The role of CD47 in neutrophil transmigration: Increased rate of migration correlates with increased cell surface expression of CD47

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Running title: CD47 regulates neutrophil migration

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Summary

CD47, a cell surface glycoprotein, plays an important role in modulating neutrophil (PMN) migration across endothelial and epithelial monolayers. Here we show that anti-CD47 mAbs delay PMN migration across collagen-coated filters or T84 epithelial monolayers towards chemoattractant fMLP. Despite delayed transmigration by anti-CD47 mAbs, the numbers of PMN migrating across in either condition was the same as in the presence of control non-inhibitory mAbs. Cell surface labeling and immunoprecipitation demonstrated an increase of CD47 to the PMN cell surface with kinetics similar to those of the transmigration response. Subcellular fractionation studies revealed redistribution of CD47 from intracellular compartments that co-sediment with secondary granules to plasma membrane containing fractions after fMLP stimulation. Experiments performed to investigate potential signaling pathways revealed that inhibition of tyrosine phosphorylation with genistein reversed the anti-CD47-mediated PMN migration delay, whereas inhibition of PI3-kinase only partially reversed anti-CD47 effects that correlated with a rapid increase in PMN cell surface CD47. Analysis of the contribution of epithelial-expressed CD47 to PMN transmigration revealed that PMN migration across CD47-deficient epithelial monolayers (CaCO2) was significantly increased after stable transfection with CD47. These results suggest that cell surface CD47 and its linked tyrosine phosphorylation signaling events regulate, in part, the rate of PMN migration during inflammation.
Introduction

In many inflammatory conditions, large numbers of PMN migrate across vascular endothelium, cell matrix and mucosal surfaces in response to chemotactic signals. Under these conditions, PMN release active oxygen species, proteolytic enzymes and inflammatory mediators that result in tissue injury. Thus, it is intuitive that large scale PMN migration and accumulation in tissues correlates with disease activity and patient symptoms (1,2).

The process of PMN movement from the vasculature, through the interstitial matrix and across mucosal surfaces requires sequential interactions between neutrophil surface molecules and counterreceptors present on tissues or cell surfaces (3-6). Previous studies have shown that the leukocyte β2 integrin CD11bCD18 is essential in this process, especially in early interactions with epithelial and endothelial cells during the transmigration response (3,7,8). While PMN migration across endothelial monolayers is only partially blocked by anti-CD11bCD18 antibodies, there is near complete inhibition of PMN migration across epithelial monolayers by the same antibodies (9). The importance of β2 integrins in PMN transmigration is manifested clinically by patients with leukocyte adhesion deficiency (LAD) who lack β2 integrins, resulting in a failure of PMN to migrate out of the vasculature and leaving high levels of circulating PMN (10,11).

A second protein that has been shown to play an important role in PMN transmigration is CD47 (12). PMN migration across both epithelial and endothelial monolayers can be inhibited by anti-CD47 antibodies (3,12,13). CD47 was originally defined as a tumor-specific marker for ovarian carcinoma but is now known to be widely expressed on all hematopoietic cells and most other cell types (14-16). The primary structure of human and mouse CD47 predicts the protein...
containing an N-terminal extracellular IgV domain, a membrane-spanning domain with multiple transmembrane α helices and an intracellular loop (15).

In addition to regulating PMN transmigration, other studies have implicated CD47 in multiple cellular functions. In particular, antibodies to CD47 have been shown to interfere with αvβ3-mediated cell functions (15), as well as to block endothelial Ca²⁺ influxes during adhesion to fibronectin- or vitronectin-coated surface (17). CD47 has been shown to bind to the C-terminus of thrombospondin-1 (18), therefore suggesting a role in platelet activation. Anti-CD47 antibodies have also been used to implicate CD47 in T cell activation (19,20), T and B cell apoptosis (21,22) and stroma-supported erythropoiesis (23). Recently, CD47 has been shown to bind to P84 (also termed SIRPα, Bit, SHPS-1 and MFR) (24-26), and this interaction has been broadly implicated in the regulation of memory formation, macrophage multinucleation, B cell aggregation and red blood cell self-recognition in mice (27,28).

Despite the diverse array of functional studies on CD47, the biochemical / molecular mechanism(s) of CD47 function remain undefined. While we have previously observed anti-CD47 antibody mediated inhibition of PMN transmigration (12), the molecular basis of this effect is not clear and is the objective of the current study to investigate the role of CD47 in PMN migration across epithelial cell monolayers and collagen-coated filters. Using a time-course transmigration assay, we demonstrate that fMLP-driven PMN migration across epithelial monolayers and collagen-coated filters is significantly delayed after cell surface CD47 is blocked by functionally inhibitory anti-CD47 mAbs. However, despite delayed transmigration caused by anti-CD47 mAbs, the number of PMN that eventually migrate is the same as that observed with isotype-matched binding controls. Cell surface labeling and immunoprecipitation experiments demonstrate increased CD47 on the PMN cell surface after stimulation with fMLP and after
transmigration. PMN subcellular fractionation experiments revealed that increased cell surface CD47 is derived from intracellular membranes that co-sediment with the secondary (specific) granules. Experiments performed to investigate potential signaling pathways revealed that inhibition of PI3-kinase partially reversed anti-CD47 mediated inhibition of migration while also directly enhancing the rate of PMN migration in a fashion that paralleled a rapid increase of PMN cell surface CD47. Additional inhibitor studies revealed that tyrosine phosphorylation inhibition by genistein reversed anti-CD47 effect in a near complete fashion. In further support of the notion that cell surface CD47 positively regulates the rate of migration, we found that transfection of CD47 into deficient epithelial cells (CaCO2) results in enhanced PMN transepithelial migration. Taken together, these results suggest that translocation of CD47 to the cell surface and tyrosine kinase-mediated signaling events are key components that determine the rate of PMN migration in an inflammatory response.

**Experimental procedures**

*Epithelial cells:* T84 cells (passages 60 to 80) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) 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 6% newborn calf serum. Cells were subcultured or harvested every 6-8 days with 0.1% trypsin and 1.0 mM EDTA in Ca²⁺ and Mg²⁺-free phosphate-buffered saline. For transmigration experiments, T84 cells were grown on collagen-coated, permeable polycarbonate filters (5µm pore size) with a surface area of 0.33 cm² (Costar, Cambridge, MA) as previously described (12). CaCO2 cells were cultured in DMEM supplemented with 10% FBS. Transfected
CaCO2 cells (CaCO2-pCB6 and CaCO2-CD47) were maintained in the same medium containing 1 mg/ml G418.

**Neutrophils:** PMN were isolated from whole blood of normal human volunteers as previously described (12). Isolated PMN were resuspended in modified Hanks balanced salt solution devoid of calcium or magnesium (HBSS(-)) (4°C) at a concentration of $5 \times 10^7$ cells/ml and used within 4 hours of isolation.

**Anti-CD47 antibodies:** C5D5, a functionally inhibitory mAb (IgG1) was produced in our laboratory as previously reported (12). B6H12.2, another functionally inhibitory anti-CD47 mAb (IgG1) (13) was purified from supernatants of hybridoma cells obtained from the American Tissue Culture Co. (ATCC). CD47 mAbs PF2.1, PF3.1, PF10.2 were produced in our laboratory by immunizing female balb C mice with CD47 immunopurified from 50g human spleen cells using previously described methods (12). Splenocytes from immunized mice were fused with P3U1 myeloma cells and cultured in selection media containing DBA-2 feeder thymocytes. Hybridoma supernatants were screened for reactivity with purified CD47 by ELISA in concert with cell surface staining by immunofluorescence and flow cytometry on T84 epithelial cells and PMN. Hybridomas were then subcloned by limiting dilution, weaned from selection media and injected into the peritoneal cavities of balb C mice for ascites fluid production. Antibodies were purified using protein-A – Sepharose affinity chromatography and dialyzed against 150 mM NaCl, 10 mM HEPES pH 7.4. Monoclonal antibody PF3.1 was coupled to CNBr-activated Sepharose at 2-3 mg/ml described by the manufacturer (Pharmacia) and used for immunoprecipitation of CD47. A polyclonal antibody (R12989) was also produced by
immunizing rabbits with synthetic peptide SNQKTIQPPRNN, corresponding to a region of the putative C-terminus of CD47 (15).

*Other antibodies and reagents:* As a non-inhibitory binding control, mAb J3F.1 (mouse IgG1) reactive with human junction adhesion molecule (JAM) was used (29). Similar to CD47, JAM is a transmembrane IgV superfamily member expressed on the surfaces of both PMN and epithelial cells (29). As a positive functionally inhibitory control, anti-CD11b mAb CBMR1/29, was used (30). Anti-β3 integrin mAbs 15, 33, 27, and LIBs-1 were kind gifts from Dr. Mark Ginsberg. LM609 was kindly provided by Dr. David Cherish. An additional Anti-β3 mAb API was purchased from GTI Inc. A functionally blocking mAb to β1 integrin, mAb 13, was kindly provided by Dr. Kenneth Yamada. Purified human thromponsbodin-1 (TS1) and anti-TS1 polyclonal antibody R1 were kindly provided by Dr. Jack Lawler (31). Rabbit polyclonal antibody against human CD11b R7928A was raised by immunizing rabbit with a peptide corresponding to the C-terminus of CD11b. Peptide 4N1K (KRFYVVMWKK) corresponding to the C-terminal cell binding domain of thromponsbodin that has previously been shown to bind to CD47 transfected cells (18,32), was synthesized and HPLC purified to >90% by the Emory Microchemical Facility.

*Transmigration experiments:* PMN transepithelial migration experiments were performed using epithelial monolayers as previously described (12). Briefly, confluent T84 or CaCO2 monolayers were washed twice with HBSS (20°C). PMN (1x10⁶) in 150 µl HBSS with or without antibody was added to the upper chamber of the monolayer setup. Transmigration was initiated by adding 1 ml of 1 µM fMLP (in HBSS) into the lower chamber followed by incubation at 37°C. PMN
migration across monolayers into the fMLP-containing lower chambers was quantified by myeloperoxidase (MPO) assay as previously described (12).

To assay the time-course of PMN transmigration, the upper chambers of transwell devices containing PMN and monolayers were gently moved to new wells containing 1 ml of 1 µM fMLP in 24-well plates at every 30 min as depicted in Figure 1. PMN migration into the lower chambers at the different time periods was then analyzed by MPO assay. To assay the PMN associated with epithelial monolayers, transmigration was stopped at different time points and monolayers were washed with HBSS and lysed with 0.5% Triton X-100. Monolayer lysates were then assayed for myeloperoxidase. Apical to basolateral PMN transepithelial migration was performed similarly except that twice as many PMN (2x10^6) were added onto the upper chamber. Additional transmigration experiments were performed using collagen-coated filters devoid of epithelial cells. For these experiments, 20 µl of a PMN suspension containing 1x10^6 cells was induced to transmigration from the upper chamber to the lower chamber containing 0.1 µM fMLP. A number of signal transduction pathway inhibitors were tested for the ability to reverse the effects of anti-CD47 mAbs on PMN transmigration. For these experiments, PMN were pre-treated for 15-60 min with the following agents prior to use in transfilter migration assays: 50-100 nM wortmannin or 20-100 µM LY294002 (PI3-kinase inhibitor (Sigma)), 0.5 µM straurosporine (PKC and other kinases inhibitor (Biomol)), 10-200 µg/ml genistein (tyrosine phosphorylation inhibitor) and its analog daidzein (both from Biomol), 0.1 µM AG126 (tyrosine phosphorylation inhibitor (Biomol)), 40 µM PP1 (Src family tyrosine kinases inhibitor (Biomol)), 20 µM SB203580 (MAP kinase inhibitor (Biomol)), 20 µM PD-98059 (MEK inhibitor (Biomol)), 10 µM U73122 (PLC inhibitor (Biomol)), 1 mM propranolol (PLD inhibitor (Biomol)), 1 µM GF109203X (PKC inhibitor (Biomol)), 20 µM AG490 (Jak kinase inhibitor (Biomol)).
Control migration was done in the presence of same dilution of vehicle (DMSO) in HBSS. For G-protein pathway inhibition experiments, PMN were pre-treated with 200-2000 ng/ml pertussis toxin (Calbiochem) for 2 h at 37°C before used in migration assay.

**PMN cell surface CD47 labeling and analysis by flow cytometry (FACS):** Surface CD47 labeling was performed by incubation of 100 µl of non-permeabilized PMN (5x10^6 cells) with anti-CD47 mAb C5D5 (20 µg/ml) for 1 h at 4°C in HBSS after blocking non-specific 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 followed by wash before flow cytometric analysis. CD47 labeling in this condition was compared to that obtained with normal mouse IgG1. To assay PMN surface CD47 expression during transmigration, PMN that remained in the upper chambers or had migrated into the lower chambers of transwell setups were cooled on ice after various migratory time periods followed by antibody labeling and flow cytometry. Cell surface CD47 on PMN was also assessed after PMN were stimulated with fMLP. In these experiments, 5x10^6 PMN in 2 ml HBSS were allowed to settle in 24-well or 6-well non-tissue culture plates at room temperature for 5 min. fMLP was then added (50 nM) followed by incubation at 37°C for various time periods. Cells were then chilled on ice and labeling performed as above.

**Cell surface biotinylation, Immunoprecipitation and Immunoblotting experiments:** PMN (1.5x10^7/ml), in suspension or adherent to 6-well plates, were stimulated with fMLP followed by cell surface biotinylation with 0.5 mg sulfo-NHS-Biotin (Pierce) in 1 ml HBSS for 1 h on ice.
Excess free Biotin was washed off and quenched with 100 mM NH₄Cl. Cells were then solublized with lysis buffer containing 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% Triton, 2 µg/ml aprotinin, 1 mM PMSF, 2 µg/ml leupeptin and 1 µg/ml pepstatin. Cell lysates (200-400 µg of total protein) were precleared with control IgG before incubation with 10 µl PF3.1-Sepharose (conjugated at 2 mg antibody / ml sepharose) for 3 h (4°C). Washed immunoprecipitates were boiled in 100 µl sample buffer and subjected to SDS-PAGE followed by transfer to nitrocellulose. Non-specific binding was blocked by 5% non-fat dry milk in TTBS. Biotin labeled cell surface CD47 was detected with streptavidin-peroxidase followed by enhanced chemiluminescence (ECL (Pharmacia)). Total CD47 was detected by blotting with polyclonal rabbit anti-CD47 antibody R12989 (1:200) and was visualized with peroxidase conjugated goat anti-rabbit secondary and ECL.

Subcellular fractionation of PMN: Freshly isolated, unstimulated and fMLP stimulated PMN (2x10⁸ / per condition) were resuspended in 5 ml nitrogen cavitation buffer (0.34 M sucrose, 10 mM Hepes, 1 mM EDTA, 0.1 mM MgCl₂, 1 mM Na₂ATP, pH 7.4) followed by lysis with nitrogen cavitation (15 min, 400 psi) (4°C). The lysates were centrifuged by low speed centrifugation (1000xg). The low speed supernatants were then subjected to isopycnic sucrose density gradient fractionation on linear 20-55% sucrose gradients in a Beckman SW-28 swinging bucket rotor (100,000xg; 3h, 4°C) exactly as previously described (33). 1.5 ml fractions were collected from each gradient and analyzed for sucrose density, protein, plasma membrane (alkaline phosphatase), primary granules (myeloperoxidase) and secondary granules (lactoferrin) as previously described (33).
Generation of CD47 transfected cells: The entire protein coding region of CD47 was PCR amplified from reverse transcribed human colonic cDNA with primers C3 (sense) (5’atgataagcttcatgtggcccctggtagcgg) and C6 (antisense) (5’agcttctagactctccaaatcggagtccat) located at the 5’UTR and 3’UTR respectively. Amplified PCR product was digested with Hind III / Xba I and cloned into pCB6 which had been pre-digested with the same restriction endonucleases. CaCO2 cells were transfected with pCB6-CD47 or pCB6 (control transfection) using lipofectin reagent as described by the manufacturer (GIBCO BRL). Stable transfectants were selected by culturing in DMEM containing 1.2 mg/ml geneticin (GIBCO BRL). Cell surface expression of CD47 was confirmed by immunofluorescence labeling and FACS analysis.

Construction of CD47 extracellular domain and alkaline phosphatase (AP) fusion protein: The putative extracellular domain of human CD47 including the signal peptide was PCR amplified by primers C3(5’atgataagcttcatgtggcccctggtagcgg) and C4 (5’tgattagatctatttggagaaaaccatga) from reverse transcribed T84 cDNA. The DNA fragment was cloned into the mammalian expression vector AP-tag2 (34) that contains the catalytic domain of human placental alkaline phosphatase (AP) at the C-terminus through Bgl II and Hind III restriction sites. After transformation, at least three independent clones were isolated and the entire protein-coding region was confirmed by DNA sequencing. Transient transfections of COS-7 were performed using a standard DEAE-dextran method (35). Protein expression and secretion was monitored by assaying AP activity in cell culture medium (34). Expressed CD47-AP fusion protein in medium was affinity purified by PF3.1 sepharose and eluted with 10 mM TEA/HCl buffer containing 100 mM NaCl, pH 10.5, followed by neutralization, concentration and dialysis.
Results

Anti-CD47 mAbs delay PMN transmigration.

Previously, we demonstrated that anti-CD47 mAb C5D5 inhibited PMN migration across T84 monolayers (12). However, we noticed that the efficiency of inhibition by C5D5 or another anti-CD47 antibody, B6H12.2 (13), was variable in the standard transmigration assays typically consisting of a single time-point (normally 2 hrs). We surmised that the variable inhibition observed might be the result of differences in PMN motility after CD47 inhibition. To address this and further investigate the mechanism of CD47-mediated migration, we modified the in-vitro transepithelial migration assay in which PMN migration was assessed at several time points during the assay (Figure 1). Using this assay setup, we tested the effect of inhibitory anti-CD47 mAbs C5D5 and B6H12.2 on a time course of PMN migration across T84 monolayers. As controls, parallel experiments were performed with binding, non-inhibitory isotype-matched anti-JAM mAb J3F.1 (29) and an inhibitory control antibody reactive with CD11bCD18 (CBRM1/29 (30)).

As shown in Figure 2A, in the presence of control non-inhibitory mAbs, less than 10% of the total applied PMN migrated across T84 monolayers during the first 30 min (5.8±0.6% for no antibody and 6.1±2.0% for J3F.1). Massive PMN migration across T84 monolayers occurred during the next 30 min period (2nd 30) with approximately 40% of the applied PMN migrating into the lower chambers (43.6±3.7% for no antibody and 38.7±4.4% for J3F.1). In the third 30 min period (3rd 30), PMN migration into the lower chamber decreased to 14.6±2.9% for no antibody and 16.8±5.2% for J3F.1. In subsequent time periods, PMN migration across the monolayers continued to decrease to <10% in the 4th 30 min and to <5% in the 5th and 6th 30 min periods, respectively. From Figure 2A, total migration of PMN into the lower chamber can be...
plotted as a function of migration time, which is shown in Figure 2B. As can be seen, under non-inhibitory conditions, approximately 65-80% of the total applied PMN (1x10^6) migrated across T84 monolayers after 3h incubation. Furthermore, the majority (>80% of migrated PMN) had migrated into the lower chamber by 1.5 hours.

Compared to control migration, the addition of anti-CD47 antibodies C5D5 or B6H12.2 (Figure 2A) resulted in reduced number of PMN migrating into the lower chamber during the first (1st) and second (2nd) 30 min time periods. In the third time period (3rd 30 min), PMN started appearing in the lower chamber but massive PMN migration into the lower chamber did not occur until the 4th and 5th 30 min time periods (20.4±3.1% and 33.8±4.3% for C5D5, 11.7±5.3% and 29.8±8.6% for B6H12.2 at the 4th 30 min and 5th 30 min, respectively). Migration continued at even later time periods with greater than 10% of PMN detected in the lower chamber after the 6th 30 min incubation.

As shown in Figure 2B, anti-CD47 treatment resulted in a significant delay in PMN migration across T84 monolayers. In particular, anti-CD47 treated PMN that migrated across T84 monolayers after 1.5 h comprised only 5-10% of the total applied cells compared to a migration of 65-80% of the total applied PMN under control conditions. However, despite the delay in PMN migration caused by anti-CD47 mAbs, the total number of PMN that migrated after 3 hours was the same as that for non-inhibitory controls. From Figure 2B, we also conclude that the inhibition observed with anti-CD47 contrasts with those observed with anti-CD11b (CBRM1/29), where there was sustained inhibition of PMN migration over the entire timecourse, a result of PMN adherence dysfunction caused by CBRM1/29.

In parallel experiments, we also assayed the PMN content within the epithelial monolayers during PMN transmigration. At various times, transmigration was terminated by
rapid cooling of the monolayers on ice, followed by washing away loosely adherent PMN. PMN within the epithelium and filters were then quantified by MPO assay. As shown in Table 1, in the first 30 min of incubation, only a few PMN migrated into the lower chamber, and this was accompanied by high values of PMN associated with T84 monolayers in both the control and anti-CD47 conditions. As predicted, monolayer-associated PMN values decreased in the control by 1.5 h (90 min), a result of massive migration into the lower chamber. However, in the presence of C5D5, the number of monolayer-associated PMN remained high for 2h, consistent with the low number of PMN migration into the lower chamber. These results agree with the previous observation of high monolayer-associated PMN values detected with a single time point migration assay when mAb C5D5 was applied (12). Thus, inhibition of cell surface CD47 with antibodies results in a delayed (shifted) timecourse, but not a decrease in the total amount of PMN migration across epithelial monolayers.

We also tested three other newly generated mAbs against CD47 (PF2.1, PF3.1 and PF10.2). All of these mAbs bound to purified CD47 and labeled non-permeabilized T84 epithelial cells, CD47 transfected CaCO2 cells, and PMN (not shown). Thus, these mAbs bind to epitopes residing in the extracellular domain of CD47. As shown in Figure 2C, all of these mAbs delayed PMN migration across T84 monolayers at different levels in the time-course transmigration assay, with mAb PF3.1 being the least effective.

In addition to assaying PMN migration across T84 epithelial monolayers, we also tested the effects of anti-CD47 mAbs on PMN transmigration across cell-free collagen-coated filters. We observed similar patterns of delayed PMN migration by anti-CD47 antibodies (including C5D5 and B6H12.2) in these assays (data not shown). These results indicate that the mechanism
of CD47 mediated PMN migration is independent of PMN CD47 interactions with cell surface CD47 or other proteins on T84 cells, but depends on PMN-specific binding / signaling events.

**Increase of cell surface CD47 during PMN transmigration and fMLP stimulation.**

To further analyze the role of CD47 in PMN transmigration, we assayed for PMN cell surface CD47 as a result of transmigration. As shown in Table 2, in the absence of anti-CD47 antibody, cell surface CD47 on non-migrated PMN (harvested from the upper chamber of transwell devices), showed only minimal increases over the 3 hr transmigration assay. In contrast, cell surface CD47 was significantly up-regulated on PMN which had migrated into the lower chamber, with the MFI values increasing over 3-fold over that of non-migrated cells (MFI of 80.3 vs. 25.0 for migrated vs. non-migrated, respectively). We also assayed cell surface CD47 expression during PMN transmigration in the presence of mAb C5D5 and observed that, despite delayed migration, this antibody did not inhibit an increase of CD47 on PMN surfaces (data not shown). These results suggest that the delayed migration by anti-CD47 mAbs treatment may be due to antibody-mediated inactivation of cell surface CD47 function.

Experiments were performed to investigate if the observed increase of PMN cell surface CD47 is related to fMLP stimulation. The expression of CD47 on neutrophil surfaces was examined during stimulation with fMLP and was compared with surface expression of CD11bCD18, which has been shown to rapidly increase upon PMN activation (36-38). As shown in Figure 3A, PMN cell surface CD47 gradually increased nearly 3-fold after stimulation with fMLP for 2 hrs. Further experiments employing cell surface biotinylation and immunoprecipitation confirmed these findings. As seen in Figure 3B, immunoprecipitation of CD47 from cell surface biotinylated PMN after fMLP stimulation, or after transmigration,
revealed significant increases in labeled CD47. In contrast, the total amount of CD47 in cell lysates, as detected by a polyclonal rabbit anti-CD47 antibody (R12989), was the same before and after fMLP stimulation. These results suggest that a significant pool of CD47, which is inaccessible by cell surface biotin labeling in resting PMN, is mobilized to the cell surface after PMN activation. Compared to the rapid up-regulation of cell surface CD11bCD18 after PMN activation (MFI reached maximal after 10 min stimulation, Figure 3A), the increase of CD47 on the PMN cell surface was a relatively slow and gradual process (Figure 3A). The difference in the kinetics of up-regulation between these two cell surface proteins suggests that they reside in distinct intracellular pools with different regulatory mechanisms.

To further identify the intracellular localization of CD47 in PMN, we performed subcellular fractionation experiments. In these studies, PMN were lysed by nitrogen cavitation followed by isopycnic sucrose density gradient centrifugation on 20-55% linear sucrose gradients. Analysis of sucrose gradient fractions, as shown in Figure 4, revealed that the membrane-bound organelles distributed between fractions 11-24 with plasma membranes sedimented between fractions 11-17 (alkaline phosphatase), secondary granules between fractions 17-20 (lactoferrin) and primary granules between fractions 20-24 (myeloperoxidase), similar to previous reports (33). Immunoblots of CD47 in these sucrose gradient fractions (Figure 4) showed that, for unstimulated PMN, the majority of CD47 co-localized with CD11b and secondary granules in fractions 17-20. Only a small amount of CD47 (<10%) was found in plasma membrane fractions (fractions 12-15). After stimulation with fMLP, about 30-50% of intracellular CD47 re-distributed into the plasma membrane fractions (fractions 12-15), which again was similar to that observed for CD11b (Figure 4). However, compared to the blots for CD11b, blotting of CD47 revealed a broader re-distribution. As shown in Figure 4, a substantial
amount of CD47 co-localized with the primary granules rather than CD11b after PMN stimulation. No CD47 was detected in fractions containing cytosolic protein in either condition (fractions #1, Figure 4).

PI3-kinase inhibition does not reverse anti-CD47 mediated inhibition but results in rapid up-regulation of cell surface CD47 and increased rates of PMN migration at early time points.

Since PI3-kinase has been shown to play an important role in PMN activation and motility (39-41), we examined the effect of PI3-kinase as a possible downstream effector of CD47-mediated regulation of PMN migration. In contrast to inhibitory effects of anti-CD47 antibodies, PMN migration across both T84 monolayers and collagen-coated filters was enhanced at early migration time points after pre-treatment with the PI3-kinase inhibitor wortmannin or LY294002. As shown in Figure 5A, wortmannin pretreatment resulted in an increase in transepithelial migration to 27.5±2.3% (of total applied PMN) during the first 30 min period in contrast to almost no migration observed for non-treated controls. Similar enhanced migration was obtained by pre-treating PMN with LY294002 (not shown). This migration enhancement by PI3-kinase inhibitors was PMN-specific, since pre-treatment of epithelial monolayers with wortmannin did not affect PMN migration (not shown). In experiments to examine the effect of PI3-kinase on CD47-mediated regulation of PMN migration, we observed that pre-treatment of PMN with wortmannin or LY294002 only partially reversed the migration delay caused by anti-CD47 antibodies (Figure 5A). This suggests that PI3-kinase is not a direct down-stream effector of CD47.

Given the positive correlation between PMN activation / migration and up-regulation of CD47 to the cell surface, we examined the role of PI3-kinase in CD47 PMN cell surface up-
regulation. Flow cytometric analysis were performed on wortmannin-treated, fMLP stimulated PMN. As shown in Figure 5B, wortmannin treatment resulted in more rapid up-regulation of CD47 on PMN cell surface compared to non-treated controls. In these experiments, CD47 was translocated to the cell surface 10 min after fMLP stimulation in wortmannin-treated condition compared to relatively minor increases of CD47 on the cell surface for untreated controls. These findings were further confirmed by surface biotinylation and immunoprecipitation experiments as shown in Figure 5C. Thus, increased cell surface CD47 expression correlates with enhanced PMN migration (Figure 5A) observed after inhibition of PI3-kinase activity.

Inhibition of specific tyrosine phosphorylation reverses anti-CD47 mediated PMN migration delay.

In an effort to identify the mechanism of anti-CD47 mediated transmigration delay, we tested a large panel of pharmacological probes / inhibitors targeted to multiple signaling pathways in our transmigration assays. To avoid epithelial contributions, we used transwells containing cell-free collagen-coated filters in these assays. Compounds that inhibit G-protein pathway (pertussis toxin), phospholipases C and D (U73122 and propranolol.HCl), PKC and other kinases (GF109203X and staurosporine), PKA/G (H-89 and H8), and the MAP kinase pathway (SB203580 and PD98059) were used alone or in combination with anti-CD47 mAb C5D5 in migration assays. While some of these compounds had direct inhibitory effects on fMLP-driven chemotaxis, there was no reversal of anti-CD47 mediated effects (data not shown). Tyrosine kinase inhibitor AG-126 inhibited PMN migration (>90%) differently from anti-CD47 mAbs (not shown), while the Src-family tyrosine kinase inhibitor PP1 did not affect PMN transmigration and anti-CD47 antibody inhibition (data not shown).
However, pre-treatment of PMN with the tyrosine kinase inhibitor genistein (42) enhanced PMN transmigration at early time point in a fashion similar to wortmannin or LY200039. As shown in Figure 6A, genistein pretreatment resulted in an increase in PMN transmigration to 67.7±17.1% (of total applied PMN) after the 1st 30 min, compared to <10% of PMN transmigrated in the control condition (Figure 6a). In contrast to the effect observed with PI3kinase inhibitors, pre-treatment of PMN with genistein did not result in rapid increase of CD47 on PMN cell surface (data not shown). However, this treatment dramatically reversed anti-CD47 mAb C5D5 inhibition in PMN migration in a near complete fashion (Figure 6A and B). As can be seen, C5D5-mediated transmigration delay was reversed by pre-treating PMN with genistein (100 µg/ml for 10 min), and resulted in an increased PMN transmigration to 37.2±5.0% after the 1st 30 min in the presence of the same concentration of antibody (Figure 6A and B). In addition, genistein reversed C5D5 inhibition in PMN transmigration in a dose-dependent manner. As shown in Figure 6C, genistein enhanced PMN transmigration at concentrations as low as 10 µg/ml while it completely reversed the C5D5 (20 µg/ml) effect at 100 µg/ml. To confirm that the effect of genistein was tyrosine kinase-specific, we compared the effect of genistein with its inactive analog daidzein. Daidzein is a structurally similar compound which shares many other effects of genistein but specifically lacks the inhibition of tyrosine phosphorylation (42,43). As shown in Figure 6A and B, daidzein also showed effects of enhancing PMN migration but failed to reverse the anti-CD47 mediated migration delay. Comparing other tyrosine kinase inhibitors, AG-126 and PP1, which either directly inhibited PMN migration (>90%) or had no effect, the effect of genistein was different and unique. This indicates that genistein may have different kinase specificity compared to the other inhibitors. Overall, our results suggest that specific
protein tyrosine phosphorylation, which can be inhibited by genistein, serves as downstream signals of CD47 mediated regulatory events.

**Epithelial-expressed CD47 positively facilitates PMN migration.**

Because of the abundant epithelial expression of CD47 (12) and our results suggesting that cell surface CD47 facilitates PMN migration, we tested whether epithelial expression of CD47 could also dynamically regulate PMN migration. In these experiments, T84 monolayers were pre-incubated with anti-CD47 mAb C5D5 or B6H12.2 followed by thorough wash before use in PMN transmigration assays. Anti-CD11bCD18 mAb CBRM1/29 was used as a control in these experiments to demonstrate that, after the same wash, no sufficient free antibodies were left to inhibit PMN transmigration by binding to PMN. As shown in Figure 7, blockade cell surface CD47 on T84 epithelial cells resulted in a delay of PMN migration across the monolayers. As can be seen, in the presence of C5D5 or B6H12.2, almost no PMN were detected in the lower migration chamber until after the 3rd 30 min of incubation. The specificity of this effect is demonstrated by the lack of inhibition with a binding control mAb, J3F.1 compared to migration in the absence of any antibody. This isotype-matched antibody binds to the extracellular domain of junctional adhesion molecule (JAM), a transmembrane Ig superfamily member which is richly expressed on the basolateral membrane of T84 cells similar to CD47 (29). Lastly, no inhibition of PMN migration was detected for monolayers pre-incubated with CBRM1/29.

In our studies of the role of epithelial CD47 in PMN transepithelial migration, we found that the intestinal epithelial cell line CaCO2 expressed very little, if any, CD47 (Figure 8). CaCO2 cells, in contrast to the only other CD47-deficient cell line (OV10) in use (44), form confluent, highly polarized monolayers that are amenable to transmigration studies as described
here. As shown in Figure 8a, after CD47 was stably transfected into CaCO2 cells, FACS analysis revealed a high level of CD47 expression on CaCO2-CD47 cells compared to mock-transfected controls. Confocal microscopy demonstrated that the majority of expressed CD47 localized along the basolateral surfaces of CaCO2 monolayers (Figure 8b), as is observed in natural epithelia (12). Transfection of CD47 into CaCO2 cells did not appear to grossly alter the functional characteristics of these cells since CD47 expressing CaCO2 cells formed polarized epithelial monolayers with trans-monolayer electric resistances of 400-500Ω.cm², which was indistinguishable from that observed with non-transfected monolayers.

The effect of epithelial CD47 on PMN migration was thus studied by performing migration assays across CaCO2-CD47 monolayers. As shown in Figure 9, PMN migration across CaCO2-CD47 monolayers was increased compared to migration across mock-transfected controls. In these assays, 11.5±4.1% of total applied PMN migrated across CaCO2-CD47 monolayers after the first hour compared to 4.3±1.9% of PMN that migrated across control monolayers. In the second hour, 20.6±6.7% PMN migrated across CaCO2-CD47 monolayers, whereas fewer PMN (12.4±2.9%) migrated across the mock-transfected controls. Pre-treatment of CaCO2-CD47 monolayers with anti-CD47 mAbs, followed by washing away unbound antibody, resulted in reducing PMN migration to an extent comparable to that observed across mock-transfected controls (Figure 9).

These data suggest that, although it is not necessary for PMN migration (as observed in PMN trans-filter experiments), CD47 on epithelial cell surfaces positively facilitate PMN transepithelial migration. In support of these observations, we also observed a 15-20% increase in PMN migration across cell-free filters that had been pre-coated with a purified recombinant CD47 fusion protein consisting of the extracellular domain fused to alkaline phosphatase (CD47-
AP, coated at 10 μg/ml) (data not shown). These observations lend further support to the hypothesis that PMN migration is enhanced by interactions mediated by cell surface CD47.
Discussion

Previous studies have suggested a role of CD47 in fMLP-driven PMN migration across endothelial and epithelial monolayers (12,13). However, these CD47-mediated effects are not fMLP specific since anti-CD47 mAbs can inhibit PMN migration driven by a range of other physiologically relevant chemoattractant such as LTB4 and IL8 (unpublished results). In this study, our analysis of PMN migration across epithelial monolayers (T84 and CaCO2) and collagen-coated filters provides new insights into the mechanism of CD47-mediated regulation of PMN transmigration.

The time course of PMN transmigration was analyzed to better understand the inhibitory effects of anti-CD47 mAbs. In these experiments, we observed the majority of PMN migration across T84 epithelial monolayers into the lower chamber occurred between 30 and 90 minutes. By blocking cell surface CD47 with inhibitory antibodies, PMN migration into the lower chamber was significantly delayed and was preceded by increased PMN accumulation in epithelial monolayers (Figure 2 and Table1). Surprisingly, inhibition of CD47 did not halt migration, and the total number of PMN that eventually migrated was the same as that observed for uninhibited controls. From these results, it can be inferred that CD47 positively regulates the rate of PMN transmigration.

The anti-CD47-mediated delay in PMN migration we observed is not secondary to low (unsaturating) antibody concentrations in our experiments, since incorporation of increased amounts of multiple inhibitory antibodies (up to 100 µg/ml) in our assays, or supplementation with additional antibody during migration, yielded the same results (data not shown). In addition, the effect caused by anti-CD47 antibodies was not mediated through Fc receptors as C5D5 F(ab)2 delayed PMN migration to the same extent as C5D5 IgG (not shown).
We observed that CD47 from both neutrophils and epithelial cells is able to facilitate PMN migration. Functional inhibition of CD47 in either cell type resulted in delayed PMN transmigration (Figure 2 and Figure 7) (12). Expression of CD47 on CaCO2 cells, a CD47-deficient intestinal epithelial cell line, enhanced PMN migration across the monolayers (Figure 9). Since CD47 is universally expressed on most cell types (15,16), it is possible that this pattern of broad tissue expression of CD47 plays a role in positively regulating leukocyte migration during the inflammatory response. Previous studies with CD47 deficient mice demonstrated enhanced susceptibility to *E. coli* peritonitis (45). This decreased host defense response was associated with an early defect (4 hrs after bacterial injection) in PMN accumulation at the infection site. However, in agreement with our in vitro findings, PMN were able to accumulate at the infection site at later time points (the PMN number at the infection site were similar for CD47 -/- and CD47 +/- mice at 24 hrs) (45). Presumably, the observed delay in PMN migration resulted in enhanced susceptibility to bacterial infection. These observations suggest that CD47 regulated PMN migration plays a crucial role in the recruiting and the timely arrival of PMN to inflammatory sites in vivo.

In other cell systems, CD47 has been shown to functionally and physically associate with β3 and β1 integrins (15,44) (32,46). However, we have not been able to detect a direct association of CD47 with β1, β2 or β3 integrins in PMN using similar co-immunoprecipitation techniques (not shown). Furthermore, we observe no significant effect on PMN transepithelial migration in the presence of a panel of functionally inhibitory mAbs against β3 integrin (AP1, mAb15, mAb33, mAb27, LIBS-1, R2-8L8 and LM609, used at 20-100 µg/ml) or β1 integrin (mAb13, 20-100 µg/ml) (data not shown). These observations suggest that the mechanism of CD47-
mediated regulation of PMN transmigration may be independent of previously described CD47-integrin interactions.

CD47 has also been shown to bind to thrombospondin 1 (TS1) through interaction with the C-terminal cell-binding domain (CBD) of TS1 (18). A peptide 4N1K (KRFYVVMWKKK) that putatively mimics this binding has been shown to have similar effects as TS1 (47,48). In particular, previous studies have shown that TS1, the CBD, or 4N1K can activate platelet α2/β1 and α2b/β3 integrins via CD47, resulting in platelet spreading and aggregation (47,48). We tested purified TS1, thrombospondin antibodies, and the peptide 4N1K in our transmigration assays and found no inhibitory or stimulatory effect at concentrations that were effective on platelets (data not shown). Furthermore, we have not observed specific adhesion of CD47 transfected epithelial cells or recombinant CD47 (CD47-AP) to either thrombospondin or 4N1K – coated surfaces (not shown). Most recently, SIRPα has been shown to bind to CD47 (24,25). Since this protein has been recognized as a signaling molecule negatively regulating cellular functions, it is possible that CD47-SIRPα interactions may play an important role in regulating CD47-mediated functions in PMN. Further studies await the availability of human SIRPα–specific reagents to help answer this important question.

How does CD47 positively regulate PMN chemotaxis? Unlike β2 integrin-mediated functions that serve to regulate initial firm attachment (3), our findings suggests that CD47 positively regulates PMN transmigration via other mechanisms that are more compatible with intracellular signaling events regulating protein-protein interactions. In particular, we now show involvement of tyrosine kinase linked signaling events in the mechanism of CD47-mediated regulation of PMN migration. We demonstrated that a specific tyrosine kinase inhibitor, genistein, reverses the delay in PMN transmigration mediated by anti-CD47. The specificity of
this effect is confirmed by the failure of the closely related compound daidzein, which has no tyrosine kinase inhibitory ability, to reverse the inhibitory effects of anti-CD47. Although both genistein and daidzein enhanced PMN transmigration, the ability to reverse anti-CD47 effects was only displayed by genistein suggesting that tyrosine phosphorylation is a key event occurring down stream of antibody-mediated CD47 ligation.

Our findings also implicate a role of cell surface CD47 in regulating the rate of PMN transmigration. In particular, while increasing functional molecules on PMN cell surface upon stimulation has been reported for CD11bCD18 (36-38,49-51), CD47 is upregulated to the PMN cell surface with a timecourse that is distinct and closely parallels the kinetics of cell migration observed in these studies. In contrast, upregulation of CD11bCD18 on the PMN cell surface occurred at early time points after stimulation, preceding the translocation of intracellular CD47 (Figure 3a). Interestingly, our subcellular fractionation results indicate that CD47 co-sediments with markers of secondary granules and CD11bCD18 in unstimulated PMN, and is re-distributed to plasma membrane and other granule containing fractions upon stimulation. Conversely, CD11bCD18 is only partially translocated to plasma membrane from the secondary granules after fMLP stimulation. The differences between these two membrane proteins suggest that CD47 may reside in a membrane-bound organelle that is distinct from that containing CD11b/CD18.

Also consistent with a role of cell surface CD47 in facilitating migration are the results of our PI3-kinase inhibition experiments. Treatment of PMN with wortmannin or LY294002 resulted in rapid fMLP-dependent increases of CD47 on the PMN cell surfaces that correlated with an increased rates of transmigration. Previous studies have shown that PI3Kγ-deficient mice have impaired PMN migration (40,41,52). Since wortmannin inhibits all PI3-kinase
isoforms, our results suggest that other PI3-kinase isoforms, such as PI3Kα, β and δ, may play important roles in modulating PMN migration.

Based on these results, we propose a hypothetical model of CD47 function in PMN (Figure 10). During PMN chemotaxis, CD47 is translocated to the PMN cell surface, where it becomes activated, possible through changes in its association with other proteins or in its conformational status. In its activated state, CD47 then induces downstream signaling events, which result in an enhanced rate of PMN migration. PI3-kinase, on the other hand, is involved in a negative regulation pathway which both limits translocation of CD47 to the cell surface and inhibits PMN migration. Thus, inhibition of PI3-kinase with wortmannin or LY294002 results in rapid increases in cell surface CD47 along with an increased rate of migration. The net effect of PI3-kinase inhibition is thus a partial reversal of the inhibition observed with anti-CD47 antibodies (Figure 5). Since many studies have shown that PI3-kinase is activated after PMN stimulation (39,53,54), our results suggest that this activation results in the delivery of a negative signal which counters positive regulatory pathways in the regulation of the rate of PMN migration. Protein tyrosine phosphorylation / de-phosphorylation, on the other hand, serves as a downstream signal which most likely occurs after fMLP stimulation and CD47 translocation. We hypothesize that fMLP-induced activation of CD47 causes a shift of specific signaling elements from a tyrosine phosphorylated to a tyrosine de-phosphorylated status. This in turn can enhance actin polymerization and cytoskeleton re-organization, that ultimately enhance PMN migration. Inhibition of CD47 function by antibodies would then inhibit this pathway resulting in a decreased rate of PMN migration. Under this scenario, inhibition of tyrosine kinase by genistein results in a state favoring tyrosine de-phosphorylation, which, in turn, enhances PMN migration and reverses anti-CD47 mediated inhibition of migration. While the proposed
mechanism explains our results, other possibilities are likely to exist. Further studies will serve to more clearly elucidate the intracellular signaling events through protein tyrosine phosphorylation / de-phosphorylation, and the define relationship of these tyrosine phosphorylated states with CD47 function, in the process of PMN migration. Studies are currently underway to identify CD47-responsive protein kinases / phosphatases and the downstream phosphoproteins that may serve to “fine tune” the acute inflammatory response. Understanding these mechanisms will shed new insights into cellular events that regulate PMN migration and may provide new ideas for anti-inflammatory agents.

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Abbreviations: IgV, immunoglobulin variable domain; PMN, polymorphonuclear leukocyte; MPO, myeloperoxidase; HBSS(-), hank’s balanced salt buffer devoid Ca²⁺ and Mg²⁺.
Figure legends

**Figure 1.** Schematic illustration of the time-course PMN transmigration assay. Epithelial cells were cultured to confluency on porous polycarbonate filters (transwell) as described in the methods. PMN were placed in the upper chamber of the transwell and induced to migrate into fMLP (1 µM for transepithelial migration and 0.1 µM for trans-filter migration) containing lower chamber. At 30 minute intervals after initiation of migration, transwells were moved into a new lower chamber containing the same concentration of chemoattractant. PMN that had migrated into the lower chamber were quantified by MPO assay.

**Figure 2.** CD47-mediated effects on the time-course of PMN transepithelial migration. A) A time-course assay of basolateral-to-apical PMN migration across T84 monolayers after application of 1x10⁶ PMN. As detailed in Figure 1, PMN that migrated into the lower chamber after each 30 minute time-point were quantified. In these experiments, 20 µg/ml of anti-CD47 antibodies C5D5 and B6H12.2, non-inhibitory binding antibody J3F.1 and inhibitory anti-CD11bCD18 antibody CBRM1/29 were included in the upper assay chamber. Data represents one of over ten experiments with three individual monolayers in each condition ± SD. Identical results were obtained using a range of concentrations of C5D5 or B6H12.2 from 1-100 µg/ml, or supplementation with additional antibodies at every 30 min during PMN migration assay (not shown). B) Results of transmigration at different time points in panel A plotted as a function of total migrated PMN. Thus, the migration at 90 minutes, for example, represents the sum of PMN in the lower chambers from the first, second and third 30 minute time periods. C) Effects of anti-CD47 mAbs PF2.1, PF3.1 and PF10.2 on PMN transmigration across T84 monolayers. Antibodies were used at 20 µg/ml.
Figure 3. Up-regulation of CD47 on the PMN cell surface by fMLP stimulation and after transmigration. A) Flow cytometric determination of PMN cell surface CD47 and CD11bCD18 after fMLP stimulation. 1x10^6 PMN were allowed to settle in 24-well tissue culture wells containing 1 ml HBSS followed by fMLP stimulation (50 nM, 37°C) for various times as indicated. To terminate stimulation, cells were washed with ice-cold HBSS followed by labeling with antibodies against CD47 (C5D5) and CD11bCD18 (CBRM 1/29) before FACS analysis as described in the methods. Mean fluorescence intensity (MFI) was determined relative to labeling observed with isotype-matched normal mouse IgG and presented as folds of increases above the basal level labeling of CD47 (basal MFI at 23.4) and CD11bCD18 (basal MFI at 63.5) before PMN were activated. B) Immunoprecipitation of cell-surface labeled CD47 after fMLP stimulation or transmigration. In the left panel, 5x10^6 PMN were stimulated with fMLP as described in Figure 3a. Cooled PMN were surface biotin labeled and lysed as detailed in the Methods. In the right panel, 1x10^7 PMN were subjected to trans-filter migration assays. After 1 h migration, PMN were collected from the upper chamber and the lower chamber followed by surface labeling with biotin and detergent lysis. Protein concentration was assayed by BCA protein assay reagent (Pierce). CD47 was then immunoprecipitated from cell lysates containing 200 µg of total protein using mAb PF3.1 conjugated Sepharose followed by SDS-PAGE and Western Blot. Cell surface CD47 was detected by probing blots with streptavidin-peroxidase followed by ECL. Total cellular CD47 was detected by probing with anti-CD47 antibody R12989 as detailed in the methods.
Figure 4. Localization of CD47 in unstimulated and fMLP stimulated PMN by subcellular fractionation. PMN (2x10^8) unstimulated, or stimulated with fMLP in petri dishes were lysed by nitrogen cavitation subjected to isopycnic sucrose gradient centrifugation and fractionated as detailed in the methods. In the top panels, the distribution of subcellular organelles and sucrose density is plotted for each fraction. Marker activities are plotted as a percentage of total recovered from all gradient fractions. As can be seen, plasma membrane is in fractions 11-17, secondary granules are in fractions 17-20 and primary granules are in fractions 20-22. The lower panels represent immunoblots of CD11bCD18 and CD47 from subcellular organelle-containing sucrose fractions (30 µl protein samples) in the panels above using polyclonal rabbit anti-CD11b (R7928A) and anti-CD47 mAb PF3.1.

Figure 5. Effects of PI3-kinase inhibition on PMN migration and CD47 expression. A) PMN (1x10^6) were pre-treated with 100nM wortmannin in HBSS for 15 min at room temperature followed by use in time-course basolateral-to-apical transepithelial migration assays as described in Figure 1. PMN migration into the lower chamber at various time periods was quantified by MPO assay. The data represents the results of six independent experiments with three-four monolayers per condition ± SD. B) FACS analysis PMN cell surface CD47 after inhibition of PI3-kinase with wortmannin (100 nM) and fMLP stimulation for the times as indicated. C) Immunoprecipitation of CD47 from cell surface biotinylated PMN after wortmannin treatment and fMLP stimulation demonstrating marked up-regulation of CD47 on PMN cell surfaces.

Figure 6. Tyrosine phosphorylation inhibition by genistein reverses anti-CD47 mediated delay of PMN migration. PMN (1x10^6) were induced to migrate across cell-free collagen-coated filters
as detailed in the methods. A) To inhibit tyrosine phosphorylation, PMN were pre-treated with 100 \( \mu \text{g/ml} \) genistein before initiation of transmigration in the presence or absence of CD47 mAb C5D5 (20 \( \mu \text{g/ml} \)) and compared to untreated PMN migration in the presence of same amount of C5D5. Control experiments were performed in the presence of the same amount of daidzein (genistein analog) or vehicle only (DMSO). Data represents the mean \( \pm \) SD of four independent experiments.  

B) Results of transmigration at different time points in panel A were plotted as a function of total migrated PMN as detailed for figure 2. C) Genistein reversed anti-CD47 mediated PMN migration delay in a dose-dependent manner. In this experiment, PMN were pre-treated with genistein at concentration of 10, 20, 40, 100, 150 and 200 \( \mu \text{g/ml} \) or correlated same amount of daidzein or vehicle. PMN transmigration assay was performed in the presence or in the absence of 20 \( \mu \text{g/ml} \) C5D5.

**Figure 7.** Blockade of T84 epithelial CD47 delayed PMN migration across the monolayers.

T84 epithelial cell monolayers that were cultured on permeable supports were pre-incubated with anti-CD47 antibody C5D5 or B6H12.2 (20 \( \mu \text{g/ml} \) of each) for 30 minutes followed by extensive washing with HBSS before use in PMN transmigration assays. To ensure no residual unbound antibody remained after washing, parallel control experiments were performed with monolayers that were pre-incubated with the inhibitory anti-CD11bCD18 mAb CBMR1/29. A non-inhibitory isotype matched binding mAb J3F.1 that reacts with human junction adhesion molecule was also used as a negative control. Data represents the mean \( \pm \) SD with three monolayers per condition of four independent experiments.
**Figure 8.** Transfection and expression of CD47 on CaCO2 intestinal epithelial cells. cDNA corresponding to the protein coding region of CD47 was amplified from T84 epithelial cells by RT-PCR and cloned into pCB6 vector. The DNA construct was transfected into CaCO2 epithelial cells and positive cells were selected with 1 mg/ml G418. **A**) Flow cytometric analysis of control (mock, vector only transfection) and CD47-transfected CaCO2 cells (CaCO2-CD47). Cells were elicited by treatment with 5 mM EDTA in PBS followed by FITC labeling with mAb C5D5 as detailed in the methods.  **B**) Confocal immunofluorescence microscopy (en face or x-y plane) of CD47 expression on CaCO2-CD47 cells and mock vector-only transfected controls. Monolayers were fixed in ethanol followed by staining with C5D5 and FITC goat anti-mouse IgG as previously described (12). Scale bars; 20μ.

**Figure 9.** CD47 expression on epithelial cells facilitates PMN transepithelial migration. CaCO2-CD47 or mock-transfected CaCO2 cells were cultured on permeable supports and used in PMN transmigration assays as detailed in the methods. To inhibit CD47 on CaCO2-CD47 monolayers, anti-CD47 antibody C5D5 or B6H12.2 (20 µg/ml of each) was added to monolayers 30 minutes prior to initiating transmigration assays. Monolayers were then washed free of antibody and PMN transmigration initiated. Data represents the mean ± SD with six monolayers per condition of three independent experiments.

**Figure 10.** A hypothetical model of CD47 linked signaling pathways in regulating PMN chemotaxis. fMLP stimulation causes a translocation of CD47 from the intracellular pool to PMN cell surface, where it is activated and delivers signal(s) to induce specific protein dephosphorylation which in turn positively regulate chemotaxis. Conversely, fMLP induced PI3-
kinase activation conveys negative signals which negatively regulate both CD47 translocation and PMN migration. On contrast, genistein inhibits protein tyrosine phosphorylation down stream of CD47 that either maximizes the dephosphorylation status or inhibits tyrosine phosphorylation-linked other negative signaling pathways, which in turn reverses anti-CD47 inhibition and enhances PMN migration.

References


Table 1. PMN association with T84 monolayers during transepithelial migration.
PMN migration across T84 monolayers (from basolateral to apical direction) was terminated at various time points and PMN within monolayers were quantified by MPO assay (n=6).

<table>
<thead>
<tr>
<th>Total migration time (min)</th>
<th>PMN associated with monolayers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Ab</td>
</tr>
<tr>
<td>30</td>
<td>47.7±3.5</td>
</tr>
<tr>
<td>60</td>
<td>36.4±2.0</td>
</tr>
<tr>
<td>90</td>
<td>12.1±4.9</td>
</tr>
<tr>
<td>120</td>
<td>6.6±4.4</td>
</tr>
<tr>
<td>150</td>
<td>5.2±1.3</td>
</tr>
</tbody>
</table>
Table 2. Detection of CD47 up-regulation on PMN surfaces after transmigration by flow cytometry. 1x10^6 PMN were induced to migrate across collagen-coated filters followed by termination at various time points. PMN in the upper chambers (non-migrated PMN) or those that migrated into the lower chambers (migrated PMN) were assayed for cell surface CD47 by flow cytometry as described in the methods. Results are depicted as mean fluorescence intensity (MFI). Data represents results of three independent experiments.

<table>
<thead>
<tr>
<th>Migration time (min)</th>
<th>Mean fluorescence intensity (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMN in the upper chamber</td>
</tr>
<tr>
<td>0</td>
<td>25.0</td>
</tr>
<tr>
<td>5</td>
<td>29.7</td>
</tr>
<tr>
<td>30</td>
<td>32.7</td>
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<tr>
<td>60</td>
<td>30.6</td>
</tr>
<tr>
<td>90</td>
<td>33.4</td>
</tr>
<tr>
<td>120</td>
<td>38.9</td>
</tr>
<tr>
<td>180</td>
<td>37.4</td>
</tr>
</tbody>
</table>
Fig 1
PMN migration (% of applied cells)

0 10 20 30 40 50 60 70

1st 30'
2nd 30'
3rd 30'
4th 30'
5th 30'
6th 30'

No Ab
J3F-1
(anti-CD47)
C5D5
(anti-CD47)
B6H12.2
(anti-CD47)
CBRM1/29
(inhibitory ctl)
HBSS
B)

Fig2b

- No Ab
- J3F.1 (neg.ctl)
- C5D5 (20µg/ml)
- B6H12.2 (20µg/ml)
- CBMR1/29 (inhibitory ctl)
- HBSS

Migration Time

Total migrated PMN (% of applied)
Migration Time

Total migrated PMN (% of applied)

Fig2c

C)
Cell surface expression (observed MFI / basal MFI)

- CD47
- CD11bCD18

fMLP stimulation (min)

0 30 60 90 120

Cell surface CD47

A) B)

63kD
47.5kD

fMLP stimulation

- 10 min
- 60 min
- before migration
- after migration

WB: avidin
WB: R12989

Fig 3
**Fig 4**

**Unstimulated PMN**

- Primary granules (myeloperoxidase activity)
- Plasma membrane (alkaline phosphatase activity)
- Secondary granules (lactoferrin)
- Sucrose density

**Stimulated PMN**

- Primary granules (myeloperoxidase activity)
- Plasma membrane (alkaline phosphatase activity)
- Secondary granules (lactoferrin)
- Sucrose density

Blot for CD11b

Blot for CD47
A) Trans-epithelial migration

Migrated PMN (x10^4)

- no treatment
- +wortmannin
- +C5D5
- +C5D5 and wortmannin

1st 30' 2nd 30' 3rd 30' 4th 30'

[Graph showing the migration of PMNs under different treatments and time points]
fMLP stimulation (min)

CD47 on PMN surface (MFI)

- No treatment
- Pre-treated with wortmannin

B)

C)

fMLP stimulation (min)

- wortmannin
+ wortmannin

CD47

CD47
Fig6ab

A)

B)

Vehicle only
+CD5
+Genistein
+CD5 and Genistein
+Daidzein
+CD5 and Daidzein

1st 30'
2nd 30'
3rd 30'
4th 30'

Migrated PMN (×10^4)

0
20
40
60
80
100

Time (hr)

0.0 0.5 1.0 1.5 2.0

Total migrated PMN (×10^4)

0
20
40
60
80
100

Vehicle only
+CD5 (20µg/ml)
+Genistein (100µg/ml)
+CD5 (20µg/ml), Genistein (100µg/ml)
+Daidzein (100µg/ml)
+CD5 (20µg/ml), Daidzein (100µg/ml)
Concentration of Genistein or Daidzein (µg/ml)

Migrated PMN (x10^4)

Vehicle only (at appropriate dilution)
+Genistein
+Daidzein
+Genistein and C5D5
+Daidzein and C5D5

Fig 6c
A) 

B) 

Fig 8

Mock transfec.

CaCO2-CD47
Transepithelial migration

Migrated PMN (x10^-4)

- 1st hr
- 2nd hr

CaCO2

Mock ctl.

CaCO2

No Ab

+CD5

+B6H12.2

fig9
fMLP → receptor → Chemotaxis → (CD47 intracellular pool)

CD47 (resting) → * → fMLP

CD47 (active) → * → P → PI3kinase

*: ? Homotypic polymerization
  ? Association with other proteins
  ? Structural changes
  ? Thr/Ser/Tyr phosphorylation
The role of CD47 in neutrophil transmigration: Increased rate of migration correlates with increased cell surface expression of CD47
Yuan Liu, Didier Merlin, Stephanie L. Burst, Mildred Pochet, James L. Madara and Charles A. Parkos

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