpA patients were different with many smaller sCD18 complexes or fragments (Fig. 9E, 9H) compared with SF from the RA patients (Fig. 9D, 9G). The size distribution of the eluted total protein also showed evidence of protein degradation in SF from SpA patients with profiles greatly skewed toward smaller protein species (Fig. 9B). SF from RA patients had elution profiles for the total protein content almost identical to the plasma profile (Fig. 9A).

**GPC analysis of rsCD11a/CD18**

To compare the CD18 complexes found in plasma and SF with a more well-defined sample of integrin, the rsCD11a/CD18 was applied to GPC. The protein content measured by the absorption at 280 nm was compared with TRIFMA signal from the KIM185–KIM127, KIM185–TS1/18, and TS1/18–KIM127 assays (Fig. 10A, 10B). A strong signal was found with the KIM185–KIM127 assay, a weaker signal with the KIM185–TS1/18, and a nearly undetectable signal with the TS1/18–KIM127 assay (Fig. 10B). As recorded by the UV absorption (Fig. 10A), the majority of the rsCD11a/CD18 complexes were distributed in two populations with $R_H$ of 7.6 and 8.8 nm. Interestingly, the KIM185–KIM127 assay easily detected the 7.6-nm rsCD11a/CD18 complexes. The KIM185–KIM127 also detected a small population of rsCD11a/CD18 complexes with an $R_H$ of ~5.5 nm. An analysis of the GPC fractions by SDS-PAGE and silver staining showed a staining pattern similar to an earlier report (60) with a strongly stained CD11a chain and a weaker staining of the CD18 chain (Fig. 10C).

The separation of the CD11a and CD18 chains into monomers with $M_R$ of 10 M (61), or, 10 5-fold higher than the concentration measured by our assays. In other words, the sensitivity of the assays

**Discussion**

In this study, we demonstrate the presence of soluble complexes of CD11 and CD18 in human plasma as well as in SF from patients with arthritis. These complexes are functionally active with the ability to bind immobilized ICAM-1. The complex formation is both quantitatively and structurally changed by inflammation and appears to provide information on the type of synovial inflammation as demonstrated by the reported differences between RA and SpA patients. Specifically, by combining mAbs with distinct epitopes, we observed that three assays targeting the same Ag, namely, CD18, produced different correlations with inflammatory markers. Furthermore, the assays had a surprising sensitivity with the ability to measure sCD18 concentrations in the femto molar range. This should be compared with the limitation in assay sensitivity originating from the “affinity ceiling” for the interaction between Abs and their epitopes, which has been proposed to correspond to a $K_D$ of $10^{-10}$ M (61), or, 10 3-fold higher than the concentration measured by our assays. In other words, the sensitivity of the assays
seemed vastly to exceed what would be expected with a simple 1:1 binding between mAb and its cognate CD18 epitope.

A similar point can be made from considering the binding of sCD11/CD18 to ICAM-1. By titrating HV plasma, we estimated that the half-maximum binding of CD11 integrins to ICAM-1 was obtained with as little as 1.2 fM CD11a/CD18 integrin applied to the wells. The monovalent interaction between CD11a/CD18 and ICAM-1 has been investigated in many studies through application of the ligand binding I domain of CD11a/CD18 mutated to assume the high-affinity conformation. Several strategies for stabilizing this high-affinity conformation reported $K_D$ values for the interaction of 200 nM (62, 63) in a mode of binding consistent with a 1:1 stoichiometry (64), as expected from structural studies of the binding (63). However, the high affinity of the sCD11/CD18 complexes for ICAM-1 in our assays exceed the affinity of the monovalent interaction with as much as 108-fold using the half-maximum saturating concentration of sCD11/CD18 as an approximation of the $K_D$ for the interaction. These findings prompted us to undertake a structural analysis of the sCD11/CD18 complexes with the aim of identifying the source of the strong binding to ligands and the variability in epitope exposure.

Using GPC and the assays detecting CD18 epitopes, we found that the sCD18 integrins in HV plasma eluted in two distinct peaks, with $R_H$ at ~8.8 nm and 6.4 nm. Based on the recent crystallographic analysis by Xie et al. (26), a single pair of CD11 and CD18 chains in the bent conformation would have a radius of 5.4 nm (Fig. 11A, 11B) and, in the extended conformation, an $R_H$ of ~6 nm as shown in Fig. 1. Some of the CD18 complexes in plasma are consequently larger than what can be accounted for by a single pair of CD11 and CD18 chains. The KIM127 Ab epitope was specifically mapped to a few side chains of the CD18 chain (41). The finding that at least two KIM127 Abs bound the same integrin complex strongly suggests that these complexes must contain more than one KIM127 epitope, and hence more than one CD18 chain. In the same fraction, we could also detect complexes containing the KIM127 epitope together with a CD11a epitope. We considered the possibility that the CD18 integrin oligomerization derived from embedding of CD11/CD18 in ectosomes or other lipid vesicles as described by others (65). However, ultracentrifugation prior to assay analysis could not reduce the sCD18 signal in the plasma samples as would be expected if the integrins were contained in lipid vesicles (50). This finding together with the sizes of the complexes, which are at least 10-fold smaller than microparticles from inflammatory cells (66), clearly point to other explanations for the oligomerization of sCD11/CD18.

To more clearly define the possibility of CD18 integrin oligomerization and the role of protein–protein interactions, we applied

![FIGURE 8. Analysis of the sCD11/CD18 complex size distribution by high-resolution GPC. A. The HV standard plasma was applied to GPC on a Superdex 200 column. The total protein concentration in the fractions was measured by the OD at a wavelength of 280 nm, stated in arbitrary units (AU). The void ($V_0$), total ($V_t$), and $V_e$ of calibration markers with known $R_H$ are indicated with arrows. B. The sCD18 concentration in the fractions was monitored by three assays with either KIM185 Ab-coated wells and biotinylated KIM127 or TS1/18 Ab used for development, or TS1/18 Ab-coated wells and biotinylated KIM127 Ab used for development of the TRIFMA signal. The signal from each fraction was stated as the mean value of duplicate measurements with error bars showing the minimum and maximum values. C. The concentration of sCD11a/CD18 complexes in the fractions was determined in an assay with KIM185 Ab-coated wells and biotinylated CD11a Ab to develop the signal. D. Analysis of the sCD18 concentration with KIM127 Ab used as both capture and developing Ab. E. Experiment with isotypic control Ab coated in the wells and KIM127 as developing Ab. In B–E, hatched lines indicate the $V_e$ for the two major CD18-containing complexes and $R_H$ calculated according to Equation 3 ($\pm$ the SD for the regression prediction). F. TRIFMA assay for the detection of sCD18 complexes with multiple KIM127 epitopes in HV and LAD-1 plasma. Wells were coated with KIM127 Ab, followed by development of signals with biotinylated KIM127 Ab. TRIFMA signals are indicated on the ordinate axis as the mean of duplicate measurements with error bars showing the minimum and maximum values.](http://www.jimmunol.org/)


are labeled with the RH eluate from the applied HV standard plasma. Peaks in the TRIFMA signal in SF and plasma samples from two SpA patients. In D–H, the levels of sCD18 in the samples applied to the GPC analysis are indicated. The column was calibrated with the markers also used for the Superdex 200 column (Fig. 8); for simplicity only the Vₐ of thyroglobulin and HSA are indicated. D and G, Analysis of the sCD18 complex size distribution in SF and plasma samples from two RA patients. Fractions from the GPC were applied to wells coated with KIM185 Ab and developed with biotinylated KIM127 Ab; the TRIFMA signal was plotted as a function of the elution volume. E and H, TRIFMA analysis of the sCD18 complex size distribution in SF and plasma samples from two SpA patients. In D–H, the levels of sCD18 in the samples applied to the GPC analysis are indicated. C and F, The total protein concentration (C) and TRIFMA signal (F) in the eluate from the applied HV standard plasma. Peaks in the TRIFMA signal are labeled with the R_H predicted from linear regression using Equation 3 and the Vₐs of the calibration markers (± the SD for the regression prediction).

rsCD11a/CD18 to GPC, followed by analysis with TRIFMA. By combining these data with the UV absorption profile, we found that rsCD11a/CD18 eluted in three peaks. Quantification of the R_H suggested a size for the eluted complexes of ~8.8, 7.6, and 5.5 nm. This is direct evidence that at least the integrin CD11a/CD18 ecto domain form complexes in an environment with physiologic salinity. Surprisingly, the report by Xie et al. (26) on the structure of integrin CD11c/CD18 also appears to explain this distribution in the complexes as thermodynamically stable (67). Calculations of the R_H (Fig. 11A, 11C, 11D) from the PDB file coordinates with the HYDROPRO algorithm (68) showed an extraordinarily good agreement with the radii determined experimentally for rsCD11a/CD18. The rsCD11a/CD18 was made with an electrostatically charged coils at the C-termini, which keeps the structure of integrins in the bent conformation and probably this way supports the comparison with the CD11c/CD18 complexes, also in the bent conformation. This comparison assigns the structure (Fig. 11E, 11F) of 4×(CD11c/CD18) to the complexes with an R_H of 8.8 nm, the structure (Fig. 11C, 11D) of 2×(CD11c/CD18) to the complexes with an R_H of 7.4 nm, and the structure (Fig. 11A, 11B) of a single, bent Protein Interfaces, Surfaces, and Assemblies toolbox (67). The total buried surface area in the 2×(CD11c/CD18) asymmetric unit is 246 nm² (Fig. 11C, 11D). The contribution to this area from the buried surface between the CD11c/CD18 chains is 2×106 nm² (Fig. 11A, 11B). Consequently, from simple subtraction the buried surface between adjacent CD11c/CD18 heterodimers is 34 nm² (Fig. 11B, 11D). Similarly, in the case of the 4×(CD11c/CD18) asymmetric units the buried surface attributable to adjacent heterodimer contacts is 123 nm² (Fig. 11E, 11F). Analysis of the complexes with the Protein Interfaces, Surfaces, and Assemblies toolbox classified all the complexes as thermodynamically stable (67). Calculations of the R_H (Fig. 11A, 11C, 11E) from the PDB file coordinates with the HYDROPRO algorithm (68) showed an extraordinarily good agreement with the radii determined experimentally for rsCD11a/CD18. The rsCD11a/CD18 was made with an electrostatically charged coils at the C-termini, which keeps the structure of integrins in the bent conformation and probably this way supports the comparison with the CD11c/CD18 complexes, also in the bent conformation. This comparison assigns the structure (Fig. 11E, 11F) of 4×(CD11c/CD18) to the complexes with an R_H of 8.8 nm, the structure (Fig. 11C, 11D) of 2×(CD11c/CD18) to the complexes with an R_H of 7.4 nm, and the structure (Fig. 11A, 11B) of a single, bent
heterodimer to the complexes with an $R_g$ of 5.5 nm. Complexes with an $R_g$ of 8.8 were found in plasma. With the observations from our biochemical analysis of rsCD11a/CD18, it would appear as the most parsimonious suggestion that the complexes similarly consist of four CD11a/CD18 heterodimers. Plasma samples from both HVs and patients also contained complexes with an $R_g$ of $\sim$7 nm. Based on the presence of both CD11a epitopes and two KIM127 epitopes in such complexes, we suggest that they are, if not identical, at least very similar to the 2×(CD11c/CD18) complexes.

These structural properties are also important regarding the questions raised above on the high sensitivity of the assays and strong binding of the integrins to ICAM-1. With multiple identical epitopes in the complexes and their large size, it seems reasonable to suggest that the binding to Ab-coated wells involves avidity binding with more than one Ab capturing one complex. Even with the Ab affinity for its epitope well below the “affinity ceiling” avidity binding increases the strength beyond what is required to capture complexes at a femtomolar concentration. Similarly, the presence of more than one ligand binding domain in the complexes likely supports avidity binding to immobilized ICAM-1. With the monovalent affinity (62, 63) of the CD11a chain I domain for ICAM-1 the dissociation constant for avidity binding for a 2×(CD11a/CD18) complex would be in the order of $10^{-14}$ M, clearly on a scale that would support significant fractional coverage of ICAM-1 with more than one Ab capturing one complex. Even with the Ab affinity for its epitope well below the “affinity ceiling” avidity binding increases the strength beyond what is required to capture complexes at a femtomolar concentration. Similarly, the presence of more than one ligand binding domain in the complexes likely supports avidity binding to immobilized ICAM-1. With the monovalent affinity (62, 63) of the CD11a chain I domain for ICAM-1 the dissociation constant for avidity binding for a 2×(CD11a/CD18) complex would be in the order of $10^{-14}$ M, clearly on a scale that would support significant fractional coverage of ICAM-1 ligand binding sites with the plasma dilutions used.

A recent report indicated that activated MMP-9 is important in the shedding of CD11a/CD18 from a murine macrophage cell line (7). Our data shows that shed integrin are not associated with lipid vesicles, entirely consistent with a proteolytic release of the integrin ecto domain from the cell surface. Other recent reports have linked TNF-α to the activation of MMP-1 and MMP-3, which serve a role in activation of MMP-9 (55, 56). Together with our data, this presented the testable hypothesis that TNF-α triggered the shedding of CD11a/CD18 from leukocyte membranes. Indeed, incubation of MNCs with TNF-α led to a significant increase of scCD18 in cell culture supernatants. Similarly, a K562 cell line expression recombinant CD11a/CD18 shed more integrin upon TNF-α stimulation. Upon appropriate stimulation of leukocytes cytosolic vesicles with a high concentration of CD18 integrins empty their content to the cell membrane vastly upregulating the surface expression of these receptors (69, 70). TNF-α induces the mobilization of integrins from cytosolic compartments (71, 72). Together with our finding that this stimulation actively promotes CD18 shedding, it would appear that mechanisms are in place to rapidly increase the concentration of scCD11a/CD18 in the extracellular milieu of leukocytes.

The functional activity of the scCD11a/CD18 complexes with regard to ligand binding suggest the reservoir of scCD11a/CD18 in plasma to act as a “buffer” of available adhesion sites for integrins in the leukocyte cell membrane and in this way inhibit or balance leukocyte adhesion. We found that supernatants from cultivation of MNCs containing 300–400 mU/ml of scCD18 inhibited the binding of CD11a/CD18-expressing cells to ICAM-1. Plain culture medium that had not been exposed to a leukocyte culture showed, by contrast, no such ability. The preincubation of wells with the media also caused moderate, but reproducible, secondary effects on
the cell binding affecting the unspecific binding by K562 cells. Incubation of protein-coated surfaces with media also containing protein species causes some replacement of the immobilized protein with those found in solution (73), and hence the complex composition of the culture medium is likely to affect the adhesive properties of surfaces used in our studies. However, incubation of the wells with culture media appeared to moderately increase the unspecific binding suggesting that these effects were not a part of the inhibition of the binding to ICAM-1. Consistent with ours and others’ (6) findings that shed integrin binds ICAM-1, we suggest that our experimental data on the inhibition of CD11a/CD18-mediated cell adhesion to ICAM-1 by the MNC culture supernatants reflects competition between the sCD11/CD18 complexes and the intact receptors expressed on the cell surface. Based on these findings, it is tempting to suggest that sCD11/CD18 serves an anti-inflammatory role. The CRP concentration in RA is strongly associated to disease activity in arthritis. In this perspective, the correlation in between the sCD18 concentration in SF and CRP concentration in plasma would reflect a scenario, where a highly upregulated proinflammatory mechanisms leading to disease. A upregulated anti-inflammatory mechanism fails to check also highly correlation in between the sCD18 concentration in SF and CRP associated to disease activity in arthritis. In this perspective, the anti-inflammatory role. The CRP concentration in RA is strongly natants reflects competition between the sCD11/CD18 complexes and the unspecific binding suggesting that these effects were not a part of the inhibition of the binding to ICAM-1. Consistent with ours and others’ (6) findings that shed integrin binds ICAM-1, we suggest that our experimental data on the inhibition of CD11a/CD18-mediated cell adhesion to ICAM-1 by the MNC culture supernatants reflects competition between the sCD11/CD18 complexes and the intact receptors expressed on the cell surface. Based on these findings, it is tempting to suggest that sCD11/CD18 serves an anti-inflammatory role. The CRP concentration in RA is strongly associated to disease activity in arthritis. In this perspective, the correlation in between the sCD18 concentration in SF and CRP concentration in plasma would reflect a scenario, where a highly upregulated proinflammatory mechanisms leading to disease. A upregulated anti-inflammatory mechanism fails to check also highly correlation in between the sCD18 concentration in SF and CRP associated to disease activity in arthritis. In this perspective, the anti-inflammatory role. The CRP concentration in RA is strongly.

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