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Signal-Regulatory Protein α-CD47 Interactions Are Required for the Transmigration of Monocytes Across Cerebral Endothelium

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Monocyte infiltration into inflamed tissue requires their initial arrest onto the endothelial cells (ECs), followed by firm adhesion and subsequent transmigration. Although several pairs of adhesion molecules have been shown to play a role in the initial adhesion of monocytes to ECs, the mechanism of transendothelial migration is poorly defined. In this study, we have investigated the role of signal-regulatory protein (SIRP)α-CD47 interactions in monocyte transmigration across brain ECs. CD47 expression was observed in vivo on cerebral endothelium of both control animals and animals suffering from experimental allergic encephalomyelitis. To investigate whether SIRPα-CD47 interactions are instrumental in the trafficking of monocytes across cerebral EC monolayers, in vitro assays were conducted in which the migration of monocytes, but not adhesion, was found to be effectively diminished by blocking SIRPα and CD47 on monocytes and ECs, respectively. In this process, SIRPα was found to interact solely with its counterligand CD47 on ECs. Overexpression of the CD47 molecule on brain ECs significantly enhanced monocytic transmigration, but did not affect adhesion. SIRPα-CD47-mediated transendothelial migration involved Gi protein activity, a known signaling component of CD47. Finally, cross-linking of CD47 on brain ECs induced cytoskeletal reorganization of the endothelium, a process that was Gi protein independent. These data provide the first evidence that the interaction of CD47 with its monocytic counterligand SIRPα is of importance in the final step of monocyte trafficking into the brain, a critical event in the development of neuroinflammatory diseases. The Journal of Immunology, 2002, 168: 5832–5839.
receptors (23, 25, 26). Recently, the ligation of SIRPα on macrophages by CD47 on target cells has been shown to prevent their phagocytosis (27, 28).

Because the high expression of SIRPα is merely restricted to monocytic cells, we suggest that SIRPα interacting with CD47 may contribute to the recruitment of monocytes into tissue during inflammatory diseases. In this study, we report that SIRPα is indeed involved in the transmigration of monocytes across brain endothelium through interaction with its counterligand CD47, a molecule that upon engagement induces signaling events in the endothelium.

Materials and Methods

Materials

Ham’s F12 medium, RPMI 1640 medium, endothelial serum-free medium (with 1-glutamine), penicillin-streptomycin, t-glutamine, FCS, HBSS, and trypsin/EDTA were all obtained from Life Technologies (Rockville, MD). Pertussis toxin (PTX) in 50% in glycerol, collagenase type I, collagen type 3, laminin, and gelatin were purchased from Sigma (Zwijndrecht, The Netherlands). Rhodamine-phalloidin and 2,7 bis-(2-carboxyethyl)-5(6) and 6-carboxyfluorescein acetoxymethyl were obtained from Molecular Probes (Eugene, OR). IFN-γ and IL-1β were a kind gift of U-Cytex (Utrecht, The Netherlands) and Glaxo Wellcome (Basel, Switzerland), respectively. The mAb ED9 (anti-SIRPα; IgG1 isotype) was generated in our laboratory and is commercially available from Serotec (Oxford, U.K.). OX-8, OX-19, and OX-52 mAbs were obtained from Serotec. OX101 (anti-CD47) IgG and OX41 (anti-SIRPα; IgG2a) were purified from hybridoma supernatants by protein A affinity chromatography (20, 24).

SIRPα-Fc protein generation

SIRPα-Fc proteins were constructed and purified from Chinese hamster ovary cell supernatants, as previously described (29).

Animals and induction of EAE

Acute experimental allergic encephalomyelitis (EAE) was induced in 8- to 11-wk-old male Lewis rats (200 g) obtained from Harlan (Zeist, The Netherlands), as described before (30). Rats were injected s.c. in hind footpads with 20 μg synthetic myelin basic protein 63–88 peptide, 500 μg Mycobacterium tuberculosis type 37H Ra (Difco, Detroit, MI), and 50 μl CFA (Difco) supplemented with PBS to reach a volume of 100 μl. Rats were examined daily (weight and clinical disease), and neurological aberrations were graded from 1 to 5, as described before (30). Clinical disease in EAE animals was apparent between days 10 and 19 after immunization with a maximum clinical score between days 14 and 15. Incidence of EAE was determined as the interphase on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient. Monocytes were purified from PBMCs by negative selection using FACS sorting (FACStar Plus, BD Biosciences, San Jose, CA) on the basis of EGFP expression. Cell surface expression of CD47 determined by FACS was used as a negative control (31, 32) and was maintained in RPMI 1640 medium supplemented with 10% FCS (heat inactivated), 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Construction of the retroviral construct LZRS-rCD47-IRES-EGFP and retroviral transduction of rat brain ECs

The cDNA of rat CD47 containing a V-G substitution at position 264 in a murine Ig-coated magnetic beads (PerkinElmer, Wellesley, MA), resulting as the interphase on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) (20, 24). The monocytic cell line NR8383 was obtained from the American Type Culture Collection (Manassas, VA). These nonadherent cells were cultured in RPMI 1640 medium supplemented with 10% FCS (heat inactivated), 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Flow cytometry analysis

For flow cytometric analysis, normal GP8.3 cells were detached by incubation with 1 mg/ml collagenase (type I) in PBS/0.1% BSA for 5 min in the incubator and harvested with a needle (0.8 × 340 mm). Cells were centrifuged and resuspended in PBS/0.1% BSA. The monocytic cell line and brain EC line GP8.3 were incubated with 5 μg/ml OX101, ED9, and OX41, or 25 μg/ml SIRP-Fc in 100 μl PBS in a 96-well plate. CD47 and mock-transduced GP8.3 ECs were also stained using OX101 and were screened for their SIRP-Fc-binding properties. After an incubation of 30 min at 4°C, cells were washed three times with PBS. Ab or protein binding to the cells was detected after incubating with fluorescein-labeled conjugates (Jackson Immunoresearch Laboratories, West Grove, PA) or rat anti-mouse IgG (Fab′/f(ab′)2)PE (DAKO) or anti-human IgG-biotin and streptavidin-PE (Sigma) in PBS/0.1% BSA for 30 min at 4°C. For blocking of Fc receptor binding, a preincubation was performed with 20 μg/ml anti-CD47 (OX101). Binding was detected by using FACScan flow cytometry (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Monocyte isolation

Female Wistar rats were obtained from Harlan and were kept under standard laboratory conditions with food and water ad libitum. Animals were used at a body weight of 250–350 g. Monocytes were isolated after perfusion of the rat, which was performed essentially according to Scriba et al. (33). Briefly, after anesthesia, the thorax was opened and two cannules (16G and 20G) were inserted in the left and right ventricle, respectively. The vasculature of the rat was perfused with 1 L prewarmed RPMI 1640 medium supplemented with 1% BSA and 20 mM HEPES. PBMCs were isolated as the interphase on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient. Monocytes were purified from PBMCs by negative selection using OX-8, OX-42, OX-52, and OX-58 mAbs and goat-anti-mouse IgG-coated magnetic beads (PerkinElmer, Wellesley, MA), resulting as the interphase on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) (20, 24).

Cell culture

The well-characterized immortalized Lewis rat brain EC line GP8.3 served as a model for the blood-brain barrier in vitro (31, 32) and was maintained in Ham’s F-12 medium supplemented with 20% FCS (heat inactivated), 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

The migratory capacity of monocytes to cross a monolayer of brain ECs was assayed using time-lapse videomicroscopy, as described previously (32). Briefly, monocytes (5 × 10⁵/ml) were added to 96-well plates containing nonstimulated or stimulated (48 h with 100 ng/ml IL-1 and 200 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin).
U/ml IFN-γ) brain EC monolayers. Before migration, cells were preincubated for 30 min with 10 μg/ml mAb OX101 (anti-CD47) and the anti-SIRPα mAbs ED9 or OX41. Monocyte migration was conducted in presence of specific Abs, as mentioned. Migration was also performed in the presence of 25 μg/ml SIRPα-Fc protein and 25 μg/ml ED9 Fab.

Data are expressed as the mean and SEM of at least 12 individual wells, and significant differences between groups were determined by two-way ANOVA.

To block intracellular signaling via Gi/Go proteins, brain ECs were pretreated for 2 h with PTX (200 ng/ml), after which cells were extensively washed and kept in culture medium for 2 h before the migration assay.

**Monocyte adhesion assay**

The involvement of SIRPα and CD47 in monocyte adhesion to monolayers of control or cytokine-treated brain ECs was also determined, as described previously (32). Freshly isolated monocytes or NR8383 cells were fluorescently labeled with 1 μM 2',7'-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxymethyl for 15 min at 37°C in F-12/2% BSA and were subsequently washed with medium. To block CD47 and SIRPα, cells were incubated for 30 min at 4°C with the anti-CD47 mAb OX101 and the anti-SIRPα mAbs ED9 or OX41, or with isotype-matched IgGs, all at 10 μg/ml. Activation of Gi/Go proteins was blocked by pretreatment of the brain ECs with PTX, as described above, after which cells were used in the adhesion assay.

Before the adhesion experiment, GP8.3 monolayers were washed twice with prewarmed F-12 medium supplemented with 0.1% BSA. Subsequently, fluorescently labeled cells (1 × 10⁶ cells/ml) were added to both monolayers and stimulated (for 48 h with 200 U/ml IFN-γ and 100 ng/ml IL-1) monolayers and were allowed to adhere for 30 min at 37°C and 5% CO₂ in the absence or presence of blocking Abs or Fab. After the incubation, nonadherent cells were removed by gently washing the monolayers with prewarmed F-12/0.1% BSA. The number of adhered monocytes was determined, as described previously (32). Freshly isolated monocytes or NR8383 cells were fluorescently labeled with 1 μM 2',7'-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxymethyl for 15 min at 37°C in F-12/2% BSA and were subsequently washed with medium. To block CD47 and SIRPα, cells were incubated for 30 min at 4°C with the anti-CD47 mAb OX101 and the anti-SIRPα mAbs ED9 or OX41, or with isotype-matched IgGs, all at 10 μg/ml. Activation of Gi/Go proteins was blocked by pretreatment of the brain ECs with PTX, as described above, after which cells were used in the adhesion assay.

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**Cross-linking studies and F-actin localization**

Before cross-linking experiments, monolayers of cerebral ECs were washed three times with HBSS and then left for 48 h at 37°C, 5% CO₂ in serum-free endothelial-specific medium. Endothelial monolayers were incubated for 30 min at 37°C, 5% CO₂ in either the absence or presence of 10 μg/ml OX101 (anti-CD47 mAb) in endothelial-specific medium. Cells were washed again three times with HBSS and then incubated with serum-free medium in the presence or absence of 10 μg/ml goat anti-mouse for 30 and 60 min, respectively. Control conditions with only the OX101 or goat anti-mouse were incubated for 30 min. After incubation, cells were washed three times with HBSS and fixated in 4% paraformaldehyde in PBS for 15 min, followed by gentle washing with PBS containing 1% BSA. Cells were subsequently permeabilized in 0.25% Triton X-100/PBS for 5 min and blocked for 30 min with PBS containing 10% FCS. Subsequently, cells were incubated with 0.1 μg/ml rhodamine-phalloidin for 1 h and then exhaustively washed in PBS containing 1% BSA. Cells were mounted in Fluostab embedding medium (ICN Biomedicals, Costa Mesa, CA) and viewed on an Eclipse E800 (Nikon, Badhoevedorp, The Netherlands) fluorescence microscope.

**Results**

Monocyte migration into the CNS is a crucial event in the development of neuroinflammatory diseases. The potential role of the monocyct molecule SIRPα and its counterligand CD47 in this process was studied.

**Expression of CD47 and SIRPα on spinal cords of EAE animals**

The neuroinflammatory disease EAE, the animal model for multiple sclerosis, was induced in Lewis rats. At the peak of the disease (i.e., day 15 after induction of EAE), animals were sacrificed, lumbar spinal cords were dissected, and cryostat sections were stained with mAbs directed against SIRPα and CD47, ED9 and OX101, respectively. Lumbar spinal sections of control animals were also stained for the expression of CD47 and SIRPα. Sections only incubated with the peroxidase-conjugated secondary rabbit anti-mouse IgG showed no immunoreactivity in control and EAE animals (data not shown).

CD47 was predominantly expressed at the level of the CNS capillaries, and especially on the CNS ECs, in both control and EAE animals (Fig. 1). No clear difference in cerebrovascular CD47 expression between EAE animals and control animals could be detected. Additionally, in EAE animals CD47 was also expressed on infiltrated cells in the perivascular cuffs. SIRPα was found to be clearly expressed in perivascular infiltrates, but was not detected on capillaries of either control or EAE animals (Fig. 1). Additionally, SIRPα was also expressed on neurones, as described previously (data not shown) (24).
Cellular expression of CD47 and SIRPα and CD47-dependent binding properties of SIRPα

To quantify expression levels of SIRPα, and CD47 on the brain ECs and monocytes in vitro, FACS analysis was performed. The monocytic cell line NR8383 expressed high levels of SIRPα, and CD47 compared with their conjugate control (Fig. 2A). Brain ECs express high levels of CD47 and little or no SIRPα. Upon stimulation with the proinflammatory cytokines IL-1β and IFN-γ, no significant increase in the expression of CD47 and SIRPα could be observed (Fig. 2A).

The involvement of CD47 in SIRPα binding was determined using SIRPα-Fc fusion proteins (Fig. 2B). SIRPα-Fc proteins were capable of binding to brain ECs, and the binding increased slightly upon stimulation of the cerebral ECs with IL-1β and IFN-γ, consistent with the constitutive CD47 expression on cytokine-stimulated brain ECs. Protein binding could be prevented completely by preincubation with the anti-CD47 mAb OX101, indicating SIRPα is the major ligand for CD47 expressed on brain ECs (Fig. 2B).

CD47-SIRPα mediate monocyte transendothelial migration

Monocyte trafficking across monolayers of brain EC was assessed by time-lapse video microscopy after 4 h of migration (Fig. 3). To identify whether CD47 and SIRPα contribute to monocyte transendothelial migration, the anti-CD47 mAb OX101 or anti-SIRPα mAb ED9 was used. In the presence of OX101, the migration of freshly isolated monocytes was reduced by 46 ± 6.6%, indicating that CD47 is involved in the migration process (Fig. 3A). Moreover, in the presence of anti-SIRPα mAb ED9, the number of migrated cells was reduced by 63 ± 9.2%, indicating that SIRPα also has a part in monocyte migration. The anti-SIRPα mAb ED9, recognizing a different epitope than ED9 (24), had no significant blocking effect on the migration process. In the presence of mAb ED9 was used. In the presence of OX101, the migration of freshly isolated monocytes was reduced by 46 ± 6.6%, indicating that CD47 is involved in the migration process (Fig. 3A). Moreover, in the presence of anti-SIRPα mAb ED9, the number of migrated cells was reduced by 63 ± 9.2%, indicating that SIRPα also has a part in monocyte migration. The anti-SIRPα mAb ED9, recognizing a different epitope than ED9 (24), had no significant blocking effect on the migration process. In the presence of
SIRPα-CD47 ARE REQUIRED FOR MONOCYTE MIGRATION

SIRPα-Fc protein (25 μg/ml) and ED9 Fab (25 μg/ml), transendothelial migration of freshly isolated monocytes was reduced by 33 ± 2.6% and by 39 ± 6.5%, respectively. Monocyte migration was not affected in the presence of isotype-matched control Abs (data not shown).

Additional experiments were carried with the rat monocytic cell line NR8383 (serving as an in vitro model for freshly isolated monocytes), and their migration profile was compared with that of freshly isolated monocytes. Transmigration of NR8383 monocytes across brain endothelial monolayers treated with the proinflammatory cytokines IL-1β and IFN-γ increased 2-fold (Fig. 3B). Anti-CD47 (OX101) and anti-SIRPα (ED9) inhibited the migration of monocytes across both control and cytokine-activated brain ECs to a similar degree, which was comparable with that of freshly isolated monocytes. Again, in the presence of anti-SIRPα mAb OX41, no significant reduction could be observed. Migration of freshly isolated rat monocytes and NR8383 cells in the presence of both the mAbs OX101 and ED9 revealed no further decrease in the number of migrating cells, and the percentage of inhibition was comparable with migration in the presence of ED9 alone (data not shown).

To further demonstrate the importance of CD47 in the transendothelial migration, we transfected the brain EC line GP8.3 with the LZR5-IRESGFP retroviral vector containing the rat CD47 construct, as analyzed by their EGFP fluorescence. After FACS sorting, staining of these cells with OX101 resulted in a 100-fold overexpression of the CD47 as compared with GP8.3 cells transduced with the mock LZR5-IRESCGF retroviral vector (geomean values; data not shown). The transendothelial migration of NR8383 cells across CD47-overexpressing endothelial monolayers increased 1.8-fold, whereas monocyte migration across mock-transduced GP8 cells was at similar levels compared with nontransduced GP8.3 cells (Fig. 3C). Monocyte passage over monolayers of GP8.3 cells ectotropically overexpressing CD47 could be blocked in the presence of OX101 (10 μg/ml) and by ED9 (10 μg/ml) (Fig. 3C).

Monocyte adhesion to cerebral EC is not influenced by SIRPα and CD47
Adhesion assays were conducted to determine whether impaired monocyte migration observed after the intervention of the CD47 interaction with SIRPα was accompanied by a different adhesion profile. Brain ECs and freshly isolated monocytes were pretreated with blocking mAbs directed against SIRPα and CD47, respectively. As shown in Fig. 4, there were no significant effects on monocyte adhesion to brain ECs, with the possible exception of the anti-SIRPα mAb ED9 that gave a slight inhibition of monocyte adhesion to cytokine-activated endothelium. In agreement, adhesion of the monocytic NR8383 cells to control or cytokine-stimulated monolayers of cerebral ECs was not significantly affected by mAbs directed against SIRPα and CD47 (data not shown). Furthermore, no induction of monocyte adhesion to the CD47-overexpressing nor the mock-transduced GP8 cells was found (data not shown).

CD47-SIRPα-mediated monocyte transendothelial migration depends on Gi-protein activity
Because CD47 is known to exert biological effects via Gi proteins (18), monocyte migration was performed after blocking Gi protein-dependent signaling by pretreatment of the cerebral ECs with PTX. Migration of freshly isolated rat monocytes (Fig. 5A) as well as NR8383 cells (data not shown) across cerebral ECs was significantly reduced upon treatment of the ECs with PTX. Similarly, monocyte transmigration was also inhibited in the CD47-overexp-
Monocyte adhesion to the endothelium was not influenced by the pretreatment of the cerebral ECs with PTX (Fig. 5B), indicating that only the transmigration of monocytes across CNS-ECs requires the active participation of Gi proteins in brain ECs.

**CD47 engagement induces cytoskeletal reorganization via a Gi protein-independent mechanism**

A number of studies indicate that adhesion molecules may transduce signals that lead to cytoskeletal reorganization in the brain EC, hence facilitating cellular trafficking (36, 37). To investigate whether the activation of CD47 induces such effects, the cross-linking effects of CD47 in brain ECs were determined. A clear rearrangement of F-actin leading to the formation of stress fibers was observed after the cross-linking of CD47 with the secondary Ab (Fig. 6). Within 30 min 50% of the cells, and after 60 min >80% of the brain EC, revealed the formation of stress fibers induced by CD47 activation. Incubation of the brain EC with the anti-CD47 mAb or secondary Ab alone showed no significant change in F-actin staining (Fig. 6). The brain ECs were also subjected to activation of CD47 by 25 μg/ml SIRPα-Fc protein after cross-linking with the secondary anti-human Fc Ab. Ligation of CD47 in this way also induces the formation of stress fibers in 50% of the cells after 60 min (data not shown).

To investigate whether these effects required activity of Gi proteins, similar cross-linking studies were performed on brain ECs that were pretreated with PTX. No inhibition on the CD47-induced cytoskeletal changes in PTX-pretreated cells could be observed (Fig. 6). These findings suggest that CD47 is capable of inducing cytoskeletal changes in the brain EC, which appear to be independent of Gi protein activation.

**Discussion**

Emigration of monocytes from the blood into the CNS is a key event in the development of neuroinflammatory lesions, such as observed during the chronic demyelinating disease multiple sclerosis. The last step in the diapedesis of monocytes requires the involvement of members of the Ig family. To date, not all molecules that are critical in the migration of monocytes into the brain are identified. In this study, we demonstrate that the interaction of the protein SIRPα expressed on myeloid cells and the endothelial CD47 contributes to the transmigration of monocytes across monolayers of brain EC, but that this interaction is not contributing to monocyte adhesion to ECs.

In our study, CD47 appeared to be highly expressed on brain ECs, and its expression is not significantly increased upon treatment of the cells with proinflammatory cytokines, as observed earlier (14). Moreover, our observations indicate that the CD47 molecule is highly and constitutively expressed in vivo on CNS capillary endothelium of control animals and animals suffering from EAE.

Blocking the interaction between the CD47 molecule and its monocytic counterligand SIRPα in vitro leads to a significantly diminished monocyte migration across the blood-brain barrier ECs, whereas their firm adhesion was not affected in our assays. This suggests that CD47-SIRPα interactions are only required in the final postadhesion step of the migration process, which is consistent with our observations that CD47 is localized close to the tight junctions in these brain EC monolayers (data not shown). Also, overexpression of CD47 in the brain EC line significantly enhanced monocyte transmigration, without affecting monocytic adhesion to these cells. CD47 has also been shown to contribute to the spontaneous migration of monocytes across the alveolar epithelial barrier (15, 16). However, in this study, no role for the counterreceptor of CD47, the myeloid protein SIRPα, has been investigated. A recent study suggested that the interaction between CD47 expressed on T cells and SIRPα expressed on HUVECs can also mediate the constitutive arrest of T lymphocytes on inflamed endothelium by up-regulation of α4β1 integrins on T cells (38). Although the monocytes used in our study also express CD47, preincubation of monocytes with the blocking anti-CD47 Ab could not interfere with their migration, suggesting that such an inverted event may not be involved in monocyte transmigration. The role of SIRPα-CD47 interaction in this postadhesion event is unique among that of other adhesion molecules involved in monocyte

**FIGURE 6.** CD47 activation induces stress fiber formation in cerebral ECs. Brain ECs were incubated with the mAb OX101, directed against CD47, for 30 min, and cross-linked for 60 min with the secondary Ab (C). Control cells were incubated either with only the primary Ab OX101 (B) or without (A: control). PTX treatment (200 ng/ml for 2 h at 37°C) had no inhibitory effect on stress fiber formation (D).
transmigration across brain endothelium. Previous studies in our laboratory have shown that adhesion molecules such as very late Ag-4/VCAM-1 and complement receptor type 3/ICAM-1 mediate the firm adhesion to as well as the transmigration of monocytes across brain endothelium (41).

CD47 is a widely expressed molecule that can mediate a variety of actions by triggering different transduction pathways (for review, see Ref. 12), among which are the Gi proteins. Indeed, we showed that only the final step in the CD47-SIRPα transendothelial migration process was shown to be fully dependent on Gi/Go protein activity. Further analysis of the CD47-mediated activation of Gi proteins and the role of this in the transmigration process will be necessary.

The transendothelial migration of monocytes likewise requires the active participation of the brain ECs to facilitate monocyte passage (32). Recently, evidence has emerged that adhesion molecules can mediate various intracellular signal transduction pathways (32). Until recently, the functional participation of the brain ECs to facilitate monocyte transmigration across brain endothelium was shown to be fully dependent on Gi/Go proteins and the role of this in the transmigration process will be necessary.

References


