Signal Regulatory Protein (SIRPα), a Cellular Ligand for CD47, Regulates Neutrophil Transmigration

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Recent studies have demonstrated that CD47 plays an important role in regulating human neutrophil (PMN) chemotaxis. Two ligands for CD47, thrombospondin and SIRPα, have been described. However, it is not known if SIRP-CD47 interactions play a role in regulating PMN migration. In this study, we show that SIRPα1 directly binds to the immunoglobulin variable domain loop of purified human CD47 and that such SIRP-CD47 interactions regulate PMN transmigration. Specifically, PMN migration across both human epithelial monolayers and collagen-coated filters was partially inhibited by anti-SIRP monoclonal antibodies. Similar kinetics of inhibition were observed for PMN transmigration in the presence of soluble, recombinant CD47 consisting of the SIRP-binding loop. In contrast, anti-CD47 monoclonal antibodies inhibited PMN transmigration by markedly different kinetics. Results of signal transduction experiments suggested differential regulation of PMN migration by SIRP versus CD47 by phosphatidylinositol 3-kinase and tyrosine kinases, respectively. Immunoprecipitation followed by Western blotting after SDS-PAGE under nonreducing conditions suggested that several SIRP protein species may be present in PMN. Stimulation of PMN with FMLP resulted in increased surface expression of these SIRP proteins, consistent with the existence of intracellular pools. Taken together, these results demonstrate that PMN migration is regulated by CD47 through SIRPα-dependent and SIRPα-independent mechanisms.

CD47 is a transmembrane Ig superfamily member that is expressed in most tissues, and its function has been broadly implicated in multiple cellular processes including neutrophil phagocytosis, T cell activation (1, 2), T and B cell apoptosis (3, 4), platelet activation (5, 6), and stroma-supported erythropoiesis (7). Other studies have demonstrated that antibodies to CD47 interfered with α,β3-mediated cell functions (8) and inhibited endothelial Ca2+ influxes during cell adhesion to fibronectin- or vitronectin-coated surfaces (9). Previous studies from our group and others have shown that CD47 plays an important role in the regulation of PMN migration (10–12). It was shown that monoclonal antibodies against CD47 inhibited the rate of PMN migration across both epithelial monolayers and cell matrix-coated transwell filters without reducing the total amount of PMN migration (10), suggesting a positive regulatory role of CD47 in PMN migration. The precise mechanism of CD47-mediated regulation of PMN migration is not known. However, specific tyrosine phosphorylation events appear to convey the downstream signals from cell surface CD47 to regulate the rate of PMN migration (10).

CD47 has been shown to bind to β3 and β1 integrins (8, 13–15), the C-terminal cell-binding domain of thrombospondin-1 (16), and collagen 4 (17, 18). However, these interactions do not appear to be directly involved in CD47-mediated regulation of PMN migration (10). Most recently, CD47 has been reported to bind to signal regulatory protein (SIRP) in rodents (19) and humans (20). Results of these studies have suggested that the interaction between CD47 and SIRPα may play a role in diverse processes such as macrophage multinucleation, self-recognition of red blood cells, and sickle cell clearance, etc. (21–23).

SIRPs form a family of transmembrane glycoproteins expressed in a variety of tissues (24). However, within these tissues, SIRPs are only selectively expressed in certain cell types (25). In mice, SIRPs (termed SHPS-1) are richly expressed in hematopoietic cells including macrophages and myeloid cells, but not in T and B cells (24). In humans, SIRPs are expressed in monocytes, granulocytes, dendritic cells, and CD34+ CD38− CD133+ bone marrow stem/progenitor cells but not in lymphocytes (20, 26). Through cDNA library screening, multiple homologous sequences that account for at least 15 additional SIRP members have been reported (27).

Primary structural analysis indicates that all SIRPs share common structural motifs including a single transmembrane segment and an N-terminal extracellular domain that contains three Ig-like loops connected by three pairs of disulfide bonds. The C-terminal intracellular domain structurally separates two subfamilies of SIRPs, termed SIRPα and SIRPβ (27). SIRPα has a long intracellular domain containing four tyrosine residues that form two immunoreceptor tyrosine-based inhibi-
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Intramolecular interactions between tyrosine motifs, whereas SIRPβ contains a basic lysine residue followed by a short intracellular tail that serves as a receptor for DAP12, a protein with an immunoreceptor tyrosine-based activation motif (27, 28).

Similar to other immunoreceptor tyrosine-based inhibitory motif domain-containing proteins such as CD3, T cell receptor ζ, FeRγ, and B cell receptor (29, 30), SIRPα has been shown to play an important role in regulating cellular responses to a wide variety of different stimuli. For example, treatment of tissue-cultured cells with growth factors (including platelet-derived growth factor and epidermal growth factor), growth hormone, insulin, colony-stimulating factor, lysophosphatidic acid, etc., has been shown to induce phosphorylation of tyrosines on the intracellular immunoreceptor tyrosine-based inhibitory motif domain of SIRPα, resulting in binding to Src homology 2 domain-containing tyrosine phosphatase-1 or 2 (SHP-1 and 2) (24, 27, 31). SIRPα binding to SHP-1 or SHP-2 mediates positive or negative signals that regulate a variety of cellular functions, respectively (27, 32, 33).

The recent reports of CD47 binding to SIRPα prompted us to examine the role of SIRP in CD47-mediated regulation of PMN transmigration. In this report, we confirm that human CD47 binds specifically to SIRPα and find that SIRPα–CD47 interactions partially inhibit FMLP-driven PMN chemotaxis, but with kinetics that are markedly different from the inhibition observed with anti-CD47 mAbs. Furthermore, our results suggest that SIRP and CD47 regulate PMN migration through different intracellular signaling pathways. Finally, we report that several protein bands reactive with SIRP antibodies under nonreducing conditions are present in PMN and can be upregulated to the cell surface after chemotactic stimulation. The significance of these findings is discussed in the context of PMN migration during the acute inflammatory response.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies against human SIRP including P3C4, SE5A5, SE7C2, SE8A3, SE12B6, and SE12C3 were raised as described previously (20). A Western blotting polyclonal rabbit antibody raised against the extracellular domain of SIRPα (10) was kindly provided by Dr. Axel Ullrich (27). Monoclonal antibodies against human CD47 used in this study were C5D5, B6H12.2, PF10.4, PF2.1, and PF3.1 (10). A polyclonal anti-CD47 antibody R12898 was raised by immunizing rabbits with a synthetic peptide SNQKTIQPPRNN corresponding to a region of the putative C terminus of CD47 as previously described (10). As a positive inhibitory control, mAb CBRM13/29, reactive with the β2 integrin CD11b was used (34), and anti-junction adhesion molecule (JAM) mAbs J3F.1 or J10.4 were used as the noninhibitory controls (35).

Cells—Culture and maintenance of T84 human intestinal epithelial cells (passages 50–90) were as described previously (36). Briefly, T84 cells were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with 15 mM HEPES buffer, pH 7.5, 14 mM NaHCO3, 40 μg/ml penicillin, 8 μg/ml ampicillin, 90 μg/ml streptomycin, and 5% newborn calf serum. For transmigration experiments, T84 cells were grown on collagen-coated, permeable collagen, polycarbonate filters with a surface area of 0.33 cm2 and pore size of 5 μm (Costar, Cambridge, MA) as previously described (11). Neutrophils (PMN) were isolated from whole blood of normal human volunteers by Dextran sedimentation followed by Ficol-Hypaque separation as previously described (37). Residual RBC were lysed with isotonic NH4Cl. Isolated PMN were resuspended in Hanks’ balanced salt solution devoid of calcium or magnesium (HBSS(−)) (4 °C) at a concentration of 5 × 105 cells/ml and were used within 4 h.

Purification of CD47, JAM, and CD11b/CD18—Human CD47 was purified from human RBC, PMN, spleen, and HT-29 intestinal epithelial cells (passages 50–90) were as described previously (36). Briefly, confluent inverted T84 monolayers were washed twice with HBSS (20 °C). PMN (1 × 106) in 150 μl of HBSS with or without antibody were added to the upper chamber of the monolayer setup. Transmigration was initiated by transferring of PMN-containing monolayers to 24-well tissue culture plates containing 1 ml of 1 μM FMLP in HBSS followed by incubation at 37 °C. PMN migration across monolayers into the fMLP-containing lower chambers was quantified by myeloperoxidase assay as previously described (11). PMN migration across acellular, collagen-coated filters was performed as described previously (10).

PMN Cell Surface SIRP/CD47 Labeling and Analysis by Flow Cytometry—PMN cell surface SIRP and CD47 expression was assessed by labeling nonpermeabilized PMN (5 × 106 cells) with anti-SIRP and anti-CD47 mAbs (20 μg/ml each) for 1 h at 4 °C in HBSS(−) before blocking nonspecific binding with 0.5% BSA. After washing, PMN were incubated with FITC-conjugated goat anti-mouse secondary antibody (1:200 dilution in HBSS(−)) (PharMingen) for 30 min at 4 °C before flow cytometric analysis. CD47 labeling in this condition (HBSS(−)) was compared with that obtained with isotype-matched, control IgG.

Cell Surface Biotinylation, Immunoprecipitation, and Immunoblotting Experiments—PMN (1.5 × 106/ml) in suspension or adherent to plastic Petri dishes were stimulated with FMLP, followed by cell surface labeling with 0.5 mg of sulfo-NHS-biotin (Pierce) in 1 ml of HBSS/1.5 × 106 PMN per ml. After washing, cells were solubilized as above.
10^7 neutrophils as previously described (10). Labeled cells were then solubilized with lysis buffer containing 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% Triton X-100, 2 μg/ml aprotinin, 1 mM PMSF, 2 μg/ml leupeptin, and 1 μg/ml pepstatin. Cell lysates (100–200 μg of total protein) were precleared with IgG-Sepharose before incubation with 2 μg anti-SIRP or anti-CD47 mAbs for 4 h at 4°C. Immunocomplexes were precipitated using Protein A/G-Sepharose followed by washing and nonreducing SDS-PAGE. Proteins were transferred to nitrocellulose, and biotin-labeled cell surface proteins were detected with streptavidin-peroxidase followed by ECL. Total cellular SIRPα proteins were detected using a polyclonal rabbit anti-SIRPα antibody (SIRPα1-Ex, 1:500), and CD47 was detected by immunoblot with R12989 or mAb PF3.1, followed by detection with appropriate peroxidase-conjugated secondary antibody and ECL.

RESULTS
The Extracellular Domain of SIRPα1 Directly Binds to Purified Human CD47 in Vitro—In a previous report, human leukocytes were shown to adhere to SIRPα-GST-coated surfaces via CD47-mediated events (20). We confirmed that CD47 binds directly to SIRPα1 extracellular domain by in vitro assays using purified CD47. Native CD47 was affinity-purified from human PMN, RBC, spleen, and intestinal epithelial cells (HT-29). SDS-PAGE analysis of the CD47 purified from PMN was shown in the inset of Fig. 1A, demonstrating characteristic broad protein staining bands between 45 and 65 kDa. Similar profiles were obtained for CD47 purified from human spleen, RBC, and intestinal epithelial cells (HT-29) (data not shown). B, absence of SIRPα1-GST binding to purified transmembrane Ig superfamily members JAM and CD11b/CD18, isolated as detailed under “Experimental Procedures.” C, specific inhibition of SIRPα1-CD47 binding by antibodies. In these experiments, binding assays of SIRPα1-GST to PMN CD47 were performed in the presence of HBSS containing 1% BSA and 10 μg/ml anti-SIRPα mAbs (P3C4, SE5A5, and SE7C2) or anti-CD47 mAbs (C5D5 and B6H12.2) and compared with binding in the presence of isotype-matched IgG. Data in this figure represent one of four experiments performed in triplicate ± S.D. *, p < 0.01.

![Fig. 1. SIRPα1 binds directly to human CD47 purified from different cell types. A, CD47 binds to SIRPα1 but not SIRPβ1. 96-well microtiter plates were coated with CD47 purified from human RBC, PMN, spleen, and intestinal epithelial cells (HT29) before blocking with 1% BSA and incubation for 1 h with 1 μg/ml SIRPα1-GST or SIRPβ1-GST fusion protein. Binding of SIRP-GST was then detected by incubation with a goat anti-GST antibody followed by peroxidase conjugated rabbit anti-goat secondary and ABTS assay. Control binding experiments were done by coating wells with 1% BSA alone. The inset shows analysis of CD47 immunopurified from human PMN by nonreducing SDS-PAGE where SS denotes the silver-stained protein, and WB denotes the Western blot probed with CD47 mAb PF3.1. Similar profiles were obtained for CD47 purified from human spleen, RBC, and intestinal epithelial cells (HT-29) (data not shown). B, absence of SIRPα1-GST binding to purified transmembrane Ig superfamily members JAM and CD11b/CD18, isolated as detailed under “Experimental Procedures.” C, specific inhibition of SIRPα1-CD47 binding by antibodies. In these experiments, binding assays of SIRPα1-GST to PMN CD47 were performed in the presence of HBSS containing 1% BSA and 10 μg/ml anti-SIRPα mAbs (P3C4, SE5A5, and SE7C2) or anti-CD47 mAbs (C5D5 and B6H12.2) and compared with binding in the presence of isotype-matched IgG. Data in this figure represent one of four experiments performed in triplicate ± S.D. *, p < 0.01.](image-url)
even though these two SIRP members are highly homologous in their extracellular domains (27). These results confirm those of others (20) and demonstrate that the extracellular domain of SIRPα1 binds directly to CD47. To confirm the binding specificity, additional experiments were performed. As shown in Fig. 1B, no binding was observed between SIRPα1-GST and two other transmembrane Ig superfamily members JAM and CD11b/CD18. The specificity of SIRPα binding to purified CD47 is further demonstrated in Fig. 1C. In this panel, the effects of some of our anti-SIRP and anti-CD47 mAbs on SIRPα binding to CD47 purified from PMN are shown. As can be seen, SIRP mAbs SE5A5 and SE7C2 and CD47 mAb C5D5 markedly inhibited recombinant SIRPα binding to CD47. In contrast, SIRP mAb P3C4 had little, if any, inhibitory effect.

A summary of the binding and inhibitory properties of a large panel of anti-SIRP and anti-CD47 mAbs can be found in Table I. Anti-SIRP mAbs SE5A5, SE7C2, and SE12C3, which were previously shown to inhibit CD47 expressing Jurkat cell adherence to SIRPα1-GST (20), directly inhibited binding of SIRPα1 to purified CD47. In contrast, mAbs P3C4, SE8A3, and SE12B6 either failed or only slightly inhibited this binding (10–20%). As also indicated in Table I, functionality inhibitory anti-CD47 antibodies including C5D5, B6H12.6, PF10.4, PF3.1, and PF2.1 (10) strongly inhibited CD47-SIRPα1 binding.

We also examined the effects of divalent cations, pH, and different detergents on SIRP-CD47 binding. We found that CD47-SIRPα1 binding occurred over a wide range of pH (from pH 5 to 10), was independent of Ca²⁺/Mg²⁺, and was not affected by the nonionic detergent Triton X-100 or 1% Nonidet P-40 (data not shown). However, CD47-SIRPα1 binding was markedly reduced in the presence of 1% octyl glucoside, 0.5% deoxycholate, or 0.25% SDS (data not shown).

Because human leukocyte adherence to SIRPα-coated surfaces is mediated through cell surface CD47 (20), we performed experiments to test the direct binding of the IgV loop of CD47 to SIRPα1 (Fig. 2). A recombinant CD47 IgV loop fused to alkaline phosphatase (CD47-AP) was constructed as detailed under “Experimental Procedures” and expressed in mammalian (CHO) cells to allow for proper folding and glycosylation, which was confirmed by binding assays with multiple functionally blocking anti-CD47 extracellular domain-reactive mAbs (10) (data not shown). As shown in Fig. 2, CD47-AP bound to SIRPα1-GST fusion protein resulting in a high value of AP activity (A₄05). Specificity of this interaction was confirmed by the absence of binding of another AP fusion protein containing the extracellular domain of junction adhesion molecule (JAM-AP) (35) and the lack of binding to either SIRPβ1-GST or BSA (Fig. 2B). The effects of our SIRP and CD47 mAbs on binding of CD47-AP to SIRPα1 were identical to those observed in binding assays with purified native CD47 (data not shown). These results indicate that CD47 binds directly to SIRPα1 via the extracellular IgV domain of CD47.

Anti-SIRP mAbs Inhibit PMN Transmigration—Our previous studies and those of others have demonstrated an important role of CD47 in regulating leukocyte transmigration. In particular, anti-CD47 mAbs (C5D5 or B6H12.2) inhibited PMN migration across epithelial, endothelial monolayers and collagen-coated filters (10–12). We therefore tested our anti-SIRP monoclonal antibodies for functional effects on neutrophil (PMN) migration across both intestinal epithelial monolayers and collagen-coated filters (Fig. 3). For transepithelial migration, PMN were induced to migrate from the basolateral to apical direction across inverted T84 intestinal epithelial monolayers as previously described (11). Effects of anti-SIRP mAbs P3C4, SE5A5, SE7C2, SE8A3, SE12B6, and SE12C3 were tested at concentrations ranging from 10 to 50 μg/ml. Anti-CD47 mAb C5D5 and anti-β₃ integrin mAb CBRM1/29 were used as inhibitory controls (10).

As shown in Fig. 3A, the tested anti-SIRP mAbs inhibited PMN transmigration to various degrees. After 1 h of migration, the fraction of applied PMN that had migrated into the lower chamber in the presence of antibodies was 40.1 ± 4.4% for P3C4, 24.9 ± 3.5% for SE5A5, 36.2 ± 4.6% for SE8A3, 34.4 ± 2.4% for SE7C2, 21.6 ± 0.6% for SE12B6, and 14.9 ± 2.3% for SE12C3, compared with 54.3 ± 2.7% of applied PMN migrated in the presence of control mouse IgG. In contrast to the partial inhibition by anti-SIRP antibodies, anti-CD47 mAb C5D5 and anti-CD11b/CD18 mAb CBRM1/29 inhibited PMN transmigration almost completely, which is in agreement with our previous observations (10). Similar inhibitory profiles were obtained with PMN migration across cell-free collagen-coated transwell filters in the presence of anti-SIRP mAbs (data not shown). In summary, the inhibitory effects of this panel of anti-SIRP mAbs ranged from 20 to 60%. Interestingly, mAb P3C4, which failed to inhibit in vitro CD47-SIRPα1 binding (Table I) and Jurkat cell adherence to SIRPα-GST (20), weakly affected PMN transmigration (10–20% inhibition).

To gain further insight into the potential roles of CD47 and SIRPα interactions in PMN migration, we examined the effects of our CD47 extracellular domain fusion protein CD47-AP on PMN transmigration. Recombinant CD47-AP was used instead of purified native protein because of the obligatory detergent requirements for native CD47. As shown in Fig. 3A, CD47-AP

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**Table I**

Summary of binding properties of anti-SIRP and anti-CD47 mAbs

| mAbs        | Binding to: SIRPα1 | SIRPβ1 | CD47 | Inhibition of binding: SIRPα1-GST to purified CD47 | PMN surface labeling | MFI |
|-------------|-------------------|--------|------|--------------------------------------------------|----------------------|-----|
| Ct.IgG      | –                 | –      | –    | None                                             | –                    | 3.80 |
| P3C4        | +                 | +      | –    | Strong                                            | –                    | 28.76 |
| SE5A5       | +++               | +++    | +++  | Strong                                            | –                    | 35.00 |
| SE8A3       | +++               | +++    | +++  | Slight                                           | –                    | 41.72 |
| SE7C2       | +++               | +++    | +++  | Strong                                            | –                    | 28.69 |
| SE12B6      | +++               | +++    | +++  | Strong                                            | –                    | 33.40 |
| SE12C3      | +++               | +++    | +++  | Strong                                            | –                    | 23.77 |
| C5D5        | –                 | –      | +    | Strong                                            | –                    | 49.1  |
| B6H12.2     | –                 | –      | +    | Strong                                            | –                    | 47.1  |
| PF3.1       | –                 | –      | +    | Strong                                            | –                    | 50.1  |
| PF2.1       | –                 | –      | +    | Strong                                            | –                    | ND    |
| PF10.4      | –                 | –      | +    | Strong                                            | –                    | ND    |

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fusion protein inhibited PMN migration by ~40–60%, which is essentially indistinguishable from the inhibition observed with anti-SIRP mAbs. In contrast, no inhibition was observed in the presence of another AP fusion protein JAM-AP. These results suggest that direct interaction between CD47 and SIRPα plays an important role in regulating PMN transmigration.

We next examined potential signaling pathways that might be involved in SIRP-mediated regulation of PMN migration. Previously, we observed that inhibition of PI3-kinase by wortmannin or LY2309 enhanced PMN transmigration, but only partially reversed the inhibitory effects of CD47 mAbs (10). On the other hand, the tyrosine kinase inhibitor genistein both enhanced PMN transmigration and completely reversed anti-CD47 inhibition (10). Thus, we tested whether wortmannin or genistein could reverse the inhibitory effects of anti-SIRP mAbs (Flg. 3B). In these experiments, PMN were pre-treated with 100 nM wortmannin or 200 μM genistein for 10 min (25 °C) prior to initiation of transmigration across epithelial-free collagen-coated filters (to avoid potential effects on epithelial cells). To allow for direct comparison of the ability of wortmannin or genistein to reverse the inhibitory effects of anti-SIRP and anti-CD47 mAbs, transmigration in the presence of antibody and drug was normalized with respect to that observed in the presence of drug alone. In this way, we compared the effects of wortmannin and genistein on antibody-mediated inhibition of transmigration to that observed in the absence of any drug pretreatment. As shown in Flg. 3B, in the absence of drug treatment, transmigration at 1 h was inhibited >90% by anti-CD47 mAb C5D5 and ~55% by a mixture of anti-SIRP mAbs (P3C4, SE5A5, and SE7C2, 20 μg/ml each). Inhibition of PI3-kinase by wortmannin did not decrease the inhibition observed with anti-CD47 in this condition, whereas genistein reversed the antibody-mediated inhibition as we have previously reported (10). In contrast, PI3-kinase inhibition resulted in near complete reversal of the inhibitory effects of anti-SIRP mAbs, whereas genistein had little effect. These results indicate that PI3-kinase, but not genistein-sensitive tyrosine kinase(s), is an important regulatory element in SIRP signaling during PMN chemotaxis.

The Kinetics of PMN Transmigration in the Presence of Anti-SIRP mAbs Are Distinct from Those Observed in the Presence of Anti-CD47—Although CD47 interacts directly with SIRPα, our functional studies demonstrated that anti-CD47 mAbs are more effective than anti-SIRP mAbs or CD47-AP in blocking PMN transmigration. This is clearly demonstrated in Flg. 3, where ~15–40% of the total applied PMN migrated across epithelial monolayers in the presence of anti-SIRP mAbs, compared with only 5.1 ± 3.4% of PMN in the presence of C5D5. We have recently shown that ligation of CD47 by antibody results in a delay of PMN transmigration but no inhibition of the total amount of migrated PMN (10). Thus, we examined the kinetics of PMN migration in the presence of anti-SIRP mAbs (Flg. 4A). As can be seen, the majority of control PMN migrated across the epithelial monolayers within 1 h. In the presence of anti-CD47 mAbs, PMN transmigration was minimal for 2 h, which is in agreement with our previous observations (10). Surprisingly, the transmigration time course observed in the presence of anti-SIRP mAbs had significantly different kinetics from that observed for anti-CD47, which is best appreciated in Flg. 4B. In the presence of anti-SIRP mAbs or CD47-AP, PMN migration occurred as in controls, but at reduced amounts. Thus, although anti-SIRP mAbs and CD47-AP inhibited PMN transmigration in all time periods, these mAbs did not delay PMN migration across the monolayers as was observed with anti-CD47 mAbs (Flg. 4B). Inhibition of PMN transmigration by SIRPα and soluble CD47 resulted in an overall 30–60% inhibition compared with the noninhibitory control. Thus, although CD47 binding to SIRPα clearly modulates PMN transmigration, the pathways that regulate migration downstream of CD47 and SIRPα are distinct.

Expression and Distribution of SIRP in PMN—Using hematopoietic cells, many studies on SIRP have focused on macro-
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**FIG. 3.** Anti-SIRP mAbs inhibit PMN transmigration. A, inverted T84 epithelial cell monolayers were cultured to confluence (high transepithelial resistance) on transwells as described under “Experimental Procedures.” Transmigration assays were performed by placing PMN (1 x 10⁶) into the upper chamber of transwells followed by initiation of transmigration in a basolateral-to-apical direction by adding fMLP (1 μM) to the lower chamber. After incubation for 1 h, PMN that had migrated into the lower chamber were quantified by myeloperoxidase assay. In these experiments, migration in the presence of 20 μg/ml anti-SIRP mAbs P3C4, SE5A5, SE7C2, SE8A3, SE12B6, and SE12C3 was compared with migration in the presence of isotype-matched normal mouse IgG (control IgG), as well as inhibitory mAbs against CD47 (C5D5) and CD11b/CD18 (CBRM1/29). Transmigration assays were also performed in the presence of both CD47-AP (50 μg/ml each), anti-CD47 mAb C5D5 (20 μg/ml), and isotype-matched control IgG (20 or 60 μg/ml each), and 50 μg/ml CD47-AP. Data represent one of six individual experiments with three monolayers in each condition ± S.D. The bracket designates p < 0.05 (*) for inhibited migration compared with control migration. As we have previously shown, migration in the presence of binding isotype matched mAbs J10.4 and W6/32 was not different from migration in the presence of normal mouse IgG (data not shown) (11, 35). B, effects of PI3-kinase and tyrosine phosphorylation inhibition on PMN migration. PMN (1 x 10⁶) were pre-treated with 100 nM PI3-kinase inhibitor wortmannin, 200 μM tyrosine phosphorylation inhibitor genistein, or the same dilution of vehicle (Me2SO) for 15 min at room temperature before use in migration assays across acellular collagen-coated filters as detailed under “Experimental Procedures.” PMN transmigration was performed in the presence of anti-SIRP mAbs (used as a mixture of P3C4, SE5A5, and SE7C2, 20 μg/ml each), anti-CD47 mAb C5D5 (20 μg/ml), and isotype-matched control IgG (20 or 60 μg/ml). For each drug treatment condition (no drug, wortmannin, genistein), migration in the presence of either anti-SIRP or anti-CD47 is shown as a percentage of migration in the presence of isotype matched control IgG (percentage of control). As we have previously reported (35), control migration after wortmannin and genistein treatment was increased compared with migration in the absence of any drug treatment (69.1 ± 5.2% and 72.5 ± 6.1% versus 39.4 ± 2.0% (% of total applied PMN) migrated PMN for wortmannin and genistein versus no drug, respectively). Data represent one of three individual experiments with three or four monolayers in each condition ± S.D. *, p < 0.05 compared with control migration.

**FIG. 4.** Kinetic analysis of the differential inhibitory effects of anti-SIRP, anti-CD47, and soluble CD47 on PMN transmigration. Time-course PMN transepithelial transmigration assays were initiated as detailed above in Fig. 3A. At 30-min intervals after initiation of migration, transwells were moved into new reservoirs containing the same concentration of fMLP. PMN that had migrated into the lower chamber were then quantified by myeloperoxidase assay as described previously (10). A, time course of PMN migration performed in the presence of control IgG (20 μg/ml), anti-CD47 mAb C5D5 (20 μg/ml), anti-SIRP mAb (mixture of P3C4 and SE5A5, 10 μg/ml each), and 50 μg/ml CD47-AP. ○, migration in the first 30-min time period; □, migration in the second 30-min time period; □, migration during the third 30-min time period; □, migration during the fourth 30-min time period; □, migration during the fifth 30-min time period. B, results of transmigration at different time points in A plotted as a function of total migrated PMN. Plotted in this way, the migration at 90 min (1.5 h) for example, represents the sum of PMN in the lower chambers from the first, second, and third 30-min time periods. ○, control isotype-matched IgG; ○, anti-CD47; ▽, anti-SIRP; ▼, CD47-AP. Data represent one of six experiments with each condition representing data from three individual monolayers ± S.D.
our antibodies after SDS-PAGE under reducing conditions. Thus, the presence of disulfide-linked homo- or hetero-
oligomers cannot be excluded.

Although a strong SIRP signal was detected by immunoprecipitation and Western blot experiments (Fig. 5A), cell surface fluorescence labeling revealed only modest labeling compared with that observed for CD47 and CD11b (Table I). In particular, cell surface labeling of freshly isolated, nonstimulated PMN with SIRP mAbs resulted in mean fluorescence intensity values ranging from 23 to 35, depending on which mAb was used. In contrast, staining with anti-CD47 mAb C5D5 and anti-CD11b/CD18 mAb CBRM1/29 resulted in mean fluorescence intensity values of 48 (average) and 124, respectively. Because both CD47 and CD11b/CD18 are up-regulated to the cell surface after PMN activation (10), we examined cell surface SIRP expression after stimulation of PMN with fMLP. In these experiments, PMN were stimulated with fMLP (0.1 μM), followed by cell surface biotinylation and SIRP immunoprecipitation. As shown in Fig. 5B, cell surface SIRP was significantly increased after PMN were stimulated with fMLP as detected in Western blots by probing with streptavidin. Total SIRP in cell lysates, as detected by the polyclonal anti-SIRP1 antibody, was the same before and after fMLP stimulation (Fig. 5B). Analysis of the banding pattern in the biotinylated SIRP blots revealed both an increase in the amount of cell surface protein and a shift in the molecular mass of the lower SIRP1 band (arrow) from 50–65 kDa to a slightly higher molecular mass (52–75 kDa) after fMLP stimulation (Fig. 5B, top panel).

**DISCUSSION**

In this study, we examined the expression pattern and binding characteristics of PMN SIRPs along with the role of SIRP in modulating PMN transmigration. Although the majority of previous reports have focused on studying SIRP in rodents (21, 25, 31, 40, 41), recent studies using cell adhesion assays and recombinant SIRPs have demonstrated CD47-SIRP interactions in human cells (20). In this report, we confirm these observations by demonstrating that human CD47 purified from multiple tissues binds directly to recombinant SIRP1 (SIRP1-GST) in a specific manner. Our studies clearly demonstrate that the binding to SIRP1 is mediated through the IgV loop, defined by a disulfide bond between Cys-41 and Cys-114, in the first portion of the CD47 extracellular domain. Recently, a second long range disulfide bond was reported in CD47 extracellular domain between Cys-263 and Cys-33 (42). Using CD47 mutagenesis, recombinant SIRP1, and binding assays, Rebres and co-workers (42) concluded that this long range disulfide bond between the first extracellular loop (IgV-containing) and a smaller third putative extracellular loop was important for strengthening CD47-SIRP1 interactions. However, because our construct CD47-AP lacks this long range disulfide bond and is capable of inhibiting PMN transmigration with kinetics that are indistinguishable from that observed with multiple SIRP mAbs, the functional significance of this second disulfide bond in SIRP-CD47-mediated regulation of PMN migration remains to be determined.

The specificity of the observed CD47-SIRP1 interactions was confirmed by the absence of binding to SIRP1 and inhibition of binding by anti-SIRP and anti-CD47 mAbs. We demonstrate that anti-SIRP mAbs, previously shown to inhibit Jurkat cell adherence to SIRP1-GST (20), inhibited the direct binding of CD47 to SIRP1. These findings confirm that SIRP1 binds to Jurkat cells through direct interactions with cell surface CD47. We also demonstrate that anti-CD47 mAbs
that inhibit PMN transmigration (10) also inhibit CD47-SIRPα1 binding, indicating that CD47-SIRPα interaction may play an important role in regulating PMN transmigration. In contrast to ligand binding characteristics of β3 integrins, we found that SIRP-CD47 interactions are independent of Ca2+ and Mg2+ and are stable over a broad pH range.

We have shown that SIRP is involved in the regulation of PMN transmigration by demonstrating inhibition of PMN migration across epithelial monolayers and/or cell-free, collagen-coated filters by anti-SIRP mAbs. In particular, anti-SIRP mAbs, which inhibited CD47-expressing Jurkat cells’ adherence to recombinant SIRPα, also inhibited PMN transmigration. Interestingly, anti-SIRP mAb P3C4, which did not block CD47-SIRPα binding, also partially inhibited PMN transmigration. We suspect that this may be because of the presence of other SIRP family members in PMN because this mAb, although generated against SIRPα, recognized both SIRPα1 and SIRPβ1 in vitro (Table I).

As shown in Figs. 3 and 4, recombinant, soluble CD47 extracellular domain (CD47-AP) inhibited PMN transmigration by the same amount and kinetics as the anti-SIRP mAbs. These functional effects were observed irrespective of PMN migration across epithelial monolayers or cell-free filters. It is thus likely that CD47-SIRPα interactions within PMN mediate the observed effects. Interestingly, CD47, but not SIRP, is expressed on intestinal epithelial cells (11),2 which raises the possibility of other functional interactions between epithelial CD47 and PMN SIRPα. In support of this, we have observed that PMN migration is enhanced across CD47-AP-coated filters and CD47-transfected epithelial cells (10). These observations suggest that the functional consequences of CD47-SIRPα interactions may be influenced by how the binding partners are displayed.

Our transmigration results suggest that the signaling pathways downstream of SIRPα and CD47 are distinct. Recently, we reported that antibody-mediated ligation of PMN cell surface CD47 caused a delay in PMN transmigration without diminishing the total amount of migrated PMN (10). This is in contrast to the kinetics of inhibition by anti-SIRP mAbs and CD47-AP, which did not delay PMN transmigration but inhibited the amount of PMN transmigration by 30–60%. In further support of these differences, in this report we show that the inhibitory effects by anti-SIRP antibodies were reversed by pre-treating PMN with the PI3-kinase inhibitor wortmannin, inhibitory effects by anti-SIRP antibodies were reversed by treatment of PMN to recombinant SIRPα/H9251 (Fig. 3B) but not by the tyrosine phosphorylation inhibitor genistein pretreating PMN with the PI3-kinase inhibitor wortmannin, inhibitory effects by anti-SIRP antibodies were reversed by treatment of PMN to recombinant SIRPα/H9251 (Fig. 3B) but not by the tyrosine phosphorylation inhibitor genistein.

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