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Osteopontin Mediates Macrophage Chemotaxis via α_4 and α_9 Integrins and Survival via the α_4 Integrin

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ABSTRACT

Osteopontin (OPN) is highly expressed by macrophages and plays a key role in the pathology of several chronic inflammatory diseases including atherosclerosis and the foreign body reaction. However, the molecular mechanism behind OPN regulation of macrophage functions is not well understood. OPN is a secreted molecule and interacts with several integrins via two domains: the RGD sequence binding to α_v-containing integrins, and the SLAYGLR sequence binding to $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_9\beta_1$ integrins. Here we determined the role of OPN in macrophage survival, chemotaxis, and activation state. For survival studies, OPN treated-bone marrow derived macrophages (BMDMs) were challenged with growth factor withdrawal and neutralizing integrin antibodies. We found that survival in BMDMs is mediated primarily through the α_4 integrin. In chemotaxis studies, we observed that migration to OPN was blocked by neutralizing α_4 and α_9 integrin antibodies. Further, OPN did not affect macrophage activation as measured by IL-12 production. Finally, the relative contributions of the RGD and the SLAYGLR functional domains of OPN to leukocyte recruitment were evaluated in an in vivo model. We generated chimeric mice expressing mutated forms of OPN in myeloid-derived leukocytes, and found that the SLAYGLR functional domain of OPN, but not the RGD, mediates macrophage accumulation in response to thioglycollate-elicited peritonitis. Collectively, these data indicate that α_4 and α_9 integrins interacting with OPN via the SLAYGLR domain play a key role in macrophage biology by regulating migration, survival, and accumulation. J. Cell. Biochem. 114: 1194–1202, 2013.

KEY WORDS: INFLAMMATION; APOPTOSIS; MIGRATION; MACROPHAGE ACTIVATION

OPN is a secreted glycoprotein that mediates diverse biological functions. While OPN was originally isolated from bone, it was later found to have a wider distribution. As a matricellular protein, OPN exists both as a component of the extracellular matrix and as a soluble cytokine. OPN is involved in normal physiological processes and is thought to regulate biomineralization in bone tissue, and to reduce growth and aggregation of calcium crystals in epithelial tissues [Wesson et al., 2003]. Importantly, OPN also plays a role in the pathogenesis of several diseases, including cancer, autoimmune disorders, and chronic inflammatory diseases.

The pleiotropic nature of OPN may be due to its ability to interact with a variety of cell types. OPN interacts with cells via two major binding domains that are conserved among species. Through the adhesive RGD domain, OPN interacts with $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_8\beta_1$, and $\alpha_5\beta_1$ integrins [Hu et al., 1995; Liaw et al., 1995; Denda et al., 1998; Yokosaki et al., 2005]. OPN also contains a SLAYGLR (SVVYGLR in human OPN) domain that mediates interactions with $\alpha_9\beta_1$, $\alpha_4\beta_1$, and $\alpha_4\beta_7$ integrins [Yokosaki et al., 1999; Green et al., 2001; Ito et al., 2009]. Additionally, OPN has been reported to interact with the CD44 receptor [Weber et al., 1996]. OPN is subject to extensive post-translational modification that can alter the bioactivity of the molecule. Post-translational modifications of OPN include phosphorylation, glycosylation, and proteolysis by thrombin, matrix metalloproteinases, plasmin, and cathepsin D [Agnihotri et al., 2001; Christensen et al., 2010].

While OPN is not expressed in circulating monocytes, its expression is strikingly upregulated during monocyte to macro-

Abbreviations used: BMDM, bone marrow derived macrophage; LEAF, low endotoxin, azide-free; LPS, lipopolysaccharide; OPN, osteopontin; PDL, poly-D-lysine; WT, wild type.

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phage differentiation [Krause et al., 1996]. In macrophages, OPN expression is induced by several pro-inflammatory cytokines including TNF-α, IL-1β, IFN-γ, and IL-6, as well as other factors including angiotensin-II, oxidized LDL, and phorbol-ester [Bruemmer et al., 2003; Nakamachi et al., 2007; Ogawa et al., 2005]. On the other hand, OPN expression is suppressed by Liver X receptor and peroxisome proliferator-activated receptor α antagonists [Nakamachi et al., 2007; Ogawa et al., 2005]. Studies in our labs and others have established that OPN is a potent macrophage chemoattractant [Giachelli et al., 1998; Panzer et al., 2001; Bruemmer et al., 2003]. In vivo functional inhibition of OPN and genetic ablation of OPN in mice greatly impair macrophage recruitment in several models of acute inflammation including a model of kidney obstruction [Ophascharoensuk et al., 1999], and a thioglycollate-induced peritonitis model [Bruemmer et al., 2003].

OPN also modifies chronic inflammatory responses. Reduced macrophage accumulation has been observed in OPN-null mice challenged with chronic inflammatory conditions including atherosclerosis [Matsui et al., 2003], delayed-type hypersensitivity [Yu et al., 1998], granulomatous disease [Nau et al., 1999], and biomaterial implantation [Tsai et al., 2005]. These studies suggest that OPN may play an important role in promoting migration and retention of macrophages at sites of inflammation. In vitro, peritoneal-derived OPN-null macrophages exhibit reduced basal migration and impaired migration towards MCP-1 [Bruemmer et al., 2003]. It is thus well-established that OPN regulates macrophage movement and accumulation, however to date the cell receptor(s) mediating these functions have not been defined.

Here we show that the interaction between the integrin α_4 and OPN plays a key functional role in macrophage biology regulating migration and survival. Furthermore, in vivo, the integrins α_4/α_9 binding-SLAYGLR domain of OPN mediates macrophage accumulation in sterile peritonitis. Finally, we have established that OPN has no effect on macrophage activation.

MATERIALS AND METHODS

MATERIALS

Unless otherwise noted, recombinant murine mammalian-derived OPN (rmOPN, R&D Systems, Minneapolis, MN) was used for experiments.

CELL CULTURE

OPN-null mice were generated on a C57Bl/6 background as previously described [Rajachar et al., 2008]. For the generation of primary bone marrow derived macrophages, femora were harvested from OPN-null ($OPN^{-/-}$) and wild type (WT) mice. Bone marrow was flushed from the femora using RPMI 1640 media (Gibco, Carlsbad, CA). Bone marrow cells were thoroughly dispersed and were expanded in macrophage expansion media (50% L929 cell-conditioned medium as a source of M-CSF, 30% RPMI, and 20% fetal bovine serum (FBS)). Cells were fed on Day 4 and mature macrophages were harvested on Day 7 for assays. Macrophage phenotype was confirmed by the expression of F4/80 and low expression of CD11c, thus indicating the absence of dendritic cells. All primary macrophage preparations were carried out following the appropriate University of Washington Animal Care Use protocols.

APOPTOSIS ASSAYS

Bone marrow derived macrophages (BMDMs) were pre-incubated with 10 μg/ml anti-α_V, anti-α_4, or isotype control antibodies (BioLegend, San Diego, CA) for 15 min at room temperature. BMDMs were then plated on poly-D-lysine (PDL) coated or recombinant mammalian murine OPN-coated 8-well Permanox chamber slides (Nunc, Rockester, NY) in serum-free RPMI and incubated overnight. The next day, percent apoptosis was determined by nuclear fragmentation. Cells were live stained with 4 μg/ml of Hoechst 33258, fixed in 10% formalin, and visualized under UV light illumination. Condensed or fragmented (i.e., apoptotic nuclei, Supplemental Fig. 1) were calculated as percentage of total nuclei as described and validated previously [Han et al., 1997; Scatena et al., 1998; Imanishi et al., 2002a].

TRANSWELL MIGRATION ASSAY

Migration assays were performed with WT BMDMs using 24-well Transwell inserts with 8 μm pore size polycarbonate membranes (6.5 mm insert, Costar, Corning, Lowell, MA). 2×10^5 BMDMs in RPMI-1640 medium containing 0.4% FBS were added to the upper chamber and incubated for 1 h at 37°C to allow cells to attach. For blocking experiments BMDMs were pre-incubated with neutralizing antibody to integrin α_4 (low endotoxin, azide-free (LEAF) purified anti-mouse CD49d antibody (clone R1-2), BioLegend), integrin α_V (LEAF purified anti-mouse CD51 antibody (clone RMV-7), integrin α_9 (clone 55A2C, a kind gift from Toshimistu Uede, Hokkaido University, Japan [Kanayama et al., 2009]), non-immune rat IgG isotype control (BioLegend), non-immune Armenian Hamster IgG isotype control (BioLegend), cyclo(-RGDfK) peptide (Anaspec, San Jose, CA), or cyclo(-RADfK) peptide (Anaspec) for 15 min at room temperature prior to plating. Dose response experiments were conducted to determine the concentration of neutralizing antibody required to block migration to OPN. Chemoattractant media containing macrophage-colony stimulating factor (M-CSF, 1.32 nM, R&D Systems) or mammalian recombinant murine OPN (rmOPN, 5 μg/ml, NSO-derived, R&D Systems) in RPMI/0.4% FBS was then added to the lower chamber and cells were allowed to migrate for 8 h. Non-migrating cells were removed from the upper surface of the insert using a cotton-tipped applicator. Cells were fixed with methanol and stained with May-Grunwald Giemsa stain (Sigma, St. Louis, MO) according to the manufacturer's directions. Migrating cells were manually counted per high-power field (HPF). Five randomly chosen HPFs were counted per Transwell insert.

FLOW CYTOMETRIC ANALYSIS OF BMDM INTEGRIN EXPRESSION

Integrin expression on BMDMs was tested by labeling WT BMDMs with rat anti-mouse α_4 (2.5 μg/ml, BioLegend), goat anti-mouse α_9 (5 μg/ml, R&D Systems), or rat anti-mouse α_V antibody (2.5 μg/ml, BioLegend), followed by PE-donkey anti-rat or PE-donkey anti-goat antibody (1:200, Jackson Immunoresearch, West Grove, PA).

MACROPHAGE ACTIVATION STUDIES

WT and OPN$^{-/-}$ BMDMs were shifted toward the M1 phenotype by stimulation with IFN-γ (20 ng/ml, Abcam, Cambridge, MA) and LPS (100 ng/ml, Sigma), or towards the M2 phenotype by treatment with IL-4 (60 ng/ml, R&D System) in serum-free RPMI. After 24 h, BMDMs were harvested and expression of M1 and M2 markers was assayed by flow cytometry. IL-12p40 (clone C17.8, Santa Cruz Biotech, Santa Cruz, CA) and CD86 (clone PO3.1, eBioscience, San Diego, CA) were used as markers of M1 macrophage activation, while mannose receptor (AF2535, R&D Systems) was used as an M2 marker. PE-conjugated secondary antibodies ($F(ab')_2$ fragment, Jackson ImmunoResearch) were used for detection. For the analysis of IL-12p40, BMDMs were treated with Brefeldin A (GolgiPlug, BD Bioscience, San Jose, CA) at 0.5 μg/ml in RPMI/10% FBS for the final 5 h of stimulation at 37°C. At 24 h, cells were harvested, permeabilized, and fixed using BD Cytofix/Cytoperm^{TM} kit (BD Bioscience). Cells were stained with IL-12p40 antibody (0.5 μg per tube) for 30 min in BD Perm/Wash Buffer on ice. Cells were washed with 2× with BD Perm/Wash Buffer. Cells were then stained with PE-conjugated donkey anti-rat antibody (Jackson ImmunoResearch, 1:200 dilution).

IL-12p70 PRODUCTION FROM RESIDENT PERITONEAL MACROPHAGES

Resident peritoneal macrophages were harvested from WT C57Bl/6 mice by peritoneal lavage with ice-cold PBS. Cells were treated with ACK buffer to lyse red blood cells. Cells were then plated on non-tissue culture treated 48-well plates in DMEM/10% FBS and allowed to adhere for 2 h. Adherent cells were stimulated with IFN-γ (20 ng/ml) and LPS (100 ng/ml), 5 nM rmOPN, or 100 nM rmOPN in serum-free DMEM. Conditioned media was collected after 24 or 48 h of stimulation. IL-12p70 concentration in the conditioned media was determined using the Mouse IL-12p70 ELISA (eBioscience).

CD68S-BASED RETROVIRAL CONSTRUCTS

All constructs were generated using standard molecular biology techniques and were confirmed by DNA sequencing. Mutations in the RGD and SLAYGLR functional domains of OPN were generated in the retroviral expression plasmid pBMN-IRES-Puro-OPN [Garton et al., 2002; Speer et al., 2005] using the Stratagene QuikChange II XL site-directed mutagenesis kit following the manufacturer's directions. The pBMN-IRES-Puro-OPN vector contains a 1097-bp fragment spanning the region of −18 to +1079 of mouse OPN cDNA (NM_009263). The primers used for mutagenesis were as follows: RGD → RAD, 5′-CCCAACGGCCGAGCTGATAGCTTGGCT-3′, SLAYGLR- → SLAAGLR, 5′-GGCCGAGGTGATAGCTTGGCTGCTGGACTGAGGT-3′. Nucleotides changed relative to the sequence of wild-type OPN cDNA are underlined. For the RAD SLAAGLR double mutant, the SLAAGLR mutation was introduced and a subsequent round of site-directed mutagenesis was performed to introduce the RAD mutation.

To provide macrophage-selective expression, OPN cDNA was inserted into the LZRS-CD68S-HA-EGFP vector [Gough and Raines, 2003]. PCR fragments were generated with *Not*I sites and were cloned into the LZRS-CD68S-HA-EGFP vector that had been digested with *Not*I to remove the HA-EGFP cDNA. The LZRS-CD68S-HA-EGFP vector was used as a negative control for in vivo studies.

GENERATION OF CHIMERIC OPN MICE

Stem cell transduction and transplantation were performed as previously described [Gough and Raines, 2003]. Briefly, plasmid DNA was used to transduce the ecotropic Phoenix packaging cell line using calcium phosphate-mediated transfection. High titer retroviral supernatant was produced by selection of transfected Phoenix cells in medium containing puromycin (2 μg/ml). Stem cells were isolated from OPN-null donor mice that had been injected intraperitoneally with 300 μl of 5-fluorouracil (10 mg/ml) 3 days prior to bone marrow isolation. Bone marrow cells were then cultured for 48 h in complete stem cell medium (DMEM with 15% FBS and stem cell factor (100 ng/ml), IL-3 (10 ng/ml), and IL-6 (20 ng/ml)) to stimulate proliferation. Cells were then transduced by two consecutive 24-h incubations with retroviral supernatants supplemented with 50 mM HEPES, 4 μg/ml polybrene, and stem-cell factor, IL-3 and IL-6 at the previously mentioned concentrations in fibronectin-coated dishes. After transduction, OPN protein expression was determined by ELISA (cells transduced with OPN constructs produced > 1,000 pg/ml. Further, Western Blot studies confirmed that OPN was expressed in retrovirally transduced cells (Supplemental Fig. 2). Cells were then harvested and injected intravenously into OPN-null donor mice. Donor mice were lethally irradiated 24 h prior to transplantation with 10.5 Gy. Mice were housed for 4–6 weeks following transplantation to allow for reconstitution of monocytes/macrophages. Study animals were second generation hematopoietic chimeras repopulated with bone marrow from primary transplants.

THIOGLYCOLLATE-ELICITED PERITONITIS MODEL

To induce thioglycollate-elicited peritonitis, mice were injected intraperitoneally with 1.0 ml of thioglycollate (3% solution, BD BBL, 221199). After 72 h, mice were euthanized by CO_2, and peritoneal cavities were lavaged with 5 ml ice-cold PBS/5 mM EDTA. Cell concentration was determined by manual counting using a hemocytometer. For qualitative analysis of the cell composition, peritoneal leukocytes were analyzed by flow cytometry. Peritoneal leukocytes were labeled with PE-conjugated primary antibodies against CD115 (macrophages, eBioscience, 12-1152), CD3 (T-cells, BD Bioscience, 555275), CD11b (leukocytes, BD Biosciences 553311), B220 (B-cells, BD Biosciences, 553089), Ly6G (neutrophils, BD Biosciences, 551461), rat IgG2b (isotype control, eBioscience, 12-4371), or rat IgG2a (isotype control, eBioscience, 12-4031). For staining, peritoneal leukocytes were washed 2× in FACS staining buffer (PBS/1% FBS/0.09% sodium azide) and 0.5×10^6 cells were resuspended in 100 μl FACS buffer with 1 μl BD Mouse Fc Block (rat anti-mouse CD16/CD32, BD#553142, 0.5 mg/ml). Cells were incubated at 4°C for 5 min to block FcγRII/III receptors. PE-conjugated primary antibodies (0.06 μg per tube) were then added directly to the pre-incubated cells in the presence of Mouse Fc Block and incubated on ice for 30 min. Cells were then washed 2× in FACS staining buffer, fixed in 4% paraformaldehyde for 20 min at 4°C. Following fixation, cells were washed 2× in FACS staining buffer

TABLE I. Integrin α Subunit Expression by Bone Marrow-Derived Macrophages

Integrin	Expression (% cells positive)
α_4	52.0 ± 13.0
α_9	11.0 ± 3.6
α_V	4.5 ± 0.9

WT BMDMs were analyzed for integrin expression by flow cytometry and the percentage of macrophages positive for different integrins subunits is shown. Data presented as mean ± SEM of four to five individual replicates.

and stored at 4°C until analysis. Cells were analyzed on a BD FACscan flow cytometer.

STATISTICAL ANALYSIS

Data is presented as mean ± SEM, unless otherwise indicated. ANOVA was used for statistical comparison among multiple groups. Asterisks (*) indicate $P < 0.05$.

RESULTS

OPN PROMOTES MACROPHAGE SURVIVAL PRIMARILY VIA AN α_4 INTEGRIN-DEPENDENT PATHWAY

To determine the receptors through which OPN mediates macrophage functions, we first determined the integrin profile on bone marrow-derived macrophages (BMDM). As shown in Table I BMDMs expressed predominantly integrin α_4 with less expression of integrin α_9 and little α_V. To determine the receptors through which OPN mediates macrophage survival, BMDMs were incubated with neutralizing antibodies to α_4, α_V, and α_9 integrins or non-immune IgG control before plating on OPN coated surfaces. Pre-incubation with anti-α_4 antibody neutralized the protective effect of OPN, while incubation with anti-α_V and anti-α_9 antibody showed less or no significant inhibition, respectively (Fig. 1). Further, pre-incubation with an RGD peptide failed to affect OPN protective function. These results suggest that the pro-survival effect of OPN is mediated primarily through an α_4 integrin-initiated signaling pathway in macrophages. These results also suggest that the SLAYGLR domain of OPN plays a major role in macrophages survival, although they do not exclude that other receptors may interact with the SLAYGLR OPN domain to mediate survival.

We have also determined that exogenous addition of OPN to OPN-null BMDMs was able to rescue the cell death induced by Fas ligation (Supplemental Fig. 3), a mechanism different from growth factor withdrawal and lack of attachment to the extracellular matrix [Choi et al., 1998; Imanishi et al., 2001, 2002b]. Further, Fas-induced cell death of OPN-deficient murine P388D1 macrophages [Li et al., 2010] could also be rescued by addition of OPN, but not when α_4 integrin was neutralized (Supplemental Fig. 4).

OPN PROMOTES MACROPHAGE MIGRATION VIA α_4 AND α_9 INTEGRIN-INITIATED PATHWAYS

To address the role of integrins in OPN-dependent macrophage migration we performed integrin blocking studies to identify the specific integrin α subunit involved in migration. BMDMs were pre-incubated with neutralizing antibody to integrin α_4, α_V, and α_9, prior to performing Transwell migration assays. Neutralizing α_4 and α_9 antibodies, but not α_V, blocked migration to OPN (Fig. 2A–C). Similarly, blocking with RGD peptide had no effect on macrophage migration to OPN compared to the RAD control peptide (Fig. 2D).

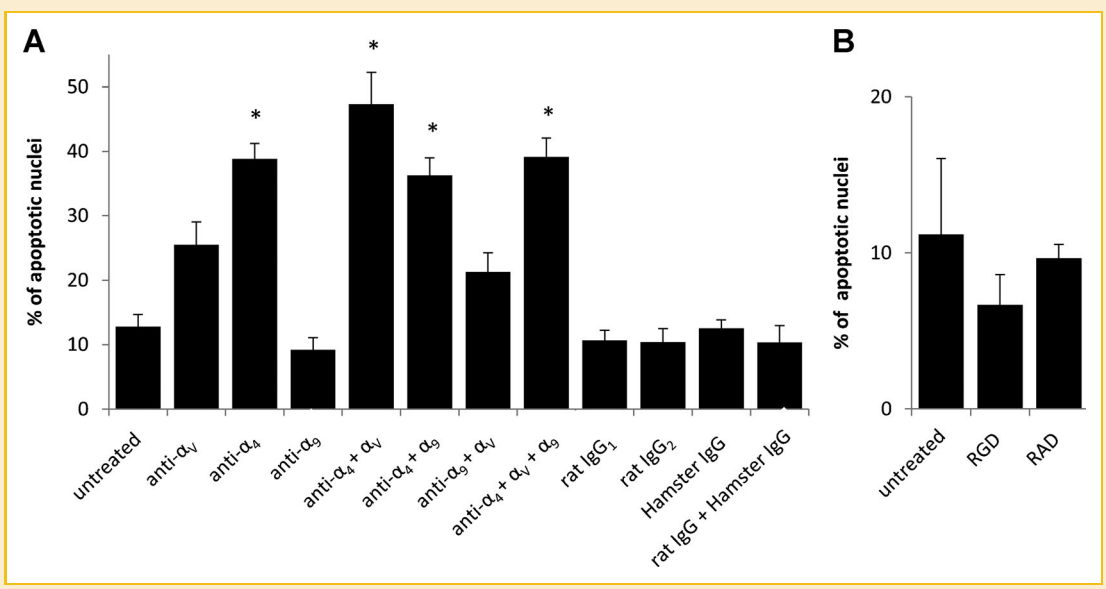

Fig. 1. OPN promotes macrophage survival via an α_4 integrin pathway. A: WT BMDMs were pre-incubated with 10 μg/ml of neutralizing α_4, α_V, or α_9 integrin antibody or control rat non-immune IgG for 15 min. Cells were then plated on OPN-coated surfaces in serum-free medium for 24 h. Cells were stained with Hoechst 33258 and percent apoptosis was determined by nuclear fragmentation. Data are presented as mean ± SEM for triplicate wells representative of three independent experiments. *$P < 0.05$ versus untreated. B: WT BMDMs were pre-incubated with 100 μM RGD or RAD peptide for 15 min. Cells were then plated on OPN-coated surfaces in serum-free RPMI for 24 h and percent apoptosis was determined. Data are presented as mean ± SEM.

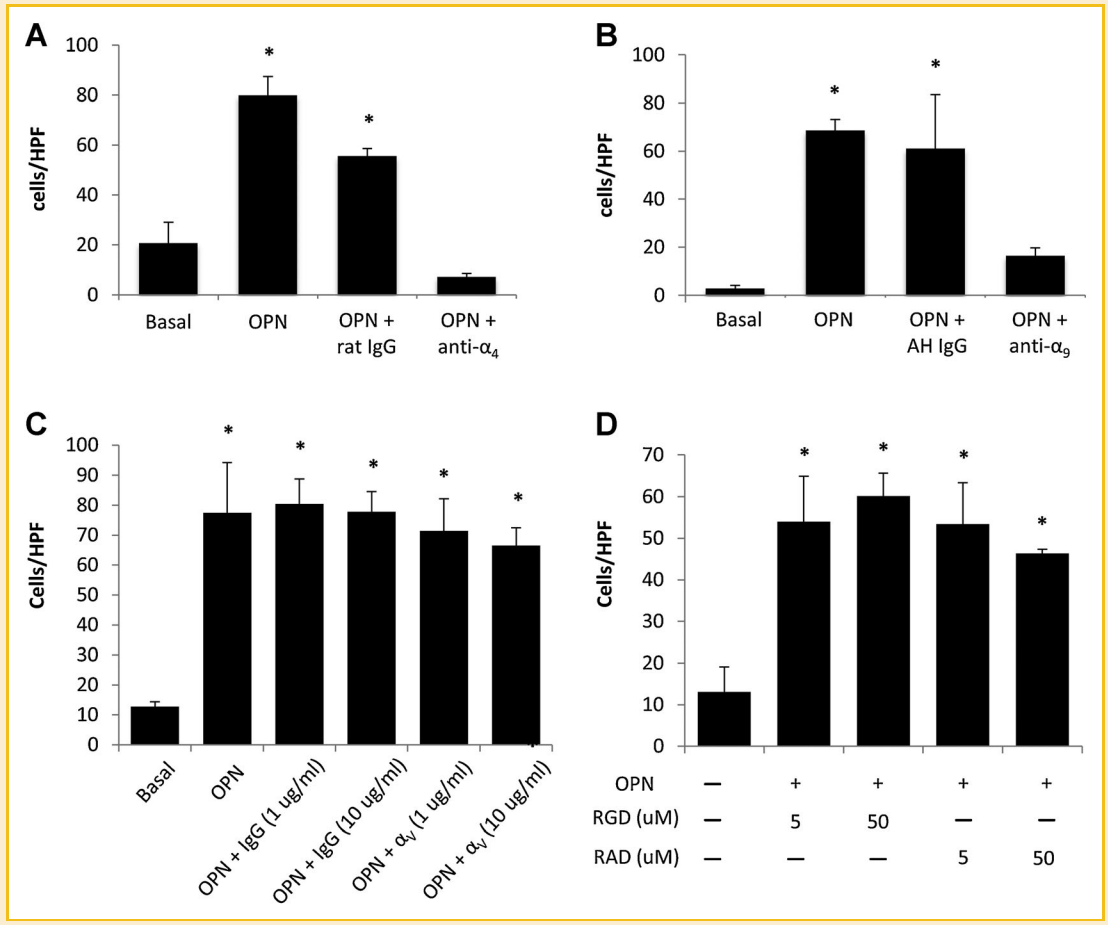

Fig. 2. OPN-induced macrophage migration is mediated by integrins α_4 and α_9. Migration of WT BMDMs to OPN was determined using a Transwell migration assay. A: BMDMs were pre-incubated with 1 μg/ml α_4 integrin neutralizing antibody or control rat IgG prior to Transwell migration assay to OPN. B: BMDMs were pre-incubated with anti-integrin α_9 antibody or hamster IgG isotype control prior to assessing migration to OPN in a Transwell migration assay. C: BMDMs were incubated with α_V integrin neutralizing antibody prior to assessing migration to OPN. D: BMDMs were pre-incubated with RGD peptide or RAD control peptide prior to migration assay. Data expressed as cell number per high power field (HPF). Data are presented as mean ± SEM of triplicate wells representative of three independent experiments. $^*P < 0.05$ versus basal.

Overall, these data indicate that integrin α_4 and α_9 mediate OPN-dependent macrophage migration.

OPN DOES NOT AFFECT PRO-INFLAMMATORY CYTOKINE PRODUCTION OR MACROPHAGE PHENOTYPE

OPN has been previously reported to stimulate IL-12 secretion from macrophages and inhibit IL-10 production [Ashkar et al., 2000; Weber et al., 2002]. Consequently, we tested the hypothesis that OPN shifts macrophages toward an M1 pro-inflammatory macrophage phenotype. M1 macrophages are defined by the upregulation of several markers in response to treatment with LPS and IFN-γ [Martinez et al., 2008], including the pro-inflammatory cytokine IL-12, and the co-stimulatory molecule CD86. However, as shown in Table II, we found that treatment with recombinant mammalian-derived OPN failed to induce IL-12 production or CD86 expression. To determine whether this effect was specific to BMDMs, we stimulated resident peritoneal macrophages with rmOPN and analyzed the induction of IL-12p70. As shown in Figure 3, stimulation with rmOPN failed to induce IL-12p70 production, even at concentrations as high as 100 nM. We also determined that OPN does not affect the M1 or M2 phenotype in $OPN^{-/-}$ macrophages (Supplemental Table I).

Finally, we evaluated the effect of OPN on NF-κB signaling pathway activation. For these studies, the macrophage-like cell line, RAW264.7, was transiently transfected with a NF-κB reporter construct. RAW264.7 cells were chosen for these experiments due to their ease of transfection compared to primary macrophages. Transfected RAW264.7 cells were stimulated with OPN for 6 h and cell lysates were collected and luciferase was assayed. As expected, stimulation with LPS, a known activator of NF-κB signaling, resulted in a significant increase in luciferase. Stimulation with either 5 or 100 nM OPN did not result in increased luciferase expression. Taken together, these results indicate that OPN does not affect macrophage activation (Supplemental Fig. 7).

THE SLAYGLR DOMAIN OF OPN MEDIATES MACROPHAGE ACCUMULATION IN THIOGLYCOLLATE-ELICITED PERITONITIS

It has previously been shown that OPN promotes leukocyte recruitment to the peritoneal cavity in response to inflammatory stimuli [Bruemmer et al., 2003]. However, the OPN domains

TABLE II. OPN Does Not Affect Pro-Inflammatory Cytokine Production

Treatment	IL-12p40 expression (% cells positive)
A	
Unstimulated	0.58
IFN-γ + LPS	50.70
rOPN	1.48

Treatment	CD86 expression (MFI)
B	
Unstimulated	28.7
IFN-γ + LPS	87.0
rOPN	21.1

(A) Bone marrow derived macrophages were stimulated for 24 h with IFN-γ + LPS, or recombinant mammalian OPN (rOPN). Cells were treated with Brefeldin A for the final 5 h of stimulation, and cells were harvested, fixed, and stained for IL-12p40. The percentage of cells positive for IL-12p40 was determined by flow cytometry. (B) WT BMDMs were treated with IFN-γ + LPS, or recombinant mammalian OPN (rOPN) for 24 h in serum-free media. Cells were harvested and stained for CD86. CD86 expression was analyzed by flow cytometry. Data expressed as mean fluorescent intensity (MFI) (B). Data from a single experiment. 10,000 events were analyzed via flow cytometry for each genotype.

responsible for leukocyte recruitment in vivo are unknown. Our in vitro data show that OPN interacts with integrin α_4 to promote macrophage survival, whereas both integrins α_4 and α_9 stimulate macrophage migration. Therefore, we hypothesized that OPN mediates macrophage accumulation in vivo through the SLAYGLR functional domain of OPN, known to bind to α_4 and α_9 integrins. We therefore generated chimeric mice expressing mutated OPN forms to test this hypothesis. Complementary cDNA encoding either full-length OPN or OPN mutants in which the RGD, SLAYGLR, or both functional domains had been mutationally inactivated were cloned into a retroviral vector for macrophage-selective expression [Gough and Raines, 2003]. Recombinant retrovirus was used to transduce $OPN^{-/-}$ hematopoietic stem cells that were then transplanted into lethally irradiated OPN-null mice. After recovery, these mice served as bone marrow donors in a secondary transplant to generate OPN-null mice expressing OPN forms in myeloid cells. To induce leukocyte migration into the peritoneal cavity, chimeric mice received an intraperitoneal injection of the sterile irritant thioglycollate and 72 h later leukocyte recruitment into the peritoneal cavity was analyzed. For comparison, non-transplanted WT mice were included in the analysis.

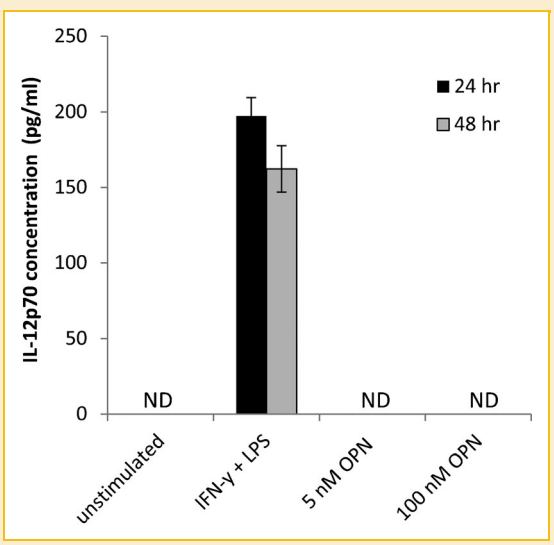

Fig. 3. OPN does not affect IL-12 production in resident peritoneal macrophages. Resident peritoneal macrophages were stimulated with IFN-γ + LPS, 5 nM OPN, or 100 nM OPN and the concentration of IL-12p70 was determined by ELISA after 24 h (black bars) or 48 h (gray bars) of treatment. ND: not detectable. Data presented as mean ± SD of triplicate wells. Data from a single experiment.

To determine whether inactivation of the RGD and/or SLAYGLR functional domains affected the cellular composition of the peritoneal lavage fluid, peritoneal exudates were analyzed with cell-specific antibodies by flow cytometry. First, peritoneal exudates from wild-type mice (WT) contained significantly higher numbers of cells compared to OPN-null mice expressing the eGFP control (Fig. 4A). Macrophage reconstitution of OPN in OPN-null mice restored the total number of cells to the WT level. Mutational inactivation of the RGD domain had no effect on cell recruitment, while inactivation of the SLAYGLR domain resulted in reduced recruitment of total cells (Fig. 4A). Second, no statistically significant differences in the numbers of CD3-positive, or B220-positive cells were observed between groups (data not shown). However, the number of CD115-cells was decreased in OPN-null mice expressing the eGFP control but it was restored with macrophage reconstitution of OPN in OPN-null mice to the WT level. Mutational inactivation of the RGD domain had no effect on CD115-positive cells recruitment, while inactivation of the SLAYGLR domain resulted in reduced recruitment of CD115-positive cells, suggesting that integrin α_4 and α_9 likely mediate macrophage recruitment into the peritoneal cavity (Fig. 4B). CD115 is the M-CSF receptor thus a specific marker for monocytes/macrophages. Further, we found that the number of CD11b-positive cells mirrors the results obtained with CD115. However, the total numbers of cells is higher than with CD115 because CD11b is expressed also by other non-lymphocyte leukocytes (data not shown).

DISCUSSION

OPN regulates the immune system at many different levels. It serves as a chemotactic molecule to promote the migration of inflammatory cells to sites of injury, and its adhesive and pro-survival properties contribute to increased cell accumulation. OPN can also modulate the immune response by enhancing expression of Th1 cytokines and matrix degrading enzymes [Weber et al., 2002; Bruemmer et al., 2003]. In this work, we demonstrate the key role of OPN in macrophage biology. While OPN does not appear to affect macrophage activation, it does promote macrophage survival and migration. We found that survival in BMDMs is mediated primarily through the α_4 integrin and OPN interaction with α_4 and α_9 induces macrophage migration. In vivo, we demonstrate that the SLAYGLR domain of OPN mediates macrophage accumulation in response to thioglycollate-elicited peritonitis.

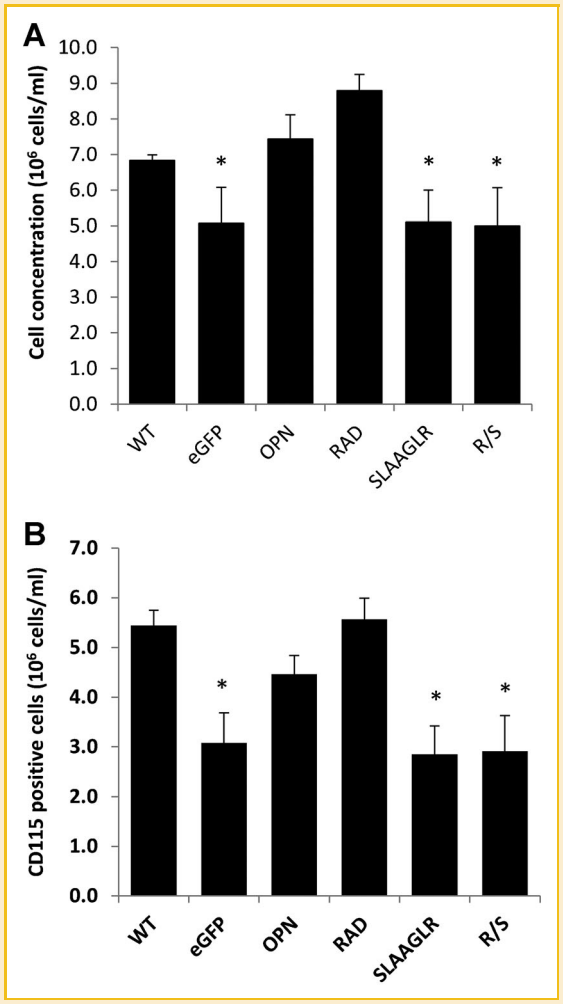

Fig. 4. The SLAYGLR domain of OPN contributes to leukocyte recruitment to the peritoneal cavity in response to thioglycollate. Sterile peritonitis was elicited in OPN hematopoeitic chimeric mice by the injection of thioglycollate intraperitoneally. After 72 h, peritoneal leukocytes were harvested by peritoneal lavage. A: Cell concentration in the peritoneal lavage fluid was determined by manual counting with a hemocytometer. B: Monocytes recruited to the peritoneal cavity in response to thioglycollate elicitation was determined by flow cytometry by staining for CD115. The number of CD115 positive cells was determined in WT and OPN-null chimeric mice expressing eGFP or OPN from a macrophage-selective retroviral vector. Additionally, monocyte recruitment was assessed in chimeric mice in which the RGD domain (RAD), the SLAYGLR domain (SLAAGLR), or both (R/S) had been mutationally inactivated. Data are expressed as mean ± SEM of between five and seven individual animals. $^{*}P < 0.05$ versus WT.

Bruemmer et al. [2003] demonstrated an in vivo effect of OPN on macrophage survival. However, in their studies the receptor mediating OPN's survival effects was not investigated. In this study, we demonstrate that when integrin binding to OPN was inhibited with specific antibodies, OPN's pro-survival effect in macrophages is mostly mediated via an α_4 integrin-initiated pathway and not via an RGD-dependent integrin. These results differ from our findings in endothelial cells where we showed that OPN's survival effect was solely mediated by its interaction with the integrins $\alpha_V\beta_3$ and was dependent on the activation of the NF-κB pathway [Scatena et al., 1998]. Together, our results suggest that distinct signaling pathways are initiated in different cell types. To our knowledge this is the first time that integrin α_4 has been implicated as a survival mediator of macrophages. However, integrin α_4 has been associated with survival of other immune cells such as B cells [Garcia-Gila et al., 2002] and transformed lymphocytes [Zucchetto et al., 2009], as well as other cells types, including lymphatic endothelial cells [Garmy-Susini et al., 2010] and retinal neurons [Leu et al., 2004].

Our data also shows that OPN-induced migration in BMDMs is mediated by the α_4 and α_9 integrins, but not by the α_v integrin or other RGD-dependent integrins. Integrin α_4 is one of the major leukocyte receptors involved in adhesion to endothelium VCAM, thus regulating trafficking. It is therefore not surprising that α_4 also mediates OPN-directed macrophage migration. The role of α9 in mediating macrophage functions is less clear; however, recent data indicate that α_9 mediates dendritic cell and macrophage regulation of Th17 responses [Kanayama et al., 2011]. Our α_4 and α_9 migration data further substantiate OPN structure–function studies showing that macrophage migration to OPN can be blocked by interfering with the SLAYGLR sequence of OPN known to interact with α_4 and α_9 integrins [Yamamoto et al., 2003].

To determine if the SLAYGLR domain of OPN mediates macrophage recruitment in vivo, we generated hematopoietic chimeric $OPN^{-/-}$ mice expressing OPN only in myeloid-derived leukocytes. The OPN constructs expressed included WT as well as forms in which the SLAYGLR domain, the RGD domain, or both were mutationally inactivated. We found that inactivation of the SLAYGLR domain results in decreased recruitment of macrophages in response to sterile inflammation. These results complement those of Yamamoto et al. demonstrating that a neutralizing antibody against the SLAYGLR domain could reduce inflammatory cell infiltration in an in vivo arthritis model [Yamamoto et al., 2003]. A similar approach was used to treat collagen-induced arthritis in non-human primates [Yamamoto et al., 2007]. Collectively, these data suggest that neutralization of the interaction between OPN and α_4 and α_9 integrin may be useful as a future therapeutic target to reduce leukocyte accumulation in chronic inflammatory conditions.

OPN has been previously reported to enhance IL-12 production and dampen IL-10 secretion in macrophages [Ashkar et al., 2000; Weber et al., 2002]. In vivo, atherosclerosis prone $ApoE^{-/-}$ mice deficient in OPN have decreased IL-12 and IFN-γ production, while IL-10 levels are enhanced [Bruemmer et al., 2003]. We hypothesized that stimulation with OPN would result in pro-inflammatory cytokine production by macrophages. Further, we hypothesized that OPN could induce a M1 pro-inflammatory macrophage phenotype paralleling its function in T-cells where OPN is believed to facilitate Th1 responses. However, we found no increase in IL-12 production in macrophages treated with OPN. Additionally, we saw no difference in macrophage phenotype between wild type and OPN-null macrophages in response to M1 pro-inflammatory agents. Finally OPN did not induce NF-κB activation in these cells, providing further evidence that OPN does not regulate macrophage activation. There are several possible explanations for the discrepancy between our observations and previously reported results. In vivo other cell types could contribute to IL-12 production

[Weiss et al., 2001] or OPN-induced pro-inflammatory cytokine production from macrophages may require co-stimulation. Indeed, O'Regan et al. demonstrated T-cell dependent IL-12 production from peripheral blood mononuclear cells (PBMCs), but found that in PBMCs alone OPN could not stimulate IL-12 production [O'Regan et al., 2000]. It is also possible that the form of OPN that was used in this study differs in post-translation modification from the one used in previous studies. Indeed, Weber et al. showed that forms of OPN which are de-phosphorylated or low phosphorylated resulted in much reduced IL-12 induction compared to the native OPN produced by MC3T3E1 cells which contains 15–17 phosphate residues and is O-glycosylated and highly sialylated [Weber et al., 2002].

The present work demonstrates the key role of OPN in macrophage biology. OPN deficiency does not appear to affect the macrophage activation phenotype, but is associated with decreased macrophage viability. OPN interaction with the integrin α_4 protects macrophages from apoptosis and interaction with α_4 and α_9 induces macrophage migration. Together, these data provide mechanistic insight into OPN regulation of macrophage retention at sites of acute and chronic inflammation.

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