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Lymphoid/Neuronal Cell Surface OX2 Glycoprotein Recognizes a Novel Receptor on Macrophages Implicated in the Control of Their Function

**Gavin J. Wright,*‖ Michael J. Puklavec,*
Antony C. Willis,† Robert M. Hoek,‡
Jonathon D. Sedgwick,‡ Marion H. Brown,*
and A. Neil Barclay*§**
*Sir William Dunn School of Pathology
University of Oxford
South Parks Road
Oxford OX1 3RE
†MRC Immunochemistry Unit
Department of Biochemistry
University of Oxford
South Parks Road
Oxford OX1 3QU
United Kingdom
‡DNAX Research Institute of Molecular and
Cellular Biology, Inc.
901 California Avenue
Palo Alto, California 94304

Summary

The OX2 membrane glycoprotein (CD200) is expressed on a broad range of tissues including lymphoid cells, neurons, and endothelium. We report the characterization of an OX2 receptor (OX2R) that is a novel protein restricted to cells of the myeloid lineage. OX2 and its receptor are both cell surface glycoproteins containing two immunoglobulin-like domains and interact with a dissociation constant of 2.5 μM and k_{off} 0.8 s^{-1}, typical of many leukocyte protein membrane interactions. Pervanandate treatment of macrophages showed that OX2R could be phosphorylated on tyrosine residues. Blockade of the OX2–OX2R interaction with an OX2R mAb exacerbated the disease model experimental allergic encephalomyelitis. These data, together with data from an OX2-deficient mouse (R. M. Hoek et al., submitted), suggest that myeloid function can be controlled in a tissue-specific manner by the OX2–OX2R interaction.

Introduction

Immunoglobulin superfamily (IgSF) domains are the most common domain type present at the surface of leukocytes, and, of around 250 leukocyte cell surface proteins that have been sequenced, about one-third contain IgSF domains, and nearly half of these contain just two IgSF domains (Barclay et al., 1997). The IgSF fold seems to be particularly suited to mediate recognition events either with other cell surface proteins such as CD2 with CD58 (Barclay et al., 1997; Davis et al., 1998) or with soluble factors such as Fc receptors with immunoglobulins (Heijnen and van de Winkel, 1997). The OX2 protein (CD200) belongs to a group of leukocyte IgSF glycoproteins including NCAM, Thy-1, and L1, which are all expressed on both lymphoid and neuronal tissue but are not generally expressed on other cell types (Clark et al., 1985; McCaughan et al., 1987; Barclay et al., 1997). N-CAM and L1 are involved in mediating interactions between cells of the nervous system, and, in the case of L1, mutants in the extracellular region are correlated with neuropathies (Brummendorf et al., 1998). In rat, OX2 is expressed on the cell surface of thymocytes, recirculating B cells, activated but not resting T cells, follicular dendritic cells, vascular endothelium, the granulosa of degenerating corpora lutea, and neurons of both the central and peripheral nervous systems (Barclay, 1981; Bukovsky et al., 1983; Webb and Barclay, 1984). Related OX2-like proteins have been identified in several human herpes viruses (Gompels et al., 1995; Nicholas, 1996) including HHV-8 (Russo et al., 1996), the virus causally linked to Kaposi's sarcoma, suggesting an important role in immunomodulation (Spriggs, 1996). Both the broad pattern of distribution and short cytoplasmic region that is devoid of any known signaling motifs make the deduction of its function difficult. However, like many other leukocyte surface proteins, OX2 contains two IgSF domains and thus seems likely to interact with another cell surface protein (Barclay et al., 1997). The IgSF domains are organized in a similar manner to the structurally related costimulatory molecules B7-1 and B7-2 that are also genetically closely linked to OX2 (Borriello et al., 1998). Indeed, both stimulatory (Borriello et al., 1997) and tolerogenic (Gorczynski et al., 1998, 1999a, 1999b, 1999c) roles for OX2 in antigen presentation have been proposed. A key question in understanding the function of OX2 is the identification and characterization of its specific binding partner or receptor (OX2R).

We have previously reported the identification of a receptor for OX2 expressed on the surface of resident macrophages obtained from the peritoneal cavity (RPCs) of both rats and mice (Preston et al., 1997). The interaction was demonstrated using a soluble recombinant form of OX2 presented as a highly multimeric array immobilized on fluorescent beads (Brown et al., 1995, 1998; Preston et al., 1997). This generates a highly avid binding reagent that addresses the technical problems encountered when detecting leukocyte cell surface protein–protein interactions that are typified by extremely low affinities due to very fast off rate kinetics (van der Merwe and Barclay, 1994, 1996). We now report the molecular identification and biochemical properties of the OX2R, a kinetic analysis of its interaction with OX2 and functional analysis in a disease model. These data provide a molecular basis for the recent finding that OX2-deficient mice (R. M. Hoek et al., submitted) have defects in their myeloid compartment and together strongly suggest a role for the OX2–OX2R interaction in the tissue-specific control of myeloid cellular biology.

Results

Production of a Monoclonal Antibody that Binds Rat Resident Peritoneal Cells and Blocks OX2 Binding

In a previous study, a receptor for OX2 was identified on both rat and mouse resident peritoneal cells (RPCs).

§To whom correspondence should be addressed (e-mail: barclay@molbiol.ox.ac.uk).
‖Present address: Imperial Cancer Research Fund, 61 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom.

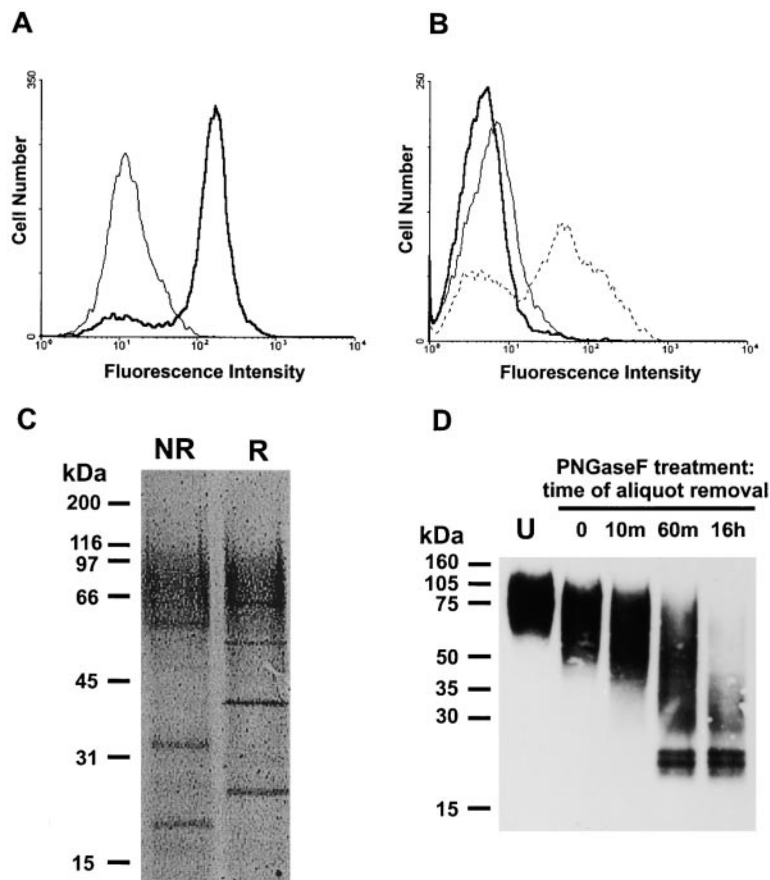


Figure 1. Generation of mAb OX102 and Biochemical Analysis of the OX2R

(A) Staining of rat RPCs with mAbs OX102 (bold) and an isotype-matched negative control (OX21).

(B) Rat RPCs were preincubated with OX45 (anti-rat CD48) or OX102 mAbs and tested for their ability to bind rOX2CD4d3+4-coated beads. rOX2 binding was blocked back to a negative control (CD4d3+4-coated beads, thin line) by OX102 (bold line) but not by the control OX45 (dotted line).

(C) The rOX2R was purified from deoxycholate solubilized crude rat spleen Tween-40 membrane preparations by immunoprecipitation and further resolved under nonreducing (NR) and reducing (R) conditions by SDS-PAGE.

(D) Purified rOX2R was left untreated (U) or was incubated with PNGaseF for the indicated times, then resolved under nonreducing conditions by SDS-PAGE, blotted onto nitrocellulose, and probed with OX102 mAb.

This interaction was demonstrated by using a recombinant soluble form of the extracellular part of rat OX2 engineered as a chimeric protein containing two IgSF domains of rat CD4(d3+4) and immobilizing this protein (rOX2CD4d3+4) via a biotinylated anti-rat CD4 mAb (OX68) to biotinylated fluorescent beads coated in streptavidin (Preston et al., 1997). A monoclonal antibody (mAb), OX88, that recognized mouse macrophages and blocked the interaction was raised (Preston et al., 1997), but all attempts to biochemically characterize the receptor using this IgM mAb were unsuccessful. In this study, further mAbs were raised against rat macrophages, and one mAb, OX102 (IgG_1), bound the majority of rat RPCs (Figure 1A) and blocked the binding of OX2-coated beads back to a negative control (Figure 1B) and presumably recognized the rat OX2 receptor (rOX2R).

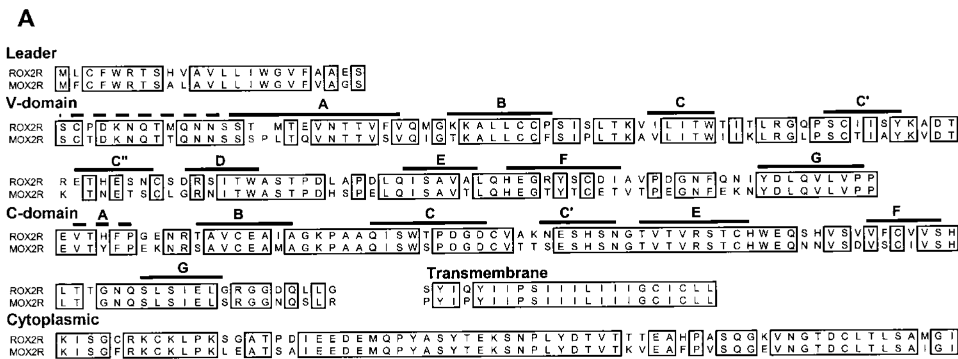
The OX102 Antigen, rOX2R, Is a Novel Macrophage Membrane Glycoprotein

The antigen recognized by mAb OX102 (rOX2R) was purified by immunoprecipitation from rat splenic lysates. Unusually, the antibody–antigen interaction was not disrupted by conventional treatments of high pH, and elution was achieved by treating the immunocomplexes with 0.5% SDS at 55°C. SDS-PAGE analysis revealed rOX2R as a broad 60–100 kDa band (Figure 1C) that correlated well with Western blots of whole rat RPC lysates (data not shown). N-linked carbohydrate moieties were enzymatically removed from purified rOX2R by PNGaseF treatment, which reduced the size of the rOX2R (as visualized by Western blot) to three bands of around 25 kDa (Figure 1D), indicating that ~70% of the mass of the glycoprotein was contributed by N-linked carbohydrate. The N-terminal amino acid sequence of the purified protein was: SCPDK-QTMQ-NSSTMTEVV. The blank cycles (−), were assumed to be asparagine due to the predicted presence of N-linked glycans. A third asparagine conforming to a potential glycosylation site was not modified, probably due to its proximity to a glycosylated site. This protein was identified as novel by screening databases of known proteins with this sequence.

Cloning and Sequencing of OX2R Show It Is a Novel Member of the IgSF

The amino terminal protein sequence was used to design an antisense degenerate primer to amplify the 5′ untranslated region and leader sequence of the rat OX2R using a RT-PCR 5′ RACE procedure, and the full-length cDNA was subsequently obtained using a 3′ RACE RT-PCR protocol. A closely related protein (mOX2R) was also identified in mouse using cross-species PCR and a full-length cDNA obtained by nested 3′ RACE PCR (see Experimental Procedures for details).

Structurally, OX2R resembles OX2, itself containing 2 IgSF domains in a typical V/C2 set arrangement, a single transmembrane region, but has a larger cytoplasmic domain than OX2. The cDNA translations (Figure 2A) contain several unusual features including additional sequence before the predicted A strand at the start of the V domain, four conserved extracellular cysteine residues distinct from the canonical B to F strand IgSF disulphides in the V set domain, and two in the C2 set domain. There are eight and ten potential N-linked glycosylation



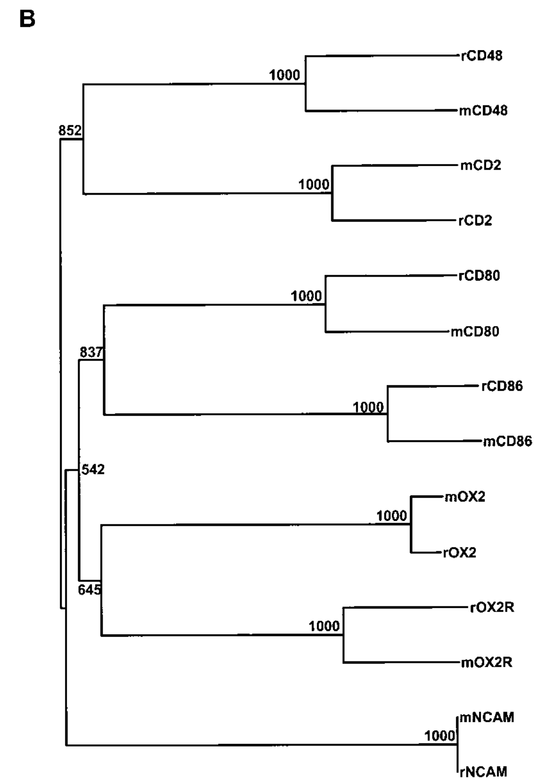


Figure 2. OX2 and Its Receptor Are Related Members of the Immunoglobulin Superfamily

(A) An alignment of the rat and mouse OX2R amino acid sequences. The two sequences have been split according to predicted domain organization. The superscripted bars in the extracellular regions predict the extent of the β sheets characteristic of the immunoglobulin fold by comparison to solved structures. The nucleotide sequences corresponding to these proteins have been deposited in the GenBank database under accession numbers AF231392 (rat) and AF231393 (mouse).

(B) Phylogenetic analysis of OX2 and its receptor. The two N-terminal IgSF domains of rat (r) and mouse (m) OX2R, OX2, CD80, CD86, NCAM, CD2, and CD48 were aligned using ClustalW (Thompson et al., 1994) and then manually refined before constructing a neighbor-joining tree with 1000 bootstrap trials.

sites in the rat and mouse, respectively, consistent with the very high level of glycosylation noted at the biochemical level (see above). Based on other IgSF interactions (Davis et al., 1998), the GFC face of the V domain is likely to be the site of OX2 binding and correspondingly is predicted to be free of N-linked carbohydrate.

The 67 amino acid cytoplasmic domain contains three conserved tyrosine residues, one of which is contained within a NPXY motif. NPXY motifs are known to bind PTB/PID domains that are present in signaling adaptor molecules such as SHC, IRS-1, and Numb (Forman-Kay and Pawson, 1999), suggesting that engagement by OX2 may lead to intracellular signals affecting macrophage function.

A phylogenetic analysis (Figure 2B) of the OX2R extracellular sequence with respect to other members of the IgSF reveals that the OX2R is closely related to OX2 itself, implying the two proteins have evolved from a common ancestral protein.

The OX102 Antigen Is the Receptor for OX2

The effective blocking of OX2 bead binding to macrophages by the OX102 mAb is a good indicator that the OX102 antigen is the OX2R. To formally prove this, the

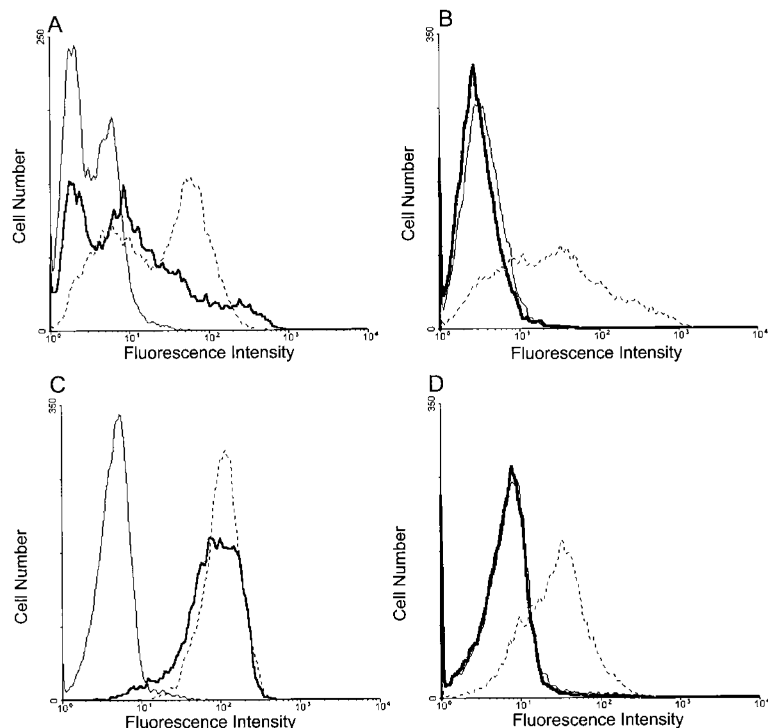


Figure 3. The OX2R Binds OX2

(A) HEK 293T cells were transfected with rat OX2R cDNA and stained for OX102 mAb (bold line), the endogenous human MHC class I with mAb W6/32 (dotted line), and a negative control mAb (OX21, thin line). Mock transfected cells stained with OX102 mAb showed no staining greater than the negative control (data not shown).

(B) rOX2R cDNA transfected 293T cells preincubated with OX102 (bold), but not W6/32 (dotted), were able to block binding of rOX2-coated beads back to a negative control (thin line). Mock transfected cells did not bind rOX2-coated beads (data not shown).

(C) Staining of rat thymocytes with mAbs OX1 (dotted line), OX2 (bold line), and an isotype-matched negative control OX21.

(D) Rat thymocytes preincubated with mAb OX2 (bold), but not OX1 (dotted), were able to block the binding of rat OX2R-coated beads back to the negative control (thin line).

The negative controls in (B) and (D) are CD4d3+4-coated beads.

full-length rOX2R cDNA was expressed in a human kidney endothelium cell line. Transfected cells could be stained with the OX102 mAb (Figure 3A) and could also bind OX2-coated beads (Figure 3B). The binding of OX2-coated beads could be prevented by preincubating the cells with OX102 but not an irrelevant antibody (W6/32) (Figure 3B), showing conclusively that the OX102 antigen is the rOX2R.

The interaction was demonstrated in the reverse orientation by producing a recombinant, soluble form of the rOX2R with a C-terminal peptide to allow specific biotinylation of the protein using the BirA enzyme and attachment to avidin-coated beads (Brown et al., 1998). Rat thymocytes, a major site of OX2 protein expression (Figure 3C), bound rOX2R-coated beads, and this binding could be blocked by prior incubation of the cells with the OX2 mAb (Figure 3D). This implies that OX2 is the only major ligand for OX2R on rat thymocytes and independently confirms that OX2 mAb recognizes the interacting face on the OX2 protein (Preston et al., 1997). A soluble form of the mouse OX2R behaved in a similar manner (data not shown), showing that mouse OX2R binds rat OX2 consistent with earlier results (Preston et al., 1997).

Kinetic Analysis of the Rat OX2–OX2R Interaction

Many of the interactions between proteins at the surface of leukocytes are very weak, but they can be quantified using surface plasmon resonance and recombinantly produced extracellular regions of these proteins (van der Merwe and Barclay, 1996). The affinity of the rat OX2–OX2R interaction was calculated at 37°C by injecting a series of different concentrations of purified soluble OX2CD4d3+4 over OX2RCD4d3+4-biotin and a negative control (CD4d3+4-biotin) immobilized on a streptavidin-coated chip in a BIAcore apparatus. The binding is represented by the difference in response units (RU) observed in the rOX2R and control flow cell (Figure 4A) once equilibrium has been reached and is plotted as a binding curve (Figure 4B). A K_D of 2.5 μM was obtained by nonlinear curve fitting to this binding curve, which agrees well with a K_D of about 2 μM obtained from a linear fit to a Scatchard plot of the same data (Figure 4B, inset).

Kinetic analysis of the interaction at 37°C yielded a k_{off} of 0.8 s^{-1} for both high and low levels of OX2R immobilization, indicating that kinetic measurements were not grossly affected by rebinding or mass transport effects (Figure 4C). k_{on} values were calculated by fitting the association binding phase to a simple 1:1 binding model using a k_{off} of 0.8 s^{-1} and were about 4×10^5 $M^{-1}s^{-1}$. These values are comparable to those measured for other known protein–protein interactions mediated by migratory cells (van der Merwe and Barclay, 1996; Maenaka et al., 1999).

rOX2R Is Expressed by Cells of the Myeloid Lineage and Can Be Phosphorylated upon Pervanadate Treatment

The rOX2R appears restricted to the myeloid lineage as shown by OX102 mAb staining. Macrophages isolated from the peritoneal cavity (Figure 1A), lung, spleen (data not shown), and leukocyte dendritic cells (Figure 5A) were highly positive. Isolated microglia, the resident macrophage of the brain (Figures 5B and 5C), and half the OX41$^+$ (SIRP) myeloid population from peripheral blood (Figure 5D) were stained, but peripheral blood B and T cells (Figures 5E and 5F), activated lymphocytes, and thymocytes (data not shown) were not stained. The lower level of OX2R expression on microglia is attributed to receptor downregulation due to the high level of OX2 expression in the CNS.

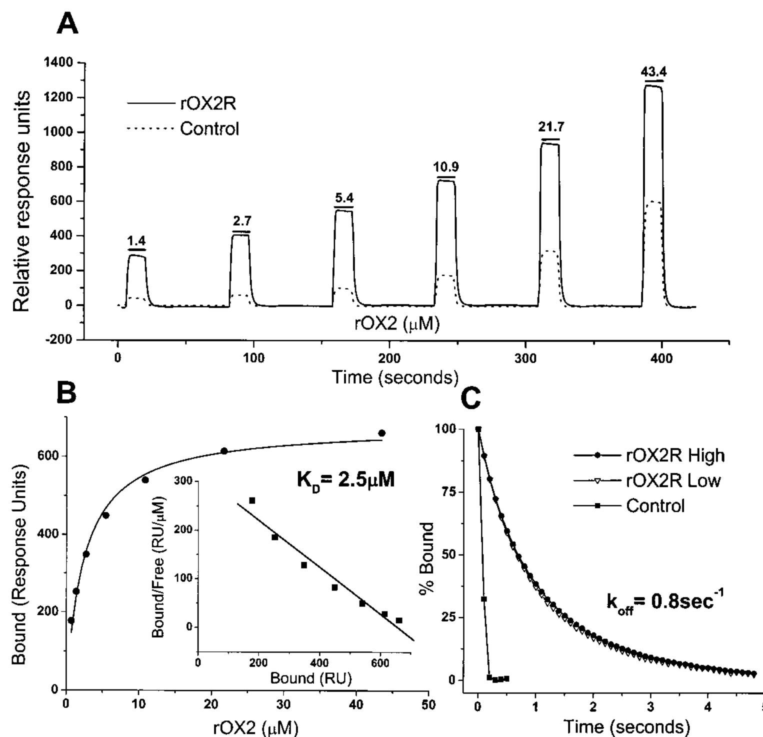


Figure 4. The Affinity and Kinetics of the Rat OX2–OX2R Interaction

All experiments were performed at 37°C. (A) The indicated concentrations of rOX2CD4d3+4 (rOX2) were injected at 20 μl/min through flow cells with 1010 RU of rOX2RCD4d3+4-biotin (rOX2R) or 1400 RU of CD4d3+4-biotin (Control) immobilized. The amount of rOX2 that bound rOX2R at each concentration was calculated as the difference between the response at equilibrium in the rOX2R and control flow cell and is plotted in (B). A Scatchard transformation of this binding data is shown inset in (B), and the linear fit shown corresponds to a K_D of about 2 μM. (C) The dissociation constant of the rat OX2–OX2R interaction was measured by injecting 5 μl of soluble rOX2CD4d3+4 (14.5 μM) at 100 μl/min over immobilized rOX2RCD4d3+4-biotin at high (1010 RU) and low (600 RU) levels and also a negative control CD4d3+4-biotin (1100 RU), and data were collected at 10 Hz. The response in the control flow cell was subtracted from the rOX2R flow cells. The data were then normalized (100% at the start of the dissociation phase), and first order exponential decay curves were fitted to the data that yield k_{off} values of 0.80 s^{-1}.

There are three tyrosine residues in the cytoplasmic domain of OX2R, suggesting that this region may be involved in the recruitment of intracellular proteins in a phosphotyrosine-dependent manner. Treatment of rat RPCs with pervanadate, which inhibits tyrosine phosphatase activity, gave a strong anti-phosphotyrosine band after rOX2R immunoprecipitation from treated but not untreated cell lysates (Figure 5G). This demonstrates that rOX2R is a substrate for tyrosine kinases present in macrophages and implies the ability to recruit intracellular signaling/adaptor molecules in a phosphate-dependent manner.

OX102 mAb Exacerbates the Disease Model Experimental Allergic Encephalomyelitis

The distribution of OX2 and its receptor point to a role in the tissue-specific regulation of macrophage biology. To test this possibility in vivo, the effect of the blocking mAb, OX102, was tested in a neural inflammation model, experimental allergic encephalomyelitis (EAE). EAE is characterized by microglial activation and perivascular infiltrates of macrophages and $CD4^+$ T cells leading to tissue damage that results in paralysis that resolves over a few days. Importantly, disease induction is dependent on both the presence (Huitinga et al., 1990) and activity (Martiney et al., 1998) of peripheral macrophages, implying a central role for macrophages in disease pathogenesis. EAE was induced in Lewis rats and the OX2–OX2R interaction prevented by administering the OX102 mAb just prior to disease onset. The treated animals developed more severe EAE compared to controls, although the duration was similar (Figure 6). These data support a role for the OX2–OX2R interaction in the regulation of macrophage/microglia activity in vivo.

Discussion

The most common structural class of leukocyte-restricted membrane proteins contains two IgSF domains and represents 15% of all proteins found at the leukocyte cell surface; OX2 and its receptor fall into this category (Barclay et al., 1997). Sequence comparison of OX2 and its receptor revealed similarities over and above those between other members of the IgSF, indicating that the proteins have evolved from a relatively recent common ancestral precursor. This close receptor:ligand evolutionary relationship has been observed in other IgSF interactions, notably within the CD2 family subset (Figure 2B) (Davis and van der Merwe, 1996; Brown et al., 1998), suggesting that receptor:ligand pairs may often evolve from a single gene product originally able to mediate homophilic binding (Williams and Barclay, 1988). The OX2 and OX2R both have characteristic and unusual patterns of additional cysteine residues in their V-like domains that distinguish them from the B7 and CD2 subsets that they resemble in topology.

One unusual feature of the OX2R is its high content of potential N-linked glycosylation sites, with eight and ten in the rat and mouse sequences, respectively (not including the unmodified site as determined by peptide sequencing) (Figure 2A). Two potential sites were glycosylated as determined by direct peptide sequencing, and many are likely to be occupied given the change in apparent Mr seen on removal of N-linked sugars by PNGaseF treatment from ~90 kDa to ~25 kDa, indicating a content of around 70% by weight of carbohydrate. This is very high for N-linked carbohydrates and is more typical of O-linked sugars found in mucins (Gum, 1992). However, both mutagenesis data (Preston et al., 1997) and a comparison to similar IgSF interactions (van der

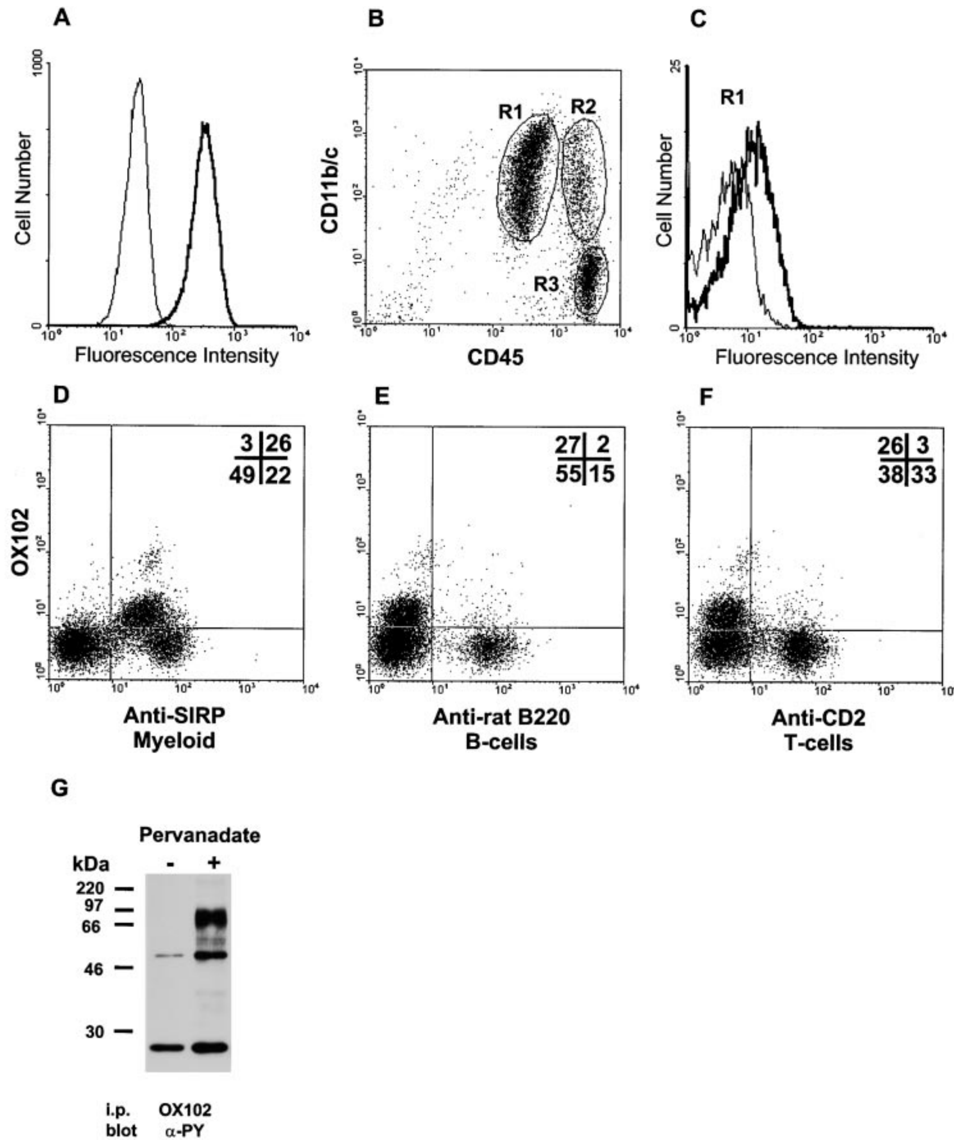


Figure 5. OX2R Is Restricted to the Myeloid Lineage in Rat and Can Be Tyrosine Phosphorylated upon Pervanadate Treatment

(A) Rat dendritic cells were defined as highly granular, OX62 mAb–positive cells in thoracic duct lymph collected from mesenteric lymphadenectomized rats (Pugh et al., 1983) and were stained with OX102 (bold line) and an isotype-matched negative control (OX21).

(B) Adult CNS leukocyte and microglial cell characterization. Gates are as follows: R1, microglia ($CD45^{lo}$, $CD11b/c^{+}$); R2, CNS-associated macrophages/monocytes/granulocytes ($CD45^{hi}$, $CD11b/c^{+}$); R3, lymphocytes ($CD45^{hi}$, $CD11b/c^{-}$).

(C) R1-gated microglia are stained with biotinylated OX102 (bold line) or an isotype-matched negative control. Around 20% of cells in R2 were $OX2R^{+}$, representing CNS vascular associated macrophages and blood granulocytes, and cells in R3, mostly lymphocytes, were not stained (data not shown).

(D–F) Peripheral blood leukocytes were stained using biotinylated OX102 and isotype-matched anti-rat lineage markers. Half the $OX41^{+}$ (SIRP) myeloid population that includes monocytes and granulocytes were OX102 positive (D), but B cells (E) and T cells (F) recognized by OX33 (Anti-rat B220) or OX34 (CD2), respectively, were not stained.

(G) Anti-phosphotyrosine (α-PY) Western blot of OX102 mAb immunoprecipitates from rat RPCs that were left unstimulated (−) or were stimulated for 10 min with 1 mM sodium pervanadate (+). The bands at 50 and 25 kDa, in both lanes, are the OX102 mAb heavy and light chain (respectively), which were visualized due to the peroxidase-conjugated anti-mouse secondary antibody.

Merwe et al., 1993) suggest that the OX2–OX2R interaction is mediated by protein and not carbohydrate. It is possible that the carbohydrate is important in preventing unwanted *cis*-interactions and maintaining the orientation of the OX2R with respect to the membrane.

Several 2-IgSF domain containing CAMs expressed by leukocytes are able to specifically interact with more than one receptor. This has been demonstrated in human and rodent models for both CD80 and CD86 (both bind CTLA-4 and CD28) (Tivol et al., 1996) and more recently for the CD48 (binding CD2 and 2B4) protein (Brown et al., 1998). It is feasible that OX2 or OX2R also have multiple ligands, although an interaction that could not be fully blocked by either OX102 or OX2 mAbs has not been detected (data not shown).

The cytoplasmic region of the OX2R contained a NPXY motif. These motifs are known to interact with the PTB/PID binding domains (Kavanaugh et al., 1995) present

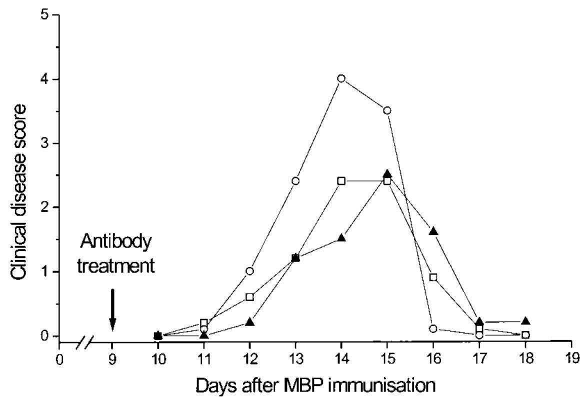


Figure 6. Administration of OX102 mAb Exacerbates a Macrophage-Dependent Neural Inflammation Disease Model

Lewis rats (five per group) were immunized with guinea pig myelin basic protein to induce EAE as described (MacPhee et al., 1989), and 2 mg of purified mAbs OX102 (open circle), OX21 isotype-matched control (open square), or PBS (closed triangle) were administered per animal just prior to disease onset. Clinical disease scores are the calculated means from each group. The experiment is representative of two separate experiments with comparable outcomes.

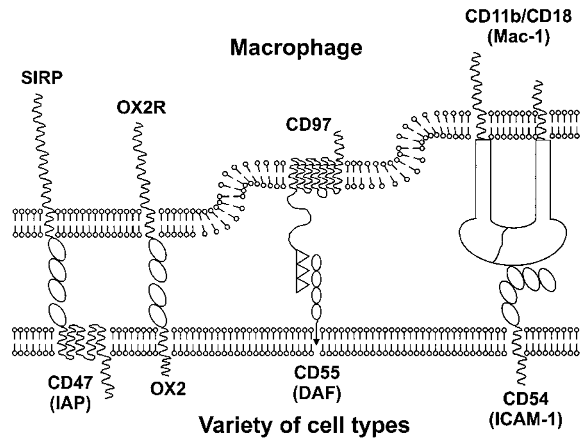


Figure 7. Scale Diagrammatic Representation of Some of the Possible Interactions between Macrophages and Nonlymphoid Cells

The dimensions of the proteins are based on known structures as described for T cell interactions (Barclay et al., 1997). The distal N-terminal Ig-like domain of SIRP contains the binding site for the single Ig-like domain of CD47 (Vernon-Wilson et al., 2000) and is thus predicted to have the shown topology. The topology of the CD55/CD97 interaction is not known, although a construct containing three EGF domains of CD97 gave the strongest binding to CD55, indicating that the two proteins may overlap rather than interact end on (Hamann et al., 1998). The integrin Mac-1 (CD11b/CD18) interacts with the third domain of CD54 (Diamond et al., 1991).

in several signaling adaptor molecules. These adaptor molecules are recruited to the cell membrane during signaling processes and therefore offer a potential biochemical mechanism to initiate signal transduction as a consequence of OX2R ligation. Several PTB/PID domains have been structurally characterized, and binding is not always dependent on the tyrosine being phosphorylated within the motif (Howell et al., 1999). NPXY motifs are found in the cytoplasmic regions of β integrins, and their mutation leads to defects in cell migration and spreading (Patil et al., 1999).

The functional consequence of a cell surface interaction can be suggested by identifying the cells and tissues that are able to interact. OX2 is expressed on the cell surfaces of a diverse range of tissues that arguably will not share the need for a recently evolved signal delivered through the OX2 protein. This is supported by the fact that the OX2 cytoplasmic tail is short (19 amino acids) and contains no known signaling motifs. In contrast, a more restricted and functionally related cell type expresses the OX2R, which contains a substantial cytoplasmic region that has been shown to support tyrosine phosphorylation and is presumably able to initiate an intracellular signal. This would imply that the role of OX2 is to "mark" certain tissues that have a common need to deliver a regulatory signal to myeloid cells expressing OX2R. The increase in severity of a macrophage-dependent inflammatory disease after blockade of the OX2–OX2R interaction implies that OX2R ligation might deliver a restrictive signal to macrophages and thus limit macrophage activation and subsequent tissue damage in inflamed sites. It is well known that macrophages isolated from different tissues exhibit a range of different morphologies and functional phenotypes, and these differences are attributed to the localized tissue microenvironment in which the macrophage resides (Gordon, 1995; Naito et al., 1996). These include the resident brain macrophages or microglia that have both a distinctive ramified morphology and exhibit a general "downregulation" of typical macrophage functions (Perry and Gordon, 1991). It is possible that the high levels of OX2 expression influence the development of macrophages in the CNS leading to the microglial phenotype. The phenotype of OX2-deficient mice is consistent with this interpretation (R. M. Hoek et al., submitted), and it is likely that OX2R signaling has similar effects on myeloid function in other OX2-positive tissues.

While many soluble factors have been shown to modify macrophage activity, only few membrane proteins have been shown to do so. The regulation of macrophage activity by direct cell–cell contact would allow a more localized control than that mediated by cytokines. The only other known myeloid cell surface proteins that have characterized membrane ligands that are broadly distributed are Mac-1 (CD11b/CD18), CD97, and SIRP. The SIRP myeloid marker (Adams et al., 1998) interacts with CD47 (Jiang et al., 1999; Seiffert et al., 1999; Vernon-Wilson et al., 2000), CD97 with CD55 (DAF) (Hamann et al., 1996), and Mac-1 with CD54 (Diamond et al., 1991). These interactions are depicted as they might occur at the surface of cells in Figure 7. Interestingly, in common with the CD2 subfamily, it is the OX2 V-like domain that binds the receptor (Preston et al., 1997), suggesting the complex is arranged with four tandem Ig-like domains spanning a distance similar to that measured for the TCR/MHC and CD2/CD48 complexes—around 15 nm. Both the molecular topography of these complexes (Wild et al., 1999), and their organization within the zone of cell:cell contact—"the immunological synapse" (Grakoui et al., 1999; Monks et al., 1998)—has been shown to be important for antigen-specific T cell activation. The finding of further interactions, such as the OX2–OX2R and SIRP–CD47, with this spatial organization suggests that this distance is important for intracellular communication in a wider context than just antigen-specific T cell activation. It is therefore likely that the formation of similar zones of contact or a "macrophage synapse" may well be critical in the tissue-specific control of macrophage functions.

Experimental Procedures

Monoclonal Antibodies

The monoclonal antibodies used were: OX1 (anti-rat CD45), OX2 (anti-rat OX2), OX21 (anti-human C3bi), OX33 (anti-rat CD45ABC), OX34 (anti-rat CD2), OX41 (anti-rat SIRP), OX42 (anti-rat CD11b/c), OX45 (anti-rat CD48), OX62 (anti-rat dendritic cells), OX68 (anti-rat CD4d3+4), W6/32 (anti-human HLA). All mAbs are referenced by the European Collection of Animal Cell Cultures (ECACC; Porton Down, Salisbury, UK).

Production of OX102 mAb

Male BALB/c mice at 6 weeks of age were immunized six times with either 0.1–0.25 mg of a crude Tween-40 membrane fraction solubilized from rat (PVG) thioglycollate-elicited peritoneal cells or unlysed whole cells. A mouse producing a good immune response to resident rat peritoneal cells (RPCs) was boosted by an intrasplenic injection of 4×10^6 unlysed RPCs. The spleen was removed 4 days later and fused to NS-1 myeloma cells to produce hybridomas using standard techniques. Hybridoma supernatants were screened for their ability to bind the rat RPC cell surface by flow cytometry and then for their ability to block the binding of rOX2CD4d3+4-coated beads. One antibody was obtained; the hybridoma was recloned three times and named OX102.

Purification of the rOX2R

The OX102 antigen was purified by affinity chromatography from rat splenic extracts as in Sunderland et al. (1979) except the antigen was not eluted by the normal high pH but required 0.5% SDS at 55°C. Amino-terminal sequencing was performed using an automated Edman degradation in an Applied Biosystems Procise 494A protein sequencer (Perkin-Elmer Ltd, UK).

Cloning and Expression of rOX2R

The cDNA for the OX102 antigen was cloned by a modification of the SMART system (Clontech, Palo Alto, CA) using cDNA primed and tagged at the 3′ end with gactcgagttgacatcgagg$(t)_{20}$v and at the 5′ end using aagcagtggtaacaacgcagagtacgcggg in a standard cDNA synthesis using Superscript II (GIBCO-BRL, UK) reverse transcriptase and poly$(A)^+$ RNA from rat (PVG) RPCs. The 5′ end of the rOX2R cDNA was amplified in a 5′ RACE RT-PCR using 5′ chemically phosphorylated oligonucleotides to the 5′ tag (aagcagtggtaacaacg cagagt) and an antisense degenerate oligonucleotide (tgcatngtytgrt tyttrtc) corresponding to amino acids DK(N)QTMQ. PCR products at a low annealing temperature (44 ± 2°C) were blunt-end cloned into SmaI-digested and phosphatase-treated PCRScript vector (Stratagene, La Jolla, CA). Sequencing of inserts revealed several clones containing the N-terminal SCP peptide sequence and an in-frame typical leader sequence. The full-length cDNA was cloned by a 3′ RACE reaction using ga*ctcgag*ttgacatcgagg (antisense) against the 3′ tag and tcatg*gcggccgc*tgctcccctac (sense) designed from the leader sequence. The oligonucleotides contain NotI and XhoI sites (in italics) that were used to clone the 3′ RACE PCR product prior to sequencing. The full-length mouse OX2R was cloned by nested 3′ RACE PCR as above but using sense oligonucleotide gagtgagcggcggaaaaccagaaaaccgaaatg initially and then gagcggc gg*gcggccgc*aaaaccgaaatgttttgct and BALB/c mouse RPC poly$(A)^+$ mRNA as a template. The full-length rOX2R cDNA was blunt-end ligated into the eukaryotic expression vector pEF-BOS and transfected by the calcium phosphate method into HEK293T cells. Both rat and mouse soluble OX2R proteins were produced by amplifying the extracellular regions of OX2R by PCR using oligonucleotides (sense) ccctacctg*tctaga*gaaagagcaccgagtgag and (antisense) cctaa taat*gtcgac*ccccctctacccagttc. Products were ligated in frame using XbaI/SalI digestion to rat *CD4d3+4* (constructs contained the sequence LGRGG*STSIT*) and a BirA biotinylation sequence at the C terminus of the protein (in vector pEF-BOS-XB). The proteins were expressed in HEK293T cells, enzymatically biotinylated, and used as described (Brown et al., 1998).

Affinity and Kinetic Analysis of the OX2–OX2R Interaction

Affinity and kinetic analysis was performed on a BIAcore 2000 (BIAcore AB, St. Albans, UK) using running buffer HBS (10 mM HEPES [pH 7.4], 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) (BIAcore AB) at 37°C. Approximately 4000 RU of streptavidin (Pierce Chemical Co, Rockford, IL) dissolved in 10 mM sodium acetate (pH 5.5) was coupled using amine coupling to a CM5 research grade chip (BIAcore AB) at 0.2 mg/ml. Biotinylated rOX2RCD4d3+4 protein and CD4d3+4 (control) were immobilized at indicated levels in individual flow cells. K_D values were obtained both by nonlinear curve fitting of the Langmuir binding isotherm and Scatchard transformations of the binding data. k_{off} values were determined by fitting a first order exponential decay curve to normalized data (Origin, MicroCal software, version 5.0) obtained from individual flow cells containing rOX2RCD4d3+4 after the subtraction of the negative control (CD4d3+4). k_{on} values were obtained by using this k_{off} value in BIAevaluation 3.0 software over the association binding phase.

Purification of rOX2CD4d3+4

Rat OX2CD4d3+4 soluble chimeric protein was purified by immunoaffinity chromatography using a OX68-Sepharose 4B column from the supernatant of a stably transfected CHO.K1 cell line (Preston et al., 1997). Purified protein was then fractionated by gel filtration on a Superdex S-200 column (Pharmacia, Uppsala, Sweden) prior to BIAcore analysis to exclude protein aggregates that are known to influence binding measurements (van der Merwe and Barclay, 1996). The extinction coefficient was determined by amino acid analysis to be 44,440 $M^{-1}cm^{-1}$. At least 90% of the rOX2CD4d3+4 protein could be immunoprecipitated using the OX2 mAb (data not shown), indicating that the bulk of the preparation was correctly folded and antigenically active.

Deglycosylation of rOX2R

Approximately 0.5 μg of purified rOX2R was incubated without or with 10 U of PNGaseF (NEB, Beverly, MA), and aliquots (3 ng) were removed at the indicated times. The aliquots were run under nonreducing conditions on a 10% SDS polyacrylamide gel, and proteins were blotted onto Hybond C-extra nitrocellulose (Amersham International plc., Bucks, UK) overnight at 30V and probed with mAbs OX102 or OX21 (negative control) for 1 hr at room temperature. A peroxidase-conjugated rabbit anti-mouse secondary serum (DAKO Immunoglobulins, Denmark) was used at 1:2000, developed using ECL+ luminescence detection system (Amersham), and detected using Kodak LS scientific imaging film (Kodak, UK).

Pervanadate Activation of Cells

We harvested 9×10^7 rat RPCs by peritoneal lavage in PBS, which were subdivided into two aliquots, and either stimulated at 37°C for 10 min with 1 mM sodium pervanadate or left unstimulated before lysis in ice-cold WOP-40 buffer (1% NP-40, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate supplemented with 0.4 mM sodium vanadate and protease inhibitors). For each immunoprecipitation, 2.25×10^7 cell equivalents were rotated with 50 μl 20% slurry of OX102 or a negative control mAb covalently attached to Sepharose overnight at 4°C. mAb-coupled Sepharose beads were pelleted and washed before boiling in 20 μl 2× SDS-PAGE sample buffer, resolved by SDS-PAGE, blotted, and probed using anti-phosphotyrosine mAb PY-20 (TCS, Bucks, UK).

Microglia Isolation

Nonperfused whole rat CNS (brain and spinal cord) from 8-week-old rats was digested with collagenase/Dnase, and cells were isolated over Percoll and subsequently stained and gated as described (Sedgwick et al., 1991).

Cell Staining Assays

Generation of the multivalent OX2 and CD2 binding reagents using either biotinylated or avidin-coated fluorescent beads (Spherotech Inc. Libertyville, IL) have been described previously (Preston et al., 1997; Brown et al., 1998). Dual labeling was performed as described previously using standard techniques using a streptavidin quantum red conjugate (Sigma, Dorset, UK) to detect biotinylated OX102 and a FITC-conjugated goat anti-mouse adsorbed against rat immunoglobulins (Serotec Ltd., Oxon).

EAE Experiments

Animals were housed according to institutional and Home Office regulations in the Sir William Dunn School of Pathology animal facility. Lewis rats (five per group) were immunized with guinea pig myelin basic protein (MBP) to induce EAE (MacPhee et al., 1989) and injected i.v. 9 days after MBP immunization (just prior to disease onset) with either 2 mg of purified mAbs in PBS or PBS alone. Disease was scored as in MacPhee et al. (1989).

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GenBank Accession Numbers

The nucleotide sequences corresponding to OX2R cDNA have been deposited in the GenBank database under accession numbers AF231392 (rat) and AF231393 (mouse).