Osteopontin in macrophage function

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The secreted phosphorylated protein osteopontin (OPN) is expressed in a variety of tissues and bodily fluids, and is associated with pathologies including tissue injury, infection, autoimmune disease and cancer. Macrophages are ubiquitous, heterogeneous cells that mediate aspects of cell and tissue damage in all these pathologies. Here, the role of OPN in macrophage function is reviewed. OPN is expressed in macrophage cells in multiple pathologies, and the regulation of its expression in these cells has been described in vitro. The protein has been implicated in multiple functions of macrophages, including cytokine expression, expression of inducible nitric oxide synthase, phagocytosis and migration. Indeed, the role of OPN in cells of the macrophage lineage might underlie its physiological role in many pathologies. However, there are numerous instances where the published literature is inconsistent, especially in terms of OPN function in vitro. Although the heterogeneity of OPN and its receptors, or of macrophages themselves, might underlie some of these inconsistencies, it is important to understand the role of OPN in macrophage biology in order to exploit its function therapeutically.

Osteopontin (OPN; encoded by the gene *SPP1*) is a secreted phosphorylated protein originally identified in cancer cells (Ref. 1) and in bone (Ref. 2). It is widely expressed in epithelial cells (Ref. 3), is a major noncollagenous protein of bone, and is found in most body fluids including milk, blood and urine (Ref. 4). It is also made by several types of cells in the immune system, including T cells, where it was originally identified as an early T-cell activation (*ETA-1*) gene (Ref. 5), and in macrophages, as described in detail below. It is overexpressed in a wide range of human cancers, and its expression is correlated with poorer prognosis (Ref. 6). This might be related to its ability to stimulate migration of many cell types (Ref. 7). It binds strongly to hydroxyapatite and regulates ectopic calcium deposition in vivo (Ref. 8).

There are two well-characterised cell-binding sites on OPN (Fig. 1). The RGD sequence binds αv (ITGAV) integrins, including $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha v\beta 1$: all these integrins bind OPN with similar affinity (Refs 13, 14). The adjacent SVVYGLR sequence in human OPN (SLAYGLR in mouse) is a ligand for $\alpha 4\beta 1$ and $\alpha 9\beta 1$, with the latter binding only the thrombin-cleaved form of OPN with high affinity (Refs 15, 16, 17). Recently, additional cell-binding sequences have been described that are distinct from these two integrin-binding sequences, although their ligands on cells are still unknown (Refs 11, 12). OPN has also been reported to bind to several other integrins, including $\alpha 5\beta 1$ (Ref. 18), $\alpha v\beta 6$ (Ref. 19) and $\alpha 8\beta 1$(Ref. 20): all these interactions are disrupted by RGD peptides, although in the case of $\alpha 5\beta 1$ and $\alpha v\beta 6$, upstream amino acid residues affect binding. Recently, OPN has been shown to bind the $\alpha x\beta 2$ integrin through a less specific acidic protein interaction that is not localised to a specific set of amino acids (Ref. 21). How many of these interactions are

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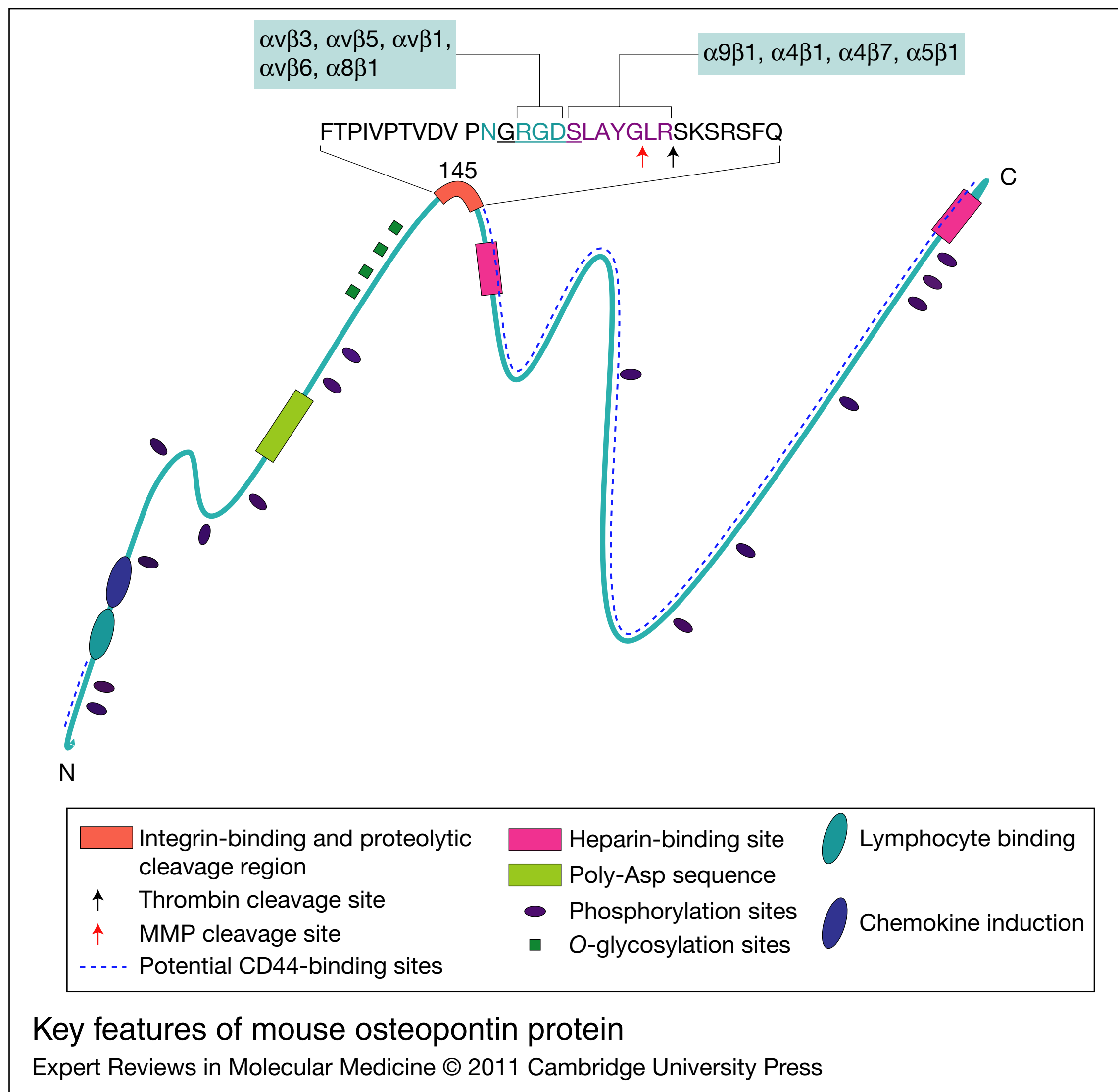

Figure 1. Key features of mouse osteopontin protein. The unstructured nature of the molecule is indicated by the blue line. The central integrin-binding sequences of mouse osteopontin are indicated: the RGD sequence is underlined in teal, and the SLAYGLR sequence is depicted in purple. The specific integrins that bind to each of these two sequences are indicated. Other features of the molecule are identified: heparin-binding sequences and the poly-Asp sequence that mediates binding to hydroxyapatite (Ref. 9); *O*-glycosylation sites and a subset of phosphorylation sites (Ref. 10); sites of cleavage by thrombin and matrix metalloproteinase (MMP); and recently identified N-terminal sequences involved in lymphocyte binding (Ref. 11) and induction of chemokine expression (Ref. 12). The binding sequence mediating CD44 interaction is controversial (see text).

physiologically relevant, and in what contexts, is still not clear and is an area that requires further investigation. OPN has also been described as a ligand for CD44 (Ref. 22), although it appears that only a limited number of isoforms of this highly variable family of molecules can bind OPN (Ref. 23), and it has been suggested that this interaction might be indirect, mediated through β1 integrins (Ref. 24). The CD44-binding sequence has been variously reported to be in the C-terminal half (Ref. 25) and at the N-terminus (Ref. 26), so the specificity of this interaction remains unclear.

Because OPN binds to many integrins as well as possibly to CD44, it has many characteristics of an adhesive protein. It is found in a soluble form in

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some tissues, which is consistent with its ability to stimulate migration (Ref. 27). It is variably phosphorylated, with the highest level of phosphates found in milk and bone OPN, and the lowest in the protein made by tumour cells (Ref. 10). It is a substrate for several proteases that regulate its integrin-binding affinity (Refs 28, 29, 30). In addition, an intracellular form of OPN has been identified in fibroblasts, macrophages and dendritic cells (Refs 31, 32, 33, 34), which might have distinct functions from the secreted protein.

Mice lacking OPN are healthy and normal in the absence of pathology (Refs 35, 36). However, the response of OPN-deficient mice to a variety of pathologies, including various forms of tissue injury, inflammation, infection and autoimmune disorders, is quite different from that of wild-type (WT) mice (Ref. 37): OPN-deficient mice are protected from some autoimmune disorders and inflammatory conditions, but are more susceptible to some infections (Ref. 4). Many of these pathologies are affected by macrophage functions, and this ubiquitous and variable cell type both expresses and responds to OPN. Given the importance of macrophages in pathology, and the frequent identification of OPN as a protein expressed by activated macrophages, it is likely that OPN has an important role in macrophage function, and thereby in the processes of inflammation and the innate and adaptive immune responses. This article reviews the expression of OPN in macrophages and summarises the reported effects of OPN on macrophage function. OPN is also expressed in, and regulates the function of, the closely related cell types osteoclasts and dendritic cells, but the role of OPN in these cell types, as well as general effects of OPN, are not covered here. Several excellent recent reviews have described the functions of OPN in other systems (Refs 9, 33, 38, 39, 40).

OPN expression in monocytes and macrophages

In vitro

OPN is expressed at a low level in monocytes, the circulating macrophage precursor cells, and is induced during their differentiation into macrophages. This has been demonstrated in vitro, where treatment of the human monocytic cell lines HL-60 and THP-1 with phorbol myristate acetate (PMA) results in large increases in OPN expression (Refs 41, 42, 43). In THP-1 monocytes, PMA, tumour necrosis factor (TNF-α/TNF), interleukin (IL)-6 and oxidised low-density lipoprotein (oxLDL) all induce OPN expression, but PMA is by far the strongest inducer (PMA, 3000-fold; TNF, 80-fold; IL-6, threefold; oxLDL, twofold). Antagonists of the nuclear receptor PPARG (peroxisome proliferator-activated receptor γ) inhibit OPN expression induced by all these compounds and even reduce basal levels (Ref. 42). In HL-60 cells, other inducers of differentiation do not increase OPN expression (Ref. 44). Mouse macrophages (RAW264.7 or peritoneal) are less sensitive to PMA, showing only a fourfold increase in OPN expression (Ref. 45), but these cells already express very high levels of OPN.

Several cytokines have been shown to increase OPN expression in monocytes and macrophages: interferon γ (IFN-γ/IFNG) stimulates expression in THP-1 cells (Ref. 46); TNF increases OPN expression in the aHINS-B3 macrophage cell line (Ref. 47) [but not in the P388D cell line (Ref. 48)]; and expression is induced by IL-10 in human blood monocytes, an effect that is suppressed by IL-4 and IL-13 (Ref. 49). IL-6 is implicated in the adipocyte-induced induction of OPN expression in U937 cells in coculture experiments (Ref. 50). In thioglycollate-elicited peritoneal macrophages, OPN mRNA levels are induced by OPN itself (Ref. 51). Coculture of human blood monocytes with tumour cells secreting colony-stimulating factor 1 (CSF1) induces OPN, suggesting that CSF1 can also regulate OPN expression in these cells (Ref. 52).

In RAW264.7 macrophage-like cells, OPN expression is increased after treatment with TNF, IL-1β or IFN-γ, in a mechanism dependent on the transcription factor AP-1, and this induction is inhibited by SERPINE1 ligands (Ref. 53). Induction of expression by IL-1β, IFN-γ or lipopolysaccharide (LPS) is dependent on nitric oxide (NO) (Refs 54, 55, 56) because blocking NO function with the arginine analogue L-nitroarginine methyl ester prevents the induction of OPN, but NO alone is not sufficient for OPN induction (Ref. 54). The mechanism of NO regulation of OPN involves heterogeneous nuclear ribonucleoprotein A/B (HNRNPA/B), which acts as a repressor of OPN transcription: *S*-nitrosylation of this protein through NO relieves this repression to increase OPN transcription (Ref. 55). In the case of LPS, a

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further increase in OPN expression results from increased expression of HNRNPU, which binds to the same promoter sequence on OPN vacated by HNRNPA/B (Ref. 57). Interestingly, recent results suggest that secretion of OPN is not regulated by LPS in RAW264.7 and mouse peritoneal macrophages: rather, intracellular OPN levels are induced several-fold (Ref. 58), suggesting the LPS exerts translational as well as transcriptional control on OPN expression. The analysis of OPN expression in transformed cell lines (such as RAW264.7) must be interpreted with caution, however, because OPN is often upregulated as a result of cellular transformation (Ref. 6). Accordingly, although OPN was identified as a constitutively expressed gene in human monocytes differentiated in vitro, its expression was not increased by LPS in these cells, as determined by array analysis (Ref. 59). Thus, further experiments are required to understand the regulation of OPN expression by LPS, in different macrophage subtypes and in different species.

Nevertheless, OPN expression has been shown to be regulated during microbial infection in human monocytes and macrophages. Its expression is induced in $CD14^+$ peripheral blood mononuclear cells (PBMCs; $CD14^+$ cells are monocytes) after coculture with microorganisms such as the opportunistic fungal pathogen *Penicillium marneffei*, in a CSF2-dependent manner (Ref. 60). However, macrophages differentiated with CSF1 and cocultured with *BCG* (*Bacillus* Calmette–Guérin, an attenuated form of *Mycobacterium bovis*) express significantly more OPN than similar cells differentiated with CSF2 (Ref. 61), implicating CSF1 in the mechanism of regulation of OPN expression in macrophages. Kupffer cells isolated from normal rats do not express OPN, but the protein is expressed in these cells isolated from rats 7 days after *Propionibacterium acnes* infection (Ref. 62). OPN is induced in THP-1 cells after infection with *M. tuberculosis* (Ref. 63), and in alveolar macrophages of mice infected with *M. tuberculosis* (Ref. 64).

Together, these results demonstrate that OPN expression is part of the response of macrophages to a variety of stimuli, suggesting that this expression is important in macrophage function (Fig. 2).

In vivo

The expression of OPN in macrophages in vivo has been reported frequently in a variety of different models of tissue injury and pathology: macrophage expression of OPN is frequently associated with inflammation. This expression has, in many cases, been detected by both immunohistochemistry and in situ hybridisation, confirming that macrophages, which are generally identified by staining with marker antibodies, synthesise the protein. Table 1 summarises some of the many studies describing expression of OPN in macrophage cells in vivo in different pathologies. Although the list of pathologies where OPN is expressed in macrophages is long, a notable exception is the kidney, where OPN expression is upregulated in tubular and other epithelial cells following injury (including ischaemia, unilateral obstruction and diabetic nephropathy) but is typically not found in macrophages. In this tissue, expression of OPN in these tubular cells is correlated with macrophage influx; for example, in rats treated with deoxycorticosterone acetate, glomeruli that express higher levels of OPN had more infiltrating macrophages (Ref. 93).

Regulation of macrophage function by OPN

Macrophage functions in the response to infection or injury can be divided into three broad areas: (1) production of cytokines or chemokines; (2) phagocytosis of bacteria or other particles, and killing of bacterial or other cells; and (3) antigen presentation. OPN has been implicated primarily in the first two of these functions. Studies on the role of OPN in macrophage function have been carried out using exogenously added protein, or the role of endogenous protein has been examined by the study of macrophages from OPN-deficient mice, or by using small interfering RNA (siRNA) or anti-OPN antibody. In some cases, unfortunately, the reported results are contradictory. To some extent, this is probably due to the heterogeneous nature of the macrophages themselves, where cells differentiated under various conditions might express different OPN receptors or have activated different signalling pathways. In addition, OPN itself is a heterogeneous protein found with different degrees of phosphorylation in different cell types (Ref. 10); the protein is

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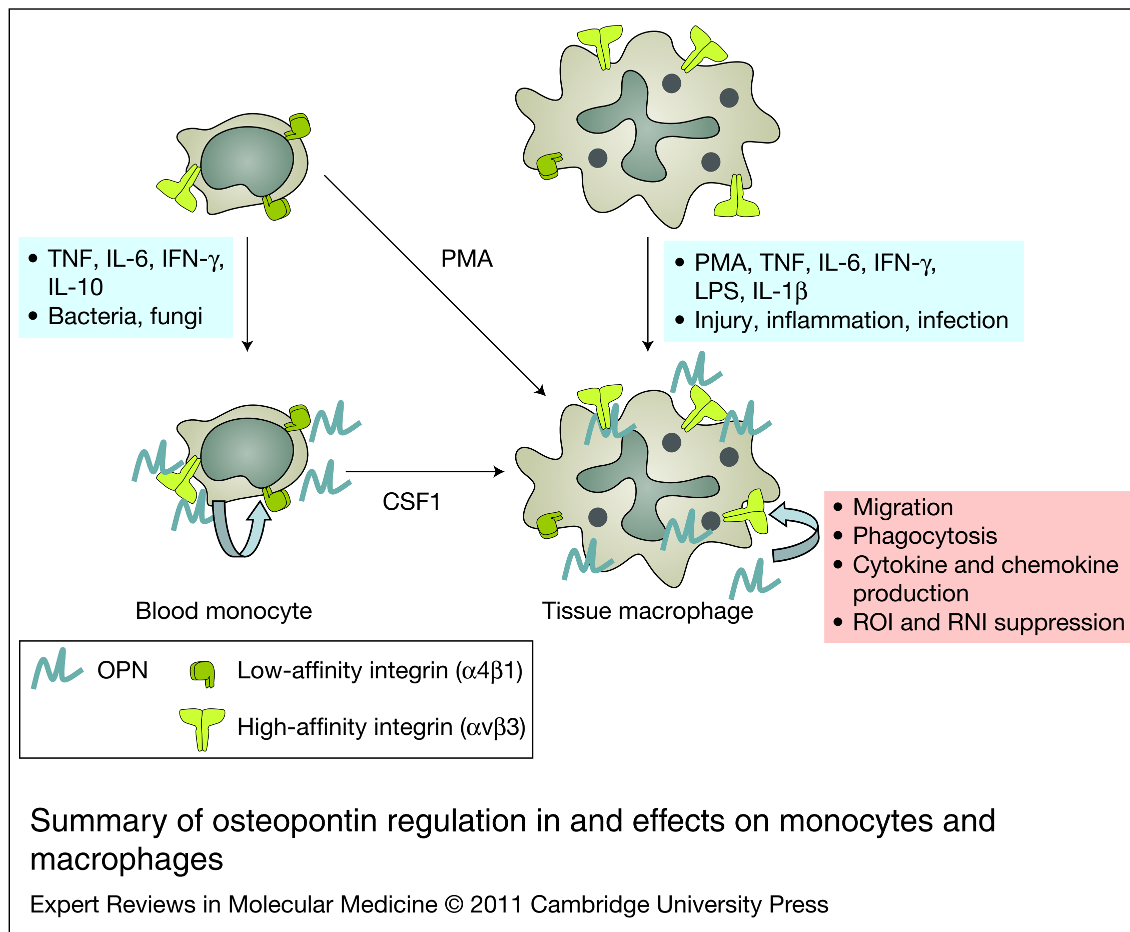

Figure 2. Summary of osteopontin regulation in and effects on monocytes and macrophages. Substances that induce osteopontin (OPN) expression in monocytes (left) and macrophages (right) are indicated in the blue boxes. Phorbol myristate acetate (PMA) induces both OPN expression and differentiation of monocytes into macrophages. The presence of intracellular OPN in macrophages is indicated. Some effects of OPN on macrophage function are listed in the pink box; although these are depicted as resulting from OPN secreted by macrophages (curved arrow), the protein could also be secreted by nearby cells. Note that the effect of OPN on reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) is suppressive. Abbreviations: CSF, colony-stimulating factor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; TNF, tumour necrosis factor.

also susceptible to proteolytic cleavage, and these cleavages can either activate (Ref. 99) or inactivate (Ref. 29) the integrin-binding ability of the protein (Ref. 19) (Fig. 1). Thus, different proteolytic fragments could have different integrin-binding properties (Refs 28, 29). Whether and how OPN phosphorylation or glycosylation regulates its function outside of mineralised tissue is still an area of active investigation (Ref. 100).

OPN receptors and expression on monocytes/macrophages

Monocytes and macrophages express a limited repertoire of integrins, but the expression of these molecules on macrophages in tissues is not well understood. Of the integrins known to bind OPN, cultured monocytes (such as THP-1, U937 and HL-60) express predominantly the $\alpha 4\beta 1$ integrin (Ref. 101). HL-60 and THP-1 cells can adhere to OPN through the $\alpha 4\beta 1$ integrin, but activation of the integrin with both Mn^{2+} and PMA is required for this effect (Ref. 15), suggesting that the affinity of this integrin for OPN is strictly regulated in monocytes. Primary monocytes isolated from human blood express low levels of $\alpha 4$, $\alpha 5$ and $\beta 1$ integrins, and these are all upregulated as the cells differentiate to macrophages, as is the $\alpha x\beta 2$ integrin (Ref. 102).

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Table 1. Osteopontin expression in macrophages in vivo

Pathology studied and models	Expression/Identification[a]	Method[b]	Ref.
Lung fibrosis			
TNF-transgenic mice with lung fibrosis	F4/80^{+} cells	ISH	47
Chronic asthma in mice	F4/80^{+} cells	Dual IF	65
Human lung diseases	BAL cells: 95% macrophages	ELISA; IH	66
Adipose tissue			
High-fat diet in mice	CD68^{+} population; F4/80^{+} cells	Cell fractionation, PCR; IH	67
Obesity and obese mouse models	Correlates with F4/80 expression	Cell fractionation, PCR	68
Carcinomas			
Human tumours, many types	Macrophages, not tumour cells	ISH, IH	69
Hepatocellular carcinoma	Tumour cells and CD68^{+} cells	IH	70
Arterial calcification			
Human atherosclerotic plaques	CD68^{+} cells	ISH, IH	71
Matrix Gla protein and OPN double KO (most OPN-expressing cells were not macrophages)	BM8^{+} cells	IH	72
Human atherosclerotic plaque macrophages (but not in alveolar macrophages or Kupffer cells)	CD68^{+} cells	ISH, IH	73
Brain injury			
LPS-injected rats (IP)	Lectin-binding microglia	ISH, IH	74
Brain injury (mechanical or toxin)	ED1^{+} cells	ISH	75
Focal ischaemia	ED1^{+} cells	IH	76
Nerve/spinal cord injury			
Spinal cord avulsion	CD11b^{+} cells	IH	77
Chemically induced demyelination	Scavenger receptor expression	ISH	78
Liver injury			
CCL_4 hepatic toxicity	Kupffer cells, hepatic macrophages	RNA from isolated cells	62
Biliary cirrhosis	CD68^{+} cells	IH	79
Galactosamine injury in obese mice	F4/80^{+} cells	IH	
Cardiac injury			
Myocardial freeze–thaw	ED1^{+} cells	IH, ISH	80
Myocardial infarct	Macrophages (F4/80^{+})	IH, ISH	81
Viral myocarditis	Mac3^{+} cells	IH, ISH	82
Arthritis/inflammation			
Inner ear calcification	CD68^{+} cells	IH, ISH	83
Experimental autoimmune uveitis	Isolectin B4^{+} microglia	IH, ISH	84
Juvenile idiopathic arthritis	F4/80^{+} cells	IH, isolated monocytes	85
Bone/skin injury			
Surgical bone injury	Morphology	Colloidal gold IH	86
Skin wound	F4/80^{+} cells	IF	87

(continued on next page)

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Table 1. Osteopontin expression in macrophages in vivo *(continued)*

Pathology studied and models	Expression/Identification[a]	Method[b]	Ref.
Digestive system disease			
Human pancreatitis	Macrophages	IH, in situ PCR	88
Crohn disease, ulcerative colitis	$CD68^+$ cells	IH	89
Pregnant endometrium	$F4/80^+$ cells	IH, in situ PCR	90
Renal disease			
Experimental glomerulonephritis	Tubular epithelial cells	IH	91
Crescentic glomerulonephritis	$CD68^+$ cells	ISH, IH	92
DOCA-salt-treated rats	Epithelial, mesangial and other cells	IH, ISH	93
Human cyclosporine toxicity	Tubules	IH, ISH	94
Subtotal nephrectomy	Tubules	ISH	95
Unilateral ureteral ligation	Tubular epithelial cells	IH	96
Infection			
Echinococcus infection	$CD68^+$ cells	IF	97
Fungal infection and resulting granulomas in iNOS-deficient mice	Morphology		98

[a]Cell types expressing osteopontin and/or the criteria used to determine that the expressing cells are macrophages.
[b]Method of detection of osteopontin expression.
Abbreviations: BAL, bronchoalveolar fluid; LPS, lipopolysaccharide; CCL_4, carbon tetrachloride; DOCA, deoxycorticosterone acetate; IF, immunofluorescence; IH, immunohistochemistry; iNOS, inducible nitric oxide; IP, intraperitoneal injection; ISH, in situ hybridisation; KO, knockout; OPN, osteopontin.

In HL-60 cells, differentiation with PMA results in expression of the αvβ3 integrin as well as β1-containing integrins (Ref. 41). In mouse bone-marrow-derived monocytes, CSF1 (used to differentiate these cells into macrophages) stimulates expression of α4β1 and α5β1 (Ref. 103). CSF1 also induces expression of the αvβ5 integrin, whereas CSF2 induces the αvβ3 integrin (Ref. 104). Myeloid-specific deletion of the αv integrin in mice results in immune defects, including development of colitis (Ref. 105), highlighting the importance of this integrin in macrophage function.

OPN effects on gene expression in macrophages

IL-12 and IL-10

OPN is widely thought to induce IL-12 expression in macrophages and to suppress the expression of IL-10, thereby regulating the T helper 1 (Th1)–Th2 bias of the adaptive immune system. Treatment of mouse resident peritoneal macrophages with purified OPN (from T cells or osteoblasts) induced secretion of IL-12 p70 directly, and suppressed expression of IL-10 induced in these cells by either LPS or IL-4 (Ref. 106). These effects of OPN on macrophages were demonstrated to be due to OPN–αvβ3 and OPN–CD44 interactions, respectively. Subsequent work showed that OPN increased expression of both IL-12 and TNF in resident peritoneal macrophages, that OPN phosphorylation was required, and that this effect required only modest (175 ng/ml) OPN concentrations (Ref. 25). Conversely, OPN produced in insect cells (and presumably post-translationally modified) had no effect on IL-12 p40 expression in resident macrophages. Similarly, there was no effect of bacterially produced OPN on IL-12 p40 secretion in murine bone marrow macrophages (Ref. 107). However, when differentiated macrophages isolated from the lamina propria of colonic mucosa from healthy patients or from those with Crohn disease were treated with OPN (native, 25 μg/ml or 0.7 μM), IL-12 production was induced only in cells isolated from the diseased mucosa (Ref. 89). This result suggests that the effect of

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OPN on IL-12 production in differentiated macrophages might require priming, perhaps involving regulation of integrin expression or affinity. The effect of OPN on IL-10 production has more support. A background-strain-specific increase in IL-10 induced by bacterial lipoprotein was reported in $OPN^{-/-}$ mice (Ref. 107), and peritoneal macrophages from OPN-overexpressing transgenic mice produced significantly less IL-10 after LPS stimulation than did WT macrophages (Ref. 108): these results are consistent with an effect of endogenous OPN in suppressing IL-10 levels. However, OPN produced in insect cells did not suppress LPS-induced IL-10 production in peritoneal macrophages (Ref. 109). The reason for these divergent results remains unclear: the phosphorylation state of OPN, the differentiation status of the macrophages and possible contaminants (discussed in more detail below) in the OPN preparations are possible explanations. Additional work using well-characterised OPN preparations and macrophage preparations with defined integrin expression could clarify this situation.

Several groups have examined the effect of OPN on IL-12 production in human monocytes from PBMCs. Very high concentrations of OPN (50 μg/ml or 1.4 μM) were required to increase IL-12 production in PBMCs (Ref. 60). Similarly, native OPN at 25 μg/ml (0.9 μM) induced IL-12 in human PBMC monocytes to a similar level as that seen with LPS (Ref. 89). In another study, OPN alone was unable to induce IL-12 in PBMCs (although the concentration used is unknown), but moderate OPN concentrations (1 μg/ml, 30 nM) could induce IL-12 in these cells when T cells were concurrently stimulated with CD3 (Ref. 110), through a mechanism dependent on CD40 ligand (CD40LG) and IFN-γ expression by stimulated T cells. PBMCs cocultured with *P. marneffei* expressed both OPN and IL-12: antibody to OPN inhibited IL-12 production in infected cells, and IL-12 production was observed only in the $CD14^{+}$ monocyte population (Ref. 60). Together, these results suggest that in some cases OPN alone might not be able to induce IL-12 expression directly, but might cooperate with other signalling molecules, perhaps through receptor or signalling pathway crosstalk. The use of micromolar concentrations of OPN raises some concerns, because low-level contamination (less than 0.1%) of such preparations with cytokines that function at nano- or picomolar concentrations (Ref. 111) could be responsible for the reported effects.

Contaminating LPS might be an additional confounding factor in these studies. Polymyxin-B affinity chromatography (which removes LPS) removed the IL-12-inducing activity on human blood monocytes from several commercially available preparations of OPN, suggesting that some of the cytokine-inducing effects of OPN resulted from endotoxin contamination (Ref. 112). Again, because OPN is often used at moderately high concentrations (1–10 μg/ml), even endotoxin levels less than 1 EU/μg could affect the results. A more extended use of polymyxin-B-treated preparations of OPN is required to prove that OPN itself regulates cytokine production in monocytes.

Other cytokines

OPN increased the expression of TNF, but not of IL-6 or IL-1β, in resident mouse peritoneal macrophages (Ref. 25). However, in LPS-treated peritoneal macrophages, OPN treatment increased the expression of all these cytokines over that seen with LPS alone, and anti-OPN reduced the expression of both IL-1β and TNF (Ref. 113). After treatment with zymosan (a yeast cell wall preparation also known as β-glucan), peritoneal macrophages from $OPN^{-/-}$ mice expressed significantly less IL-10 and IL-1β than WT cells; expression of intracellular OPN potentiated induction of these cytokines by zymosan through a mechanism involving ERK (MAPK1; mitogen-activated protein kinase 1) phosphorylation (Ref. 34). Stimulation of murine bone marrow macrophages with titanium particles resulted in lower secretion of several cytokines, including TNF, IL-1α, IL-1β, IL-6, CSF1 and CCL3 [chemokine (C–C) motif ligand 3], from $OPN^{-/-}$ cells as compared with similarly treated WT cells (Ref. 114), implicating OPN in the regulation of expression of these cytokines/ chemokines. In human monocytes isolated from PBMCs, OPN (100 nM/3.75 μg/ml) induced expression of IL-1β, TNF, IL-8 and IL-6, whereas expression of IL-10 was reduced (Ref. 115). The induction of IL-1β was dose dependent, was seen at both the protein and mRNA levels, and did not require the RGD sequence of OPN. These effects of OPN on gene expression paralleled increased phosphorylation of p38 MAPK (Ref. 115).

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Alternative alleles encoding OPN have been identified in different strains of mice; these alleles correlate with strain-specific susceptibility to *Rickettsia* infection, with susceptible mice such as C3H expressing allele B, and several resistant strains expressing allele A (Ref. 116). The proteins encoded by these two alleles differ in nine amino acids, some of which are close to integrin-binding sites (Ref. 117). Synthetic OPN translated in vitro from these different alleles significantly upregulated TNF, IL-1β and IFN-γ mRNA levels in mouse bone marrow macrophages. However, there was a substantial difference in the potency of the two alleles, with allele B having a much smaller effect than allele A (Ref. 118). Owing to the unusual in vitro translation method used to produce OPN, the protein concentration and post-translational modifications required for these effects are unknown.

OPN effects on other genes in macrophages

OPN has been implicated in the regulation of chemokine expression. Incubation of human PBMCs with synovial fluid from rheumatoid arthritic joints, which contains abundant OPN, increased the expression of chemokine CSF1 and CCL4; this effect was partially blocked by antibodies to OPN (Ref. 12). The expression of these two chemokines was seen in $CD14^+$ monocytes but not in the $CD14^-$ population, and the results could be mimicked with purified OPN at 1 μg/ml (30 nM) or less. Peptide mapping showed that this activity of OPN was not associated with the RGD or SVVYGLR sequences, but was rather found in the sequence from amino acids 50 to 83, which is immediately adjacent to a sequence that was previously implicated in OPN binding to lymphocytes (Ref. 11). The receptor for this sequence is still undefined, because anti-CD44 did not block the effects of OPN in this system.

OPN (10 nM) treatment of RAW264.7 cells results in decreased expression of the mitochondrial protein cytochrome *c* oxidase through a CD44-dependent and αvβ3-independent mechanism (Ref. 119). OPN (5–10 nM) also increases expression of CD44 by increasing its half-life in a GRGDS-dependent mechanism in both RAW264.7 and ANA-1 cells, but there is no effect on the levels of CD44 mRNA (Ref. 120). Accordingly, OPN increases adhesion of these cell types to the CD44 ligand hyaluronic acid. OPN was also shown to increase TIMP1 (TIMP metalloproteinase inhibitor 1) expression in PBMCs, an effect seen only in the $CD14^+$ population (Ref. 121). In HL-60 cells, soluble OPN increases the expression of CA2 (carbonic anhydrase II) (Ref. 122).

Taken together, these results strongly suggest that OPN can regulate the expression of many gene products, including inflammatory cytokines, in cells of the monocyte/macrophage lineage. However, the high concentrations of protein required and the potential role of LPS or other contaminants in these effects must be taken into account, and future studies should directly address these issues.

OPN in macrophage effector functions

Effector functions of macrophages include the ability of these cells to engulf and destroy bacteria, mammalian cells and particles through phagocytosis and to kill bacteria and foreign invaders through production of cytotoxic compounds such as NO and reactive oxygen species (ROS). Mobility of these cells is important in their ability to carry out these functions. In this section, the role of OPN in these functions is summarised.

OPN and iNOS expression in macrophages

The role of OPN in the production of NO and in the expression of inducible nitric oxide synthase (iNOS/NOS2) in macrophages has been extensively studied. In vitro, OPN suppresses NO production and NOS2 mRNA levels in IFN-γ- and LPS-treated RAW264.7 mouse macrophages, although these effects are seen only within a very narrow dose range and in specific physiological contexts (Refs 123, 124). Endogenous OPN also suppresses NO production in a feedback loop, because GRGDS peptide, which presumably blocks OPN binding to integrins such as αvβ3, increases NO production in LPS-treated macrophages (Ref. 54). OPN-specific siRNA but not a mismatched sequence increased the expression of NOS2 through a mechanism that involved the *NOS2* promoter (Ref. 125). Exogenous OPN (50 μM or 1.7 mg/ml) could overcome the effects of siRNA and suppress *NOS2* transcription. Chromatin immunoprecipitation (ChIP) experiments demonstrated that blocking OPN expression increased the binding of STAT1 (signal transducer and activator of transcription 1)

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to the *NOS2* promoter; the mechanism involves the ability of OPN to decrease STAT1 stability, which in turn results from increased STAT1 ubiquitination (Ref. 125). These results obtained in RAW264.7 cells were confirmed in primary murine bone marrow macrophages (Ref. 125).

In vivo, the effect of OPN on NO production is not as well defined as in the RAW264.7 cell line. A mouse model of in vivo LPS exposure – caecal ligation and puncture – was used to assess the role of OPN in NOS2 expression in vivo. In these experiments, OPN deficiency resulted in increased expression of NOS2 and phosphorylated STAT1 and reduced STAT1 ubiquitination in bone marrow macrophages or liver tissue 24 h after treatment, consistent with the in vitro results (Ref. 126). Other results from OPN-deficient mice, however, suggest the opposite effect. For instance, reduced production of NO was reported in elicited peritoneal macrophages from OPN-deficient mice as compared with WT after LPS and IFN-γ treatment (Ref. 127). In dextran sodium sulfate (DSS)-induced colitits, lower NOS2 levels were observed in the inflamed intestinal tissues of OPN-deficient mice as compared with WT (Ref. 128). These lower levels of NOS2 and NO in OPN-deficient animals are inconsistent with the idea that OPN suppresses NOS2 expression, and suggest that the effect of OPN on NOS2 expression might be context specific.

OPN and cell killing

In cytotoxicity assays, OPN-deficient peritoneal macrophages stimulated with IFN-γ and LPS had an impaired ability to kill tumour cells, an effect that correlated with reduced production of NO (Ref. 127). Resident peritoneal macrophages were not affected by OPN deficiency, providing further evidence that the state of differentiation or activation of macrophages regulates their response to OPN. Macrophages differentiated in vitro with CSF1 are resistant to *M. tuberculosis* infection as compared to those differentiated with CSF2 (Ref. 61), and OPN is one of the most highly upregulated genes correlating with resistance. Accordingly, exogenous OPN was able to inhibit the proliferation of *M. tuberculosis* in infected sensitive macrophages, and significantly increased superoxide production (Ref. 61). These results are consistent with experiments showing that *BCG* replication was more extensive in OPN-deficient peritoneal macrophages than in corresponding WT cells (Ref. 129), although no differences in NO production were noted and superoxide production was not assessed. Together, these results support a role for OPN in enhancing macrophage cytotoxicity, although further work is needed to understand the mechanism of these effects.

OPN and phagocytosis

OPN-deficient lamina propria macrophages from mice with DSS-induced colitis were impaired in the phagocytosis of fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* as compared with macrophages from WT mice (Ref. 128). Bone marrow macrophages from OPN$^{-/-}$ mice also showed impaired phagocytosis of fluorescent beads, and reduced nuclear factor (NF)-κB activation following phagocytosis (Ref. 114). Furthermore, phagocytosis in human macrophages was induced by 100 ng/ml OPN, but not by 500 ng/ml (Ref. 128). Thioglycollate-elicited mouse peritoneal macrophages from WT and OPN-deficient mice, however, had equal abilities to phagocytose FITC-labelled *Listeria monocytogenes* (Ref. 127).

Phagocytosis of the fungal opportunistic pathogen *Pneumocystis* by macrophages leads to clearance of these organisms. In the absence of an adaptive immune response (on a RAG2$^{-/-}$ background), OPN-deficient mice are susceptible to these organisms, unlike WT mice. Phagocytosis of these organisms was severely compromised in macrophages from OPN$^{-/-}$ mice, through a mechanism involving colocalisation of intracellular OPN with the pattern-recognition receptors TLR2, dectin and mannose receptor (Ref. 34). The reduced phagocytosis in the absence of OPN was accompanied by reduced killing of the phagocytosed organisms resulting from defective production of ROS.

It has also been suggested that OPN can function as an opsonin to facilitate phagocytosis. Microspheres with different chemistries were all internalised by J477A.1 macrophages more efficiently when coated with OPN than when uncoated, but in some cases the effect was similar to that seen with immunoglobulin G (IgG) coating (Ref. 130). OPN also binds two bacterial species – *Streptococcus agalactiae* and *Staphylococcus aureus* – in a Ca^{2+}-dependent manner, with maximal binding at 10 μg/ml OPN (Ref. 21). OPN-coated bacteria were somewhat more efficiently phagocytosed by PMA-stimulated U937 cells,

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whereas OPN-coated latex beads were substantially more efficiently phagocytosed (Ref. 21) in an $\alpha x\beta 2$-dependent mechanism.

Together, these results implicate OPN in the regulation of macrophage effector functions, including cell killing and phagocytosis, but suggest that the differentiation state or cellular milieu of the macrophage preparations might determine the effect of OPN. Differential expression or regulated affinity of OPN-binding integrins might underlie some of these differences. An important future direction to understand these differences is to directly compare the role of OPN in macrophage effector functions using macrophage preparations isolated from different tissues or at different stages of differentiation.

OPN and macrophage migration

Integrins are intimately involved in the regulation of cellular migration, and OPN as a small integrin-binding protein can influence this process in macrophage cells. This has been well demonstrated both in vivo and in vitro. In the following sections, exogenous OPN refers to injected or added protein, whereas endogenous OPN refers to the protein present in the whole animal or made by macrophages. The role of endogenous protein has been mostly tested by analysis of OPN-deficient mice or cells.

In vivo: exogenous OPN

Injection of purified OPN into the skin of mice (Ref. 131) or rats (Ref. 132) resulted in macrophage accumulation at the injection site; a neutralising anti-OPN antibody decreased macrophage accumulation at sites of injection of the chemoattractant fMLP (Ref. 132). Similarly, in an air pouch assay, injection of OPN (either thrombin-cleaved or intact) resulted in macrophage accumulation in the air pouch exudate (Ref. 133), which was inhibited by anti-CD44 (Ref. 133). OPN injection into the peritoneum of mice resulted in a five- to sixfold increase in total cells, 90% of which were Mac1^{+} (ITGAM^{+}) (Ref. 25). In the vitreous space of the eyes of normal mice OPN (both intact and N- and C-terminal fragments) recruited F4/80^{+} (EMR1^{+}) cells (Ref. 84).

In vivo: endogenous OPN

The role of endogenous OPN in macrophage recruitment into the peritoneum or other sites has been tested using OPN-deficient mice, but there remains a lack of consensus on the effect. Macrophages are recruited into the peritoneum after intraperitoneal thioglycollate injection. The number of total cells recruited has been reported to be either fewer (Ref. 134) or greater (Ref. 129) in OPN-deficient mice as compared with WT. In subcutaneous air pouches there was no difference in the number of macrophages that accumulated after OPN injection between the two genotypes (Ref. 133); however, the macrophages that accumulated in OPN-deficient air pouches in response to OPN injection expressed higher levels of $\alpha 4$ integrin than those from WT mice, suggesting that there are alterations in the population of elicited macrophage cells in OPN-deficient mice. If OPN promotes macrophage migration, one would predict that the number of elicited macrophages would be lower in the OPN-deficient mice. Thus, the mechanism of the observed increased macrophage accumulation in OPN$^{-/-}$ mice remains unclear.

In vitro: effect of exogenous OPN

OPN induces migration of the MH-s mouse alveolar macrophage cell line, which expresses both $\beta 3$ integrin and CD44 (Ref. 25). In chemotaxis and chemokinesis assays, OPN and its C-terminal fragment increased migration, and this effect was blocked by anti-CD44 antibody; the N-terminal fragment was ineffective in this assay. By contrast, haptotaxis, or migration on an OPN-coated surface, was induced by both N-terminal and C-terminal OPN fragments, and phosphorylation of the N-terminal fragment was required for this activity, which was blocked by RGD peptides, suggesting $\alpha v\beta 3$ interactions. Similarly, phosphorylation of the N-terminal fragment of OPN was required for MH-s cell spreading on OPN (Ref. 25). Human monocytes, when activated with IL-1, migrate towards bovine milk OPN as efficiently as to normal human serum (Ref. 21).

In vitro: effect of endogenous OPN

The role of OPN produced by macrophages themselves has been tested by examining the migratory abilities of OPN-deficient macrophages. Elicited peritoneal macrophages from OPN-deficient mice showed reduced migration (as compared with WT cells) in vitro towards mouse serum (Ref. 135), fMLP (Ref. 32) or CCL2 (Refs 32, 67, 134, 135), but not to CSF1

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or high concentrations (6.5 nM) of OPN (Ref. 32). Deficient migration was partially (Ref. 67) or fully (Ref. 32) restored by OPN coated on the surface, whereas only the highest concentration of soluble OPN tested (5 μg/ml or 140 nM) could restore migration of $OPN^{-/-}$ macrophages towards the chemoattractant fMLP to WT levels (Ref. 32). The N-terminal half of OPN is required for this effect, but cleavage by thrombin is not required (Ref. 32). An intracellular form of OPN in peritoneal macrophages identified by immunofluorescence and seen only in permeabilised cells colocalises with CD44 at the leading edge of migrating cells. OPN deficiency results in reduced cell-surface expression of CD44, which is required for macrophage migration (Ref. 32).

OPN in macrophage differentiation

An siRNA approach was used to assess the role of OPN in RAW264.7 macrophage cells (Ref. 136). OPN expression was downregulated by 90% in two stably transfected clones expressing OPN short hairpin RNA. OPN-downregulated cells were less adherent, and had reduced expression of CD44. Nonstimulated migration was reduced, and the OPN-downregulated cells were more susceptible to apoptosis in serum-free medium. The expression of the macrophage differentiation marker MSR1 (macrophage scavenger receptor 1) was downregulated, as was expression of IL-12, but the expression of IL-10 and of TNF was unaffected. Interestingly, exogenously added OPN could not compensate for the lack of OPN expression. The authors of this report suggest that together these effects support a role for endogenous, likely intracellular, OPN in macrophage differentiation.

Alterations in macrophage numbers in OPN-deficient mice

Because there is considerable evidence that OPN regulates macrophage migration, this effect should be manifest by a reduction of macrophage numbers in tissues of OPN-deficient mice in response to pathology. Indeed, this has been reported in several instances.

In renal disease

OPN is strongly implicated in renal disease, where its upregulation in tubular epithelial cells is thought to contribute to macrophage infiltration into renal tissues. This has been demonstrated in several different models of renal injury in rodents (Table 2). Together, these results are consistent in implicating OPN in supporting macrophage accumulation in various forms of renal injury, and in contributing to the pathological responses to these injuries.

In granulomas and autoimmunity

Granulomas are organised collections of macrophages formed in response to bacteria or particles that cannot be effectively removed. Intravenous injection of zymosan results in granuloma formation in the liver. In this model, OPN deficiency resulted in a reduced number of granulomas, accompanied by a reduction in the total number of macrophages. Transgenic mice overexpressing OPN in lymphoid cells responded to zymosan injection with increased granuloma numbers accompanied by increased levels of NOS2 mRNA. These results suggest that OPN regulates inflammatory cell, including macrophage, infiltration into the liver, especially at later stages of this disease (Ref. 144). Pulmonary granulomas induced in mice with *Schistosoma mansoni* eggs were also dependent on OPN: the accumulation of granulomas was delayed in OPN-deficient mice and these granulomas contained significantly fewer macrophages (Ref. 145). In experimentally induced autoimmune uveitis, an autoimmune disease of the eye, there was a reduction in the number of granulomas in OPN-deficient mice, in parallel with a decreased number of inflammatory infiltrating cells, including $F4/80^{+}$ macrophages (Ref. 84). Granulomas arising after infection with *BCG*, however, were larger in $OPN^{-/-}$ mice and were accompanied by increased bacterial load, reflecting impaired bacterial clearance in the absence of OPN (Ref. 129). Accordingly, in vitro, *BCG* grew more rapidly in macrophages from $OPN^{-/-}$ mice. Together, these results are consistent with a role for OPN in supporting macrophage accumulation in granulomas. The paradoxical appearance of larger granulomas in *BCG* infection might in fact be a consequence of reduced macrophage accumulation at early times of disease: the consequent attenuation of bacterial killing can lead ultimately to larger granulomas at later times as the host attempts to control the bacterial infection.

In tumours

OPN-producing tumours in WT mice contained more macrophages than OPN-deficient tumours,

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Table 2. Effect of osteopontin on macrophage accumulation in renal injury

Method of blocking OPN	Injury model	Effect on macrophage accumulation	Comments	Ref.
Anti-OPN	Rat glomerulonephritis	Inhibition		137
Antisense OPN	Rat Goodpasture syndrome	Inhibition		138
$OPN^{-/-}$ mice	Cyclosporine nephrotoxicity	Inhibition	20%	139
$OPN^{-/-}$ mice	UUL	Inhibition	20%; early times only	140
$OPN^{-/-}$ mice	UUL	None	Neonatal mice	141
$OPN^{-/-}$ mice	Ischaemia–reperfusion	Inhibition	Especially in glomeruli	142
$OPN^{-/-}$ mice	Angiotensin infusion	Inhibition		143

Abbreviations: OPN, osteopontin; UUL, unilateral ureteral ligation.

and more of the macrophages in OPN-producing tumours were positive for the mannose receptor (Ref. 146). However, in a model of spontaneous tumour development, where both the tumours and the hosts are OPN deficient, macrophage accumulation was independent of OPN status (Ref. 147). Migration of tumour cells with silenced OPN expression was reduced, but could be restored by coculture with human macrophages or macrophage-derived conditioned medium: OPN was shown by blocking antibodies and siRNA techniques to be required for the effects on tumour cell migration (Ref. 148). These results suggest that OPN expression in stromal macrophages might regulate tumour cell function.

In obesity

OPN-deficient mice maintained on a high-fat diet gained weight similarly to WT animals. However, OPN-deficient mice had improved insulin sensitivity, similar to that of nonobese mice, in stark contrast to results in WT mice (Ref. 67). This increased insulin sensitivity was associated with reduced macrophage accumulation in adipose tissue, together with reduced expression of IL-6, TNF, NOS2 and CCL2. Furthermore, plasma levels of inflammatory markers (IL-6, CCL2 and SERPINE1) but not adipokines (adiponectin, leptin and resistin) were reduced in obese OPN-deficient mice. Consistent with these results, neutralising antibody to OPN reduced macrophage numbers in liver and adipose tissue in obese mice, accompanied by increased insulin sensitivity and an increased proportion of apoptotic macrophages (Ref. 149). Although these results suggest that OPN protects macrophages from apoptosis, the same effect could be due to decreased macrophage infiltration if the total number of apoptotic macrophages is not altered. In genetically obese ob/ob mice treated with D-galactosamine to induce liver injury, neutralising antibody to OPN reduced liver injury, in parallel with reduced macrophage infiltration (Ref. 150).

In colitis

The role of OPN in DSS-induced colitis has been explored in a series of studies (Refs 128, 151, 152, 153). Two groups reported an increased inflammatory response to DSS in OPN-deficient mice (Refs 128, 152). Increased accumulation of neutrophils and total apoptotic cells in the colons of OPN-deficient mice in one study was suggested to be due to reduced clearance of apoptotic neutrophils by OPN-deficient macrophages (Ref. 152). In another study, although there was no difference in macrophage accumulation in WT or OPN-deficient diseased colons, NOS2 expression was strongly reduced in DSS-treated $OPN^{-/-}$ mice, further supporting the idea that impaired macrophage function in the absence of OPN increases pathology (Ref. 128). Conflicting results, however, were obtained in a third study of DSS-induced colitis, where pathology was suppressed in the absence of OPN (Ref. 153): in this case, reduced macrophage infiltration in the inflamed colonic tissue in $OPN^{-/-}$ mice was suggested to be responsible for the suppressed pathological response. Different background strains [C57BL/6 (Refs 128, 152) versus Black Swiss (Ref. 153)] were used in these studies, which might

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underlie the divergent results. Whether OPN regulates macrophage accumulation or function in DSS-induced colitis will require further studies using matched mice (preferably littermates) of well-defined genetic background.

Conclusions and therapeutic implications

There are two important conclusions to be drawn from studies of the role of OPN in macrophage function. First, it is clear that the protein is important in the migration of these cells, as shown by extensive in vitro and in vivo studies. Second, its role in other macrophage functions such as phagocytosis and bacterial/cell killing is also supported by experimental results, but more work is required to clarify the effects and the mechanism of action of OPN. Particularly compelling are experiments using OPN-deficient mice or cells, because these studies are not as susceptible to experimental variabilities as are those using purified preparations of OPN. These experimental variabilities raise concerns about the role of OPN described in experiments in vitro. For instance, the role of OPN in cytokine production in macrophages/monocytes remains controversial, and experiments with rigorously defined sources of OPN are required to resolve these conflicts. In particular, OPN preparations must be shown to be free of LPS, and the use of preparations of OPN where contaminating proteins have been inactivated (Ref. 26) is also required.

Because OPN has the potential to regulate numerous aspects of macrophage functions, various therapeutic opportunities exist to exploit these effects. However, the effect of OPN might be either helpful (in the case of infection, where macrophage function is beneficial for the innate immune response) or detrimental (in the case of injury or autoimmune disease, where macrophage function can cause tissue damage). Thus, it will be important to have a full understanding of the roles of OPN in macrophage function to take advantage of these effects therapeutically. An overarching observation apparent from the data reviewed here is the variable results that have been obtained by different investigators in defining the precise role of OPN in macrophage function. Although some of this variability might be due to artefacts, some might also be due to heterogeneity in macrophages, in terms of their expression of OPN receptors under different conditions of stimulation or differentiation, as well as heterogeneity in OPN forms used in vitro and present in vivo in terms of their post-translational modifications and proteolytic cleavage forms. These heterogeneities in OPN forms, while presenting some experimental difficulties, might also provide an important therapeutic advantage. If indeed specific forms of OPN have unique abilities to regulate macrophage function under specific circumstances, then reagents targeting these forms, as either agonists or antagonists, might be able to affect specific functions of macrophages while leaving others unaffected. The therapeutic opportunities in this area are many, and understanding these heterogeneities will be key to exploiting these opportunities.

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Further reading, resources and contacts

Publications

Sodek, J., Ganss, B. and McKee, M.D. (2000) Osteopontin. Critical Reviews in Oral Biology and Medicine 11, 279-303

This paper reviews the early literature on OPN, with particular focus on its role in bone biology.

Ramaiah, S.K. and Rittling, S. (2007) Pathophysiological role of osteopontin in hepatic inflammation, toxicity and cancer. Toxicological Sciences 103, 4-13

The role of OPN in neutrophils is discussed in this review.

Several other excellent reviews on OPN are listed in the introduction of this article.

Dale, D.C., Boxer, L. and Liles, W.C. (2008) The phagocytes: neutrophils and monocytes. Blood 112, 935-945

Geissmann, F. et al. (2010) Development of monocytes, macrophages, and dendritic cells. Science 327, 656-661

These two recent reviews discuss various aspects of macrophage biology.

Websites

Information about past and present conferences focused on OPN, as well as other information about the protein, can be found at the 2010 FASEB osteopontin conference website:

http://www.osteopontin.org

The macrophage.com website useful resource for researchers working on macrophages providing a variety of information on this heterogeneous cell type:

http://www.macrophage.com

Features associated with this article

Figures

Figure 1. Key features of mouse osteopontin protein.

Figure 2. Summary of osteopontin regulation in and effects on monocytes and macrophages.

Tables

Table 1. Osteopontin expression in macrophages in vivo.

Table 2. Effect of osteopontin on macrophage accumulation in renal injury.

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