Molecular and cellular properties of the rat AA4 antigen, a C-type lectin-like receptor with structural homology to thrombomodulin.

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Abbreviations used in this paper:

CTLD: C-type lectin-like domain; EGF: epidermal growth factor; C1qRp: phagocytic complement C1q receptor; MBL: mannose binding lectin; SPA: pulmonary surfactant protein A; CRD: carbohydrate recognition domain ; PAMPs: pathogen-associated molecular
patterns; PRR: pattern recognition receptor; TM: thrombomodulin; TMD: transmembrane domain; ISH: in situ hybridization; SP: signal peptide.
Summary:

The murine fetal stem cell marker AA4 has recently been cloned and is known to be the homolog of the human phagocytic C1q receptor involved in host defense. We herein report the molecular cloning and the cellular expression pattern of the rat AA4 antigen. Modular architecture analysis indicated that the rat AA4 is a member of C-type lectin-like family and, interestingly, displays similar domain composition and organisation to thrombomodulin. Northern blot and RT-PCR analyses indicated that rat AA4 was encoded by a single transcript of 7 kb expressed constitutively in all tissues. In situ hybridization showed that AA4 was expressed predominantly by pneumocytes and vascular endothelial cells. Using an affinity purified polyclonal antibody raised against a rat AA4-Fc fusion protein, AA4 was identified as a glycosylated protein of 100 kD expressed by endothelial cells > platelets > NK cells and monocytes (ED1+ cells). The staining was associated to the cell surface and intracytoplasmic vesicles. Conversely, erythrocytes, T and B lymphocytes, neutrophils, and macrophages (ED2+ cells) were consistently negative for AA4. As expected, the macrophage cell line NR8383 expressed weak levels of AA4. Taken together, our results support the idea that AA4/C1qRp is involved in some cell-cell interactions.
Introduction:

In 1983, McKearn and colleagues raised two monoclonal antibodies (mAb) against cell surface antigens expressed by mouse pre-B cells (1). One of them, the mAb clone AA4.1 recognized a cell surface antigen identified as a B cell lineage marker (2). In addition, it has since been shown that the AA4.1 mAb identifies multipotent hematopoietic stem cells (3-8).

Experiments have demonstrated that transfer of an AA4+B220+ subset of differentiated embryonic stem cells can reconstitute the lymphoid system of sublethally irradiated Rag-1-deficient mice, indicating that AA4 was an early marker of multipotent hematopoietic progenitors that can give rise to all mature blood cells (9). In the adult, mouse AA4 mRNA was detected in lung, heart and whole bone marrow and the AA4 antigen was found predominantly on vascular endothelial cells (10).

It is only recently that AA4 was cloned by two groups using either a murine B lymphoid retroviral cDNA library or a genomic library (10,11). AA4 antigen was identified as a type I glycosylated transmembrane protein with a C-type lectin-like domain (CTLD) (12), followed by a cysteine-rich domain composed of a tandem array of five epidermal growth factor (EGF)-like repeats. Collectively, the protein domain architecture and the cellular expression pattern seem to indicate that AA4 plays an important role in cell-cell interactions during hematopoietic and vascular development (10).

Mouse AA4 is homologous to the human phagocytic C1q receptor (C1qRp). AA4 and C1qRp possess similar domain structures and exhibit 68% amino acid identity (13). Human C1qRp is a 97 kD heavily O-glycosylated protein detected on cells of myeloid lineage, cultured endothelial cells and platelets (14,15). A mAb anti-C1qRp (clone R3, IgM subclass) was shown to abrogate moderately the C1q-mediated enhancement of monocyte
phagocytosis (16). In addition, it has been shown that the mannose binding lectin (MBL) and
the pulmonary surfactant protein A (SPA) are able to act on C1qRp and enhance phagocytic
function of human monocytes \textit{in vitro} (17). Taken together, these findings suggest that
C1qRp is a common component of a receptor for C1q, MBL and SPA, that have been shown
to recognize pathogen-associated molecular patterns (PAMPs) (18). It is now accepted that
these 'defense collagens’ can also bind to apoptotic cells or toxic cell debris and thus, would
constitute a link between a 'non-self’ target and macrophages bearing phagocytic receptors
(e.g. complement receptor type 3, CR3) (for review see Eggleton et al., (19)). It is possible
that the binding of C1q, MBL or SPA to C1qRp is instrumental in the intracellular signalling
and the clearance of invaders or toxic cell debris by phagocytes as part of an efficient innate
immune response. Of interest, particularly with respect to the role of C1q in the clearance of
potentially toxic cell debris, is the recent finding that C1q-deficient mice show a profound
impairment in the removal of apoptotic cells which then accumulate in the kidney leading to
glomerulonephritis (20). In addition, two independant groups have shown that C1q can bind
directly and specifically to surface blebs of UV-induced apoptotic cells leading to the
activation of the classical pathway of the complement system (21,22).

As yet, the major role of mouse AA4/human C1qRp remains elusive, although it seems
likely that signalling through this receptor will lead to enhancement of cellular functions and
cell-cell interactions (10,17,23). The simpliest view is that AA4/C1qRp is a critical
signalling component of a pattern recognition receptor (PRR), complex involved in the
clearance of target cells opsonised with defense collagens. It is now generally recognized that
many protein modules containing part or all of the CTLD motif serve functions other than
carbohydrate recognition (12). Conceivably, it is therefore possible that the CTLD of
AA4/C1qRp may well prove to be involved in the binding to target cells through specific
moieties, yet to be characterized.
To gain further insight into the role of AA4/C1qRp, we have cloned the rat homolog of the mouse AA4 and the human C1qRp and characterized its cellular/tissue expression pattern. In this manuscript, we present the full-length cDNA sequence and highlight the modular architecture of rat AA4. Rat AA4 bears a striking domain composition and organisation very similar to that of thrombomodulin (TM), a natural anticoagulant membrane protein (for review, see (24)). Interestingly, rat AA4 was concentrated to sites also known to express abundant levels of TM. AA4 was found on endothelial cells, platelets, undifferentiated monocytes (ED1+ cells) and, unexpectedly, on all circulating NK cells. Close examination of the immunofluorescent staining pattern, including confocal microscopy imaging of AA4-CHO transfected cells, indicated that AA4 is distributed at the cell membrane (5-30% of the staining) and is also contained within intracytoplasmic components (vesicles). Our understanding of the structure/function relationship of AA4/C1qRp together with that of TM is still in its infancy. Nevertheless, the current perception from this study and previous published observations is that AA4/C1qRp is involved in the enhancement of cell-cell interactions (e.g., phagocytosis, adhesion) leading to an efficient innate immune response against pathogens and toxic cell debris (10,17,23).
Experimental procedures:

Source of tissues and antibodies

Rat tissues were from adult male PVG obtained from the animal house of the University of Wales College of Medicine (UWCM, Cardiff, UK) unless otherwise stated. Mouse monoclonal antibodies against rat cell markers were respectively from PharMingen (Cambridge, UK): CD3, CD8a, NKR-P1A, CD45RA; Serotec (Oxford, UK): ED1; while CD11b/c (clone OX42), Thy1.1 (clone OX7) and ED2 antibodies were used as tissue culture supernatants prepared from hybrydomas grown in house. All the hybridoma cell lines were obtained from the American Type Culture Collection (ATCC). The mouse (clone OX23) and the rabbit anti-human factor H (irrelevant antibody) were generously provided by Dr M. Fontaine (INSERM U519, Rouen, France).

Cell Culture

Primary cultures of rat endothelial cells were prepared from embryonic PVG rat hearts (18 days postcoitum). Briefly, tissues were dissected and minced with a scalpel in Hanks’ balanced salt solution (HBSS). After 3 washings in HBSS, tissues were incubated for 15 min at 37°C in HBSS with DNase I (2 µg/ml), 0.025% Trypsin (w/v) and collagenase type III (5µg/ml). After enzymatic digestion, dissociated cells were resuspended and washed in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated (30 min, 56°C) fetal calf serum (FCS) and filtered through a 70 µm nylon sieve. Cells were then plated out at 10^5 cells/collagen-coated glass coverslip (16 mm in size). The culture medium was changed every day until day 3 when the cells where used for immunostaining.
The Chinese hamster ovary (CHO-K1) cell line obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK) was cultured in F-12 medium. The rat alveolar macrophage cell line (NR8383), kindly provided by Dr DP Ramji (MOMED, University of Cardiff), was cultured in F-12K medium. Cells were grown in media supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin and 1 µg/ml amphotericin B. All reagents used for cell culture were from Life technologies (Paisley, UK). Cultures were maintained at 37°C in a humidified incubator with 5% CO2.

Rat leukocytes where isolated from fresh blood (adult male Wistar) as follows. Whole blood (5 ml) in 10 mM EDTA was spun at 1300 g for 20 min. The buffy coat was harvested and washed in RPMI medium, diluted to 10 ml in RPMI, then underlayed with 5 ml of Histopaque-1083 (Sigma) and centrifuged for 30 min at 900 g at room temperature. The leucocyte-containing interface was harvested, washed in PBS/BSA three times then standardized to a cell density of 10^6 cells/ml.

**Primers**

Five oligonucleotide primers were chosen within highly conserved regions between the mouse AA4 and the human C1qRp cDNA sequence (Accession numbers AF081789 and HSU94333 respectively) using the DNAstar Software (DNASTAR Inc, Madison, USA). Their sequences from 5’ to 3’ and their positions on the mouse cDNA coding sequence were as follows: 5’TTTGTTCCTGCTGCTGGGGC3’ (18 up),

5’GCTGTGCCTTTTGCCTCTGTAGCCA3’ (637 up),

5’CACCACCCACCCAGCTGAAG3’ (336 down), 5’ACAGAGGCAAAAGGCACAGC3’
(637 down) and 5’TCAGCAGTCTGTCCCTGGTG3’ (1916 down). Primers were from Life Technologies.

**Synthesis and isolation of a rat AA4 cDNA probe**

Total RNA was extracted from rat spleen using the Ultraspec™ RNA isolation kit (Biotecx, Houston, USA) according to the manufacturer’s procedure. An aliquot of total RNA (2 µg) was reverse-transcribed at 37°C for 1 h in a 30 µl final reaction volume with 60 U RNasin (Promega, Southampton, UK), 0.5 mM dNTPs (Bioline, London, UK), 250 pmol random hexamer primers (pdN6, Amersham Pharmacia, Rainham, UK) and 400 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Life Technologies) in the reaction buffer (10 mM Tris/HCl (pH 8), 15 mM KCl, 0.6 mM MgCl₂ and 5 mM DTT). PCR was carried out with 3 µl of reverse-transcribed RNA mixture, in a 50 µl final reaction volume with 20 pmol of each AA4 primer (637 up and 1916 down), 0.2 mM dNTPs and 2.5 U of Taq DNA polymerase in 10X buffer B (Promega). The cycling parameters comprised 94°C for 45 s, 60°C for 45 s and 72°C for 90 s for a total of 30 cycles, with a final extension cycle of 72°C for 10 min. All PCR were performed using a Hybaid Omnigene (Teddington, UK) thermocycler. The RT-PCR product of 1.3 Kb was purified on agarose gel using the Geneclean® DNA purification kit (Bio 101, Anachem Ltd, Luton, UK), and subcloned into the pGEM®-T vector (Promega). This construct was used as template to PCR amplify a 1.3 Kb coding region fragment.

**Screening of rat lung cDNA library**
The cDNA library was made as previously described (25). Briefly, the library was constructed using the λZAP® II vector and poly(A)+ lung RNA isolated from rats (Wistar) injected with LPS. The library was plated out onto 12 NZY agar plates (140 mm) to give ~50,000 colonies per plate. Plates were incubated at 37°C for 8 h then duplicate colony lifts were taken from each plate onto Hybond-N nylon membranes (Amersham Pharmacia). The membranes were sequentially washed in 1.5 M NaCl / 0.5 M NaOH, 1.5 M NaCl/ 0.5 M Tris/HCl (pH 8) and 0.2 M Tris/HCl (pH 7.5)/2X SSC. Membranes were then air dried on Whatman paper and the DNA cross-linked to the membrane using the Stratalinker 2400 UV crosslinker (120,000 µJ of UV energy for 30 s) from Stratagene (Cambridge, UK). Membranes were prehybridized for 1 h in Rapid-Hyb buffer (Amersham Pharmacia) at 65°C, then incubated overnight at 65°C with labeled rat AA4 1.3 kb cDNA probe diluted in the same buffer. Membranes were washed twice in 1X SSC/0.1% (w/v) SDS at room temperature for 15 min and exposed on X-ray film (Kodak, Sigma) overnight at -70°C. Positive colonies were identified by comparison of the colony lifts and agar plugs (~10 mm diameter) were taken from these areas on the primary plates. Phages were eluted by incubating the plugs in SM buffer (100 mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris/HCl (pH 7.5), 0.01% (w/v) gelatin) overnight at 4°C.

Screening, isolation of single phage clone and phagemid isolation

Eluates (0.2 ml) from 44 positive agar plugs were centrifuged to remove debris. The supernatants were ethanol precipitated and the pellets, after centrifugation, were resuspended in 10 mM Tris. Phage solutions (20 µl) were PCR amplified as described above using 20 pmol of each 18 up and 336 down primers. The cycling parameters comprised 94°C for 45 s,
60°C for 45 s and 72°C for 60 s, for a total of 35 cycles, with a final extension cycle of 72°C for 10 min. PCR products were resolved on a 1% (w/v) agarose gel in 1X Tris Borate EDTA (TBE).

Three original positive phage clones were isolated by secondary and tertiary screening. They were transferred from the UNI-ZAP vector to SOLR bacteria by in vivo excision using the ExAssist/SOLR system (Stratagene) according to the manufacturer’s instructions. Bacteria containing the desired phagemid were then grown overnight in 10 ml Luria-Bertani (LB) broth. Bacterial lysates were PCR screened using 18 up and 336 down primers as described above. Positive samples were then selected for further analysis. The phagemid was isolated from the SOLR bacteria using the QIAprep Spin Miniprep Kit (QIAGen Ltd., Crawley, UK) and used for sequencing using an automated sequencer ABI model 373A (Perkin and Elmer, Buckinghamshire, UK) and Prism labelling kit (Applied Biosystems, Warrington, UK).

**Northern blotting**

A small AA4 specific cDNA fragment (18-637) subcloned into pGEM-T vector was PCR amplified using T7 and SP6 primers. The cDNA was labeled using the Megaprime DNA labelling system and [α-32P]dCTP (Amersham Pharmacia). Unincorporated nucleotides were removed using a ProbeQuant G-50 Micro Column (Amersham Pharmacia). The probe was denatured at 95°C for 5 min before hybridization.

For Northern blot analysis, total RNA (20 µg) from various rat tissues (adult male Wistar) were prepared by Trizol (Life Technologies) according to the manufacturer’s instructions, heat denatured for 15 min at 65°C and then separated by electrophoresis into 1% (w/v) agarose formaldehyde gel. The RNA was transferred overnight in 20X SSC onto Hybond-N+...
nylon membrane (Amersham Pharmacia) and UV cross linked to the membrane. The membrane was prehybridized at 65°C for 1 h in QuickHyb solution (Amersham Pharmacia) and hybridized in the presence of the radio-labeled probe at 65°C. After washing once in 2X SSC, 0.1% (w/v) SDS for 10 min at room temperature and twice in 0.1X SSC, 0.1% (w/v) SDS for 15 min at 65°C the membrane was exposed to X-ray film (Sigma) for 2.5 days at -80°C.

**RT-PCR (Reverse Transcription and Polymerase Chain Reaction)**

RT was performed using the same amount (2 µg) of total RNA from each tissue using the protocol as described above. PCR was performed using the following specific primers:

5’GCCACAGGCAGCCGACTCAT3’ (rat 1646 up) and

5’TCAGCAGTCCGTCCCAGGTG3’ (rat 1913 down). These rat specific primers were selected on the basis that they will overlap the boundary between exons 1 and 2 as recently described for the mouse AA4 gene. The mouse AA4 genomic sequence (NCBI accession number AF074856) reveals a unique intronic sequence of 248 bp localized at position 1911 on the cDNA sequence (11). All samples were subjected to RT-PCR for GAPDH as a housekeeping gene and positive control. GAPDH primers were:

5’GAACGGGAAGCTTGTCATCA3’ (sense) and 5’TGACCTTGCCCACAGCCTTG3’ (antisense). RT-PCR products were resolved into 1% (w/v) agarose gel (containing 25 µg ethidium bromide/100 ml gel) in 1X TBE buffer and visualized under UV light. RT-PCR products were sequenced as described above to assess the specificity.

*Generation of digoxigenin-labeled UTP riboprobes and In Situ Hybridization (ISH)*
Sense and antisense riboprobes were prepared by in vitro transcription of a rat AA4 cDNA fragment (112-637) cloned into pGEM-T in the presence of digoxigenin-11-UTP (Boehringer Mannheim, Lewes, UK) according to the manufacturer’s instructions. A test strip was used to confirm the equal digoxigenin label incorporation between sense and antisense probes. Rat brain was isolated and snap frozen in isopentane. Frozen sections were cut on a cryotome® SME (Shandon, Cheshire, UK) and adhered to super-frost glass slides (Surgipath Europe Ltd., Peterborough, UK). Rehydrated sections, 12 µm thick, were fixed in 4% (w/v) paraformaldehyde for 15 min in PBS and washed three times in PBS. Sections were hybridized and developed as previously described (26,27). The sections were allowed to air dry then rapidly dehydrated in alcohol, cleared in xylene and mounted in a neutral mounting medium (XAM) from BDH (Poole, UK).

Expression of the cloned cDNA in mammalian cells

The coding region for rat AA4 was amplified using a 5’ specific primer containing a XbaI site (in bold) and a 3’ specific primer containing a BamHI site (in bold). The sequences of these primers were as follow : 5’GCTCTAGAGAATGGTCACCTCAACTGGTTTGC3’ (sense) and 5’CGGGATCTCAGCAGTCCGTCGGGTG3’ (antisense). The ATG start codon is underlined. The PCR product was subcloned into XbaI and BamHI sites of the expression vector pDR2ΔEF1-α kindly provided by Dr I. Anegon (INSERM U437, Nantes, France). This vector containing the insert or without insert (vector control) was transfected into CHO using the LipofectAmine Plus™ Reagent (Life Technologies) according to the manufacturer’s procedure. Stable transfectants were selected in F-12 medium containing 400 µg/ml of hygromycin B (Life Technologies) for two weeks and maintained in medium containing 100 µg/ml of hygromycin B. The level of expression of AA4 was tested by FACS
(cell in suspension) and by immunofluorescent staining of adherent cells cultured on glass coverslips (see above).

**Generation and purification of rat AA4-Fc fusion protein**

In order to generate a soluble recombinant form of the rat AA4, a cDNA encoding the first 235 (amino terminus) amino acids of the mature protein including the whole CTLD was amplified by PCR using Pwo DNA proof-reading polymerase (Hybaid GmbH, Ashford, UK). The plasmid containing the full length rat AA4 cDNA was used as a template. Primers used for amplification incorporated restriction sites enabling ligation into the expression vector Signal pIgplus (R&D systems, Abingdon, UK) following digestion of the PCR product with XbaI and BamHI (Amersham Pharmacia) and the vector with NheI and BamHI. Primers were as follows: 5’TTCTCTAGAGCTGCTGCTGATTCAGAGGCT3’ (sense) and 5’TTGGGATCCCTGACACAGAGGCGCTGAGCTGC3’ (antisense).

The PCR product was ligated into the expression vector Signal pIgplus according to the manufacturer’s instructions, ensuring that it was in frame with DNA encoding the hinge and Fc regions of human IgG\(_1\). Expression would result in soluble form of rat AA4 consisting of 235 amino acids, from Ala21 to Val255 (Fig. 2), linked to the Fc domain of human IgG\(_1\) (AA4-Ig). Ligation into the multiple cloning site resulted in addition of five amino acids to the amino terminus of the mature protein (Asp-Lys-Leu-Ala-Arg-). In order to obtain high level of secretion, DNA encoding the AA4-Fc fusion protein was subcloned into the expression vector pDR2\(_{\Delta EF1-\alpha}\). Sequencing of the entire cDNA construct confirmed that no errors had been introduced by PCR. CHO cells were transfected with this plasmid as described above. A stable cell line was generated and culture supernatant was harvested.
AA4-Ig construct was purified from the supernatant by protein A (Prosep A from Bioprocessing, Consett, UK) affinity chromatography, a 0.1 M citrate buffer (pH 5) wash was used to remove weakly bound contaminants (bovine Ig present in the fetal calf serum) and fusion protein was eluted using 0.1 M glycine/HCl (pH 2.5). Fractions containing AA4-Ig were pooled and neutralized with 1 M Tris. After SDS PAGE, Coomassie staining was used to estimate the concentration and the purity of the fusion protein.

Generation of rabbit anti-rat AA4

A multiple array, rat AA4 peptide VCGDEAESKTNYYLCKETTAGVFHW (MAP-AA4 peptide) presenting the least homology between the mouse AA4 and the human C1qRp and corresponding to amino acid residues 223 to 247 (Fig. 2) was synthesized on a Synergy synthesizer (Applied Biosystems, Warrington, UK). Anti-peptide antiserum was raised in New Zealand White rabbit by repeated s.c. immunization (nine in total) with MAP-AA4 peptide (100 µg/immunization) and AA4-Ig (200 µg/immunization) in complete Freund’s adjuvant for the first time and followed by incomplete Freund’s adjuvant (ICN pharmaceuticals Ltd, Oxford, UK).

Affinity purification of the rabbit anti-rat AA4 antiserum

Contaminant rabbit Abs (anti-bovine Ig and anti-human IgG1 Fc portion) were immunoabsorbed from the antiserum. For this purpose, 10 mg of bovine IgG (Sigma) and 10 mg of human IgG (made in house) were coupled to 4 g of cyanogen bromide (CNBr)-activated Sepharose (Amersham Pharmacia), according to the manufacturer’s specifications. The antiserum was loaded into this column and the breakthrough collected for further purification. Rat AA4-Ig (12 mg) was coupled to 2.5 g of CNBr-activated. The rabbit anti-
rat AA4 'bovine and human IgG free' antiserum was loaded into the column at 4°C. After washing with PBS, specific Abs against the CTLD of AA4 were eluted using 50 mM diethylamine. The eluate was concentrated (100 µg/ml final concentration) and buffer exchanged into PBS by ultrafiltration using an Amicon concentrator (Amicon Corp., Danvers, USA).

**Preparation of cell lysates and Western blot analysis**

Mock-transfected or AA4 transfected CHO cells were solubilized on ice for 1 h in 1 ml lysis buffer (2% (v/v) NP-40, 10 mM EDTA, 10 µg/ml pepstatin A, 10 µg/ml leupeptin, 1 mM PMSF). An aliquot of the supernatant was mixed with an equal volume of non-reducing sample loading buffer (100 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/20% (v/v) glycerol/0.002% (w/v) bromophenol blue) and subjected to 10% (w/v) SDS-PAGE. Gels were Western blotted onto nitrocellulose which was subsequently blocked with PBS containing 5% (w/v) non-fat dried milk (PBS/milk). Blots were probed with the affinity purified rabbit anti-rat AA4 (25 ng/ml) for 1 h at room temperature (RT), washed 3 times in PBS containing 0.1% (v/v) tween-20 (PBS/tween) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hemel Hempstead, UK) in PBS/milk. Following 3 washes in PBS/tween, the presence of bound HRPO-conjugated secondary Ab was detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce and Warriner, Chester, UK) and exposed to X-ray film (Sigma). No bands were detected on blots incubated with secondary Ab only (data not shown).

**Immunofluorescence analysis (double staining)**

Rat mononucleated cells were prepared as described above. Cells (10^5/100 µl) were cytopspun on super-frost glass slides (Surgipath Europe Ltd) at 500 rpm for 5 min. The cells were fixed
in acetone, 2 min at RT, washed in PBS and incubated for 1h at RT with primary Ab diluted in PBS/BSA. Sections were washed in PBS and incubated (1h, RT) with fluorescein-conjugated donkey anti-mouse IgG and rhodamine-conjugated donkey anti-rabbit IgG (Jackson Laboratories, Statech Sci., Luton beds, UK). In some cases, cells were counterstained with DAPI (final concentration 50 ng/ml, Sigma, 1h, RT). After intensive washings in PBS, slides were mounted with the Vectashield mounting medium (Vector Laboratories, Peterborough, UK).

**Image analysis**

Immunostaining were observed using an upright fluorescent/bright field microscope (DMLB, Leica, Heidelberg, Germany) and photographed using a photo camera (MPS60, Leica). Printed images were further analysed after scanning and computer-assisted image analysis using the Image-pro+ software (Media Cybernetics, Silver Spring, USA). Surface plot was used to create a three dimensional representation of the intensity of the staining (see Figures 6 and 8).
Results:

*Rat AA4 is a type I transmembrane protein composed of a C-type lectin-like domain (CTLD) and 5 epidermal growth factor (EGF)-like domains*

We have cloned the rat homolog of the mouse AA4 and human C1qR to gain further insight into the molecular and cellular properties of this novel transmembrane protein with a lectin-like domain. Oligonucleotides chosen in region of high homology between the mouse and the human coding sequences were used to generate a specific rat AA4 cDNA fragment of 1.3 Kb. This fragment was used as a probe to screen a rat lung cDNA library and yielded forty-four positive clones after primary screening of 600,000 plaques. These colonies were subjected to PCR screening using oligos (18 up and 336 down) which amplify ∼300 bp in the 5’ end. Four colonies were positive by PCR. The complete cDNA was 4779 bp long and comprised 196 bp of 5’ flanking sequence, a 1932 bp open reading frame (ORF) and 2651 bp of 3’-UTR including the poly-A-tail.

The deduced amino acid sequence of AA4 consisted of 643 amino acids (Fig. 1A). The domain composition and organisation were analysed using the SMART software (Simple Modular Architecture Research Tool, web site http://smart.embl-heidelberg.de/) and the results are represented in a schematic format in Figure 1B. The rat AA4 was composed of a twenty two amino acid putative signal peptide predominantly composed of hydrophobic residues, a CTLD, five EGF-like domains including three calcium-binding EGF-like domains and a single transmembrane domain (TMD) (Fig. 1B). The TMD was boarded by a serine/threonine rich region (mucin-like, aa 462 to aa 577) and a short cytoplasmic tail presenting a tyrosine phosphorylation consensus motif R/KX2-3D/EX2-3Y that is generally recognized by tyrosine kinases (Fig. 2). A putative N-linked glycosylation site is indicated
within the second EGF domain (Fig. 1B). Comparison of predicted amino acid sequence of rat AA4 with mouse AA4 and human C1qR was carried out using the ClustalW software (web site: http://www2.ebi.ac.uk/clustalw/). The highest degree of similarity between the three species was observed in the EGF domains followed by the TMD and the CTLD (Fig. 2). The predicted molecular mass of the rat AA4 was 69 kD.

Rat AA4 belongs to the C-type lectin-like (CTLD) family and is structurally related to TM

Database comparison using SMART revealed that the rat AA4 exhibits striking domain composition and organisation very similar to that of rat TM (data not shown). Both molecules display extracellular domains composed of a single CTLD and a tandem arrangement of EGF-like domains. Interestingly, TM has one additional EGF-like domain (six in total) that is known to be required for thrombin binding and protein-C cofactor activity to mediate anticoagulant activity (28,29). The domain structure of the rat AA4 CTLD is similar to that of the rat TM, rat mannan binding protein (MBP-A, MBP-C), mouse mannose receptor, rat CD23, rat galactose lectin receptor and rat E-selectin (Fig. 3). The CTLDs are aligned with the positions of the "invariant" residues and includes short intervening stretches of amino acids that are not homologous to one another. The four cysteine residues which are essential for the correct folding of the carbohydrate recognition domain (CRD) are shown in Fig. 3 (black background). Interestingly, AA4 and TM do not have the residues that are part of the consensus involved in calcium binding and sugar specificity (Fig. 3, arrow) (30). For example, the glutamate-proline-asparagine (EPN) motif is an essential feature of the calcium-dependent carbohydrate (mannose-specific) recognition domain and which is clearly missing in rat AA4 CTLD. The rat AA4 and the rat TM exhibit 4 additional cysteine residues (8 in total). The function of these supplementary cysteines is unknown, but they could be involved in intermolecular disulfide bridge formation that will probably affect the
overall folding pattern of the domain (30). The WIGL/I motif depicted in grey in the Figure 3 is found to be highly conserved among membrane receptors that modulate endocytosis (13).

**Rat AA4 is encoded by a single mRNA of 7 kb which is constitutively and broadly expressed in rat tissues**

The expression of AA4 mRNA in rat tissues was analysed by Northern blot. Hybridization was carried out with a 639-bp radio-labeled rat cDNA specific probe, corresponding to approximately 33% of the coding region, and revealed a single band of 7 Kb in lung and heart (Fig. 4A). The expression of AA4 mRNA was not detectable in brain, thymus, liver, spleen, intestine, kidney, adrenal gland and testis by Northern blot. The GAPDH probe was used as a positive control showing the integrity of total RNA (Fig. 4A). RT-PCR, a more sensitive method for the detection of transcripts, was therefore employed. Total RNA was isolated from several tissues to generate cDNA then used as template in PCR reactions to amplify a 287-bp fragment of AA4. Primers (rat 1646 up and rat 1913 down) were carefully chosen to be able to discriminate from the amplification of potential contamination by genomic DNA. Amplification of genomic DNA would result in the amplification of a PCR product of 535-bp due to the presence of a 5’ terminal intronic sequence (unpublished observations). RT-PCR analysis indicated that AA4 mRNA is constitutively and broadly expressed in all tissues studied. The expression of the rat AA4 mRNA was more pronounced in lung and heart that in adrenal gland, brain, brainstem, cerebellum, spinal cord, kidney, spleen, liver, intestine, stomach, muscle, testis and thymus (Fig. 4B). No bands were detected when the template or the oligonucleotides were omitted from the PCR. Sequencing confirmed the specificity of rat AA4 RT-PCR products (data not shown).

**Rat AA4 mRNA is expressed by pneumocytes in lung and endothelial cells in heart**
In situ hybridisation (ISH) was carried out to characterize the cellular expression pattern of the AA4 mRNA. Rat lung and heart frozen sections were hybridized with a specific digoxigenin-labeled antisense riboprobe for AA4 and the results are presented in Figure 5. Alveolar pneumocytes but not the bronchial epithelial cells were strongly stained with the antisense probe (Fig. 5a). Due to the limitation in resolution of the ISH staining, it was difficult to assess whether resident lung macrophages were expressing the AA4 mRNA. In heart, we consistently found that endothelial cells as well as smooth muscle cells of the arteries (Fig. 5c) and capillaries (Fig. 5d) were strongly stained for AA4 mRNA. Neither lung (Fig. 5b) nor heart (Fig. 5e) sections were stained after hybridization with the sense riboprobe.

Rat AA4 is a 100 kD heavily glycosylated protein distributed at the cell membrane and contained within intracytoplasmic vesicles

To gain insight into the biochemical nature, the posttranslational processing of rat AA4 and its subcellular distribution, the ORF of the rat AA4 cDNA was cloned into the eukaryotic expression vector (pDR2ΔEF1-α) and transfected into CHO-K1 cells. Stable clones were selected and AA4 expression was demonstrated by flow cytometry analysis using the affinity purified rabbit anti-rat AA4. This antibody was raised against a rat AA4 (CTLD)-Fc fusion protein (see above). Rat AA4-transfected CHO (CHO/AA4) cells were strongly stained in a dose dependent manner while no staining was obtained with mock-transfected CHO cells or CHO cells transfected with an empty vector (φ, Fig. 6A). Moreover, the antibody was able to detect a single band (100 kD under non-reducing conditions) following Western blotting of CHO/AA4 (Fig. 6B). The faint band (~140kD) detected from untransfected or transfected CHO cell lysates probably represents the hamster AA4. CHO/AA4 cells cultured on glass slides were fixed using acetone and immunostained for AA4 using the rabbit antibody. The
AA4-transfected cells were strongly stained whereas mock transfected cells remained negative (Fig. 6C). Surface plot analysis of the staining pattern indicated that AA4 was localized to the membrane (5-30% of the staining) and to intracellular vesicles (Fig. 6D). This staining pattern was confirmed by confocal microscopy imaging (data not shown).

**AA4 is expressed by endothelial cells, alveolar macrophage cell line, monocytes, platelets and NK cells**

To characterize the cellular pattern of expression of AA4, immunofluorescence analysis of acetone-fixed cells was performed using the affinity purified polyclonal Ab (Fig. 7). First, cells obtained from primary culture of embryonic rat heart were double stained for AA4 and Thy1.1. Endothelial cells (Thy1.1 negative cells) were strongly stained for AA4 while fibroblasts (Thy1.1 positive cells) were negative for AA4 (Fig. 7a). The rat alveolar macrophage cell line NR8383 was immunopositive for AA4 (Fig. 7b) and ED1 (Fig. 7c). Rat leukocytes and platelets were cytospun onto glass slides and double stained for AA4 and ED1 (Fig. 7d,e), ED2 (Fig. 7f), CD11b/c (Fig. 7g), NKR-P1A (Fig. 7h,i), CD3 (Fig. 7j,k), CD8 (Fig. 7l,m) and CD45RA (Fig. 7n,o). Monocytes (ED1+ cells, Fig. 7e), natural killer cells (NKR-P1A+ cells, Fig. 8i) as well as platelets (Fig. 7d,f,g,h,l), were strongly stained for AA4. In contrast, differentiated monocytes (ED2+ cells, Fig. 7f), neutrophils (CD11b/c+ cells, Fig. 7g), T-cells (CD3+ cells, Fig. 7k) including cytotoxic T-cells (CD8+ cells, Fig. 7m), and B-cells (CD45RA+ cells, Fig. 7o) were negative for AA4 (Fig. 7f,g,h,j,l,n respectively).

**Subcellular distribution of AA4: cell membrane and intracellular vesicles**

Cells were cytospun onto glass slides, fixed with cold acetone (5min) and were analysed by immunofluorescence for AA4. Platelets (Fig. 8a), NK cells (Fig. 8b) and monocytes (Fig. 8c)
concentrated AA4 at the cell membrane (5-30% of the total staining) and the remaining was localized to intracellular vesicles, identified by co-localization with the transferrin/transferrin receptor as being endosomes (data not shown). Surface plot analysis of the staining clearly indicated that AA4 was localized to intracytoplasmic components in platelets (Fig. 8d), NK cells (Fig. 8e) and monocytes (Fig. 8f).
Discussion:

In this manuscript, we describe for the first time the molecular characterization of the rat AA4. In addition, we present key data regarding the modular architecture, the subcellular distribution and the tissue expression pattern of AA4, which together shed further light on the putative role of this C-type lectin-like receptor in cell-cell interactions as discussed below. The rat AA4 was found to be highly conserved with the mouse AA4 and human C1qRp. The predicted rat AA4 protein displays 86.8% identity to the mouse AA4 and 65.7% sequence identity to human C1qRp (Fig 2). Interestingly, rat AA4 has a domain composition and organisation very similar to that of TM. TM (CD141) is a transmembrane glycoprotein composed of a unique CTLD, a repeat of six EGF-like domains, a serine/threonine rich (mucin-like) region, a single TM followed by a short cytoplasmic tail (24,28,31). TM is a receptor for thrombin and serves as a cofactor for thrombin-catalyzed activation of protein C. Protein C is a plasma protein that functions as a physiological anticoagulant in its activated form (24). Thrombin interacts with TM through the last two of the six EGF domains but EGF domains 4-6 are essential to accelerate protein C activation. Thrombin interaction with TM is further stabilized by a chondroitin sulfate moiety located in the O-linked mucin-like region (31). Rat AA4 exhibits a unique CTLD followed by a tandem of only five EGF domains followed by a mucin-like (serine/threonine rich) region, a TMD and a short cytoplasmic tail. Interestingly, the rat AA4 comprised a carboxy-terminal tyrosine phosphorylation motif (R/KX2-3D/EX2-3Y) recognized by tyrosine kinases and which may be involved in signal transduction pathways (32). This motif is present in human C1qRp but not in mouse AA4 antigen and is absent from TM.

The predicted molecular mass of the mature rat AA4 protein is 69 kD while the apparent size
seen in SDS-PAGE is 100 kD (Fig. 6B). This difference may be due to glycosylation of the potential N-glycosylation site present in the second EGF domain and/or O-glycosylation of the mucin-like region as recently reported for the human C1qRp (15). We have no evidence yet that a chondroitin sulfate moiety is present in the O-linked mucin-like region of AA4. It would be of interest to assess whether thrombin can bind to the EGF domains of AA4 and mediate activation of the protein C-anticoagulant system.

The CTLD of AA4 and TM bears distant homology to the CRD of the C-type lectin family. For example, the group of type I surface lectins includes members of the macrophage mannose receptor-like family (mannose receptor, DEC205) that bind and internalize carbohydrate-bearing antigens by receptor-mediated endocytosis (33). Other members include selectins (CD62), that are implicated in adhesion events on endothelial cells and which mediate the tethering and rolling of leukocytes via oligosaccharide groups (34). Given that rat AA4 is expressed on the surface of a wide variety of cells, some of which are organized into tissues, such as endothelial cells, and others of which interact with different cells only under specific conditions of inflammation or blood clotting, it is possible that this lectin-like molecule behaves like a platelet-endothelial cell adhesion molecule (PECAM, for example like CD31, PECAM-1)(10,35). Regardless of the mechanism by which AA4 promotes adhesion, its presence in relatively large quantities on platelets, leukocytes, and endothelial cells suggests that it may be involved in a wide variety of vascular processes in physiological and pathological conditions.

Interestingly, undifferentiated circulating monocytes but not macrophages (ED2+ circulating cells and tissue macrophages) expressed rat AA4, suggesting that the previously reported enhancement of phagocytosis following signalling through this receptor is restricted to immature phagocytes (Fig 7). We have recently stained various human tissues and we were surprised to find abundant expression of C1qRp on circulating monocytes but not on tissue
macrophages (E.P. McGreal et al., manuscript in preparation). Together, these observations seem to indicate that the expression of AA4/C1qRp is tightly regulated during embryogenesis as well as later, in adult life.

We herein report for the first time that AA4 is expressed by NK cells (Fig 7h,i). NK cells are known to express various type II lectins (NKG2 complex, NKR-P1A/CD161, CD94) and which are mainly involved in inhibition of cellular functions to protect from undesired killing of ‘self’ cells (36). We postulate, particularly in analogy to the role of AA4/C1qRp on monocytes, that AA4 is involved in controlling NK-mediated innate immune response. For instance, it would be interesting to ascertain whether NK cell display increased cytotoxicity toward target cells opsonised with C1q, MBL or SPA. Experiments along these lines are highly warranted.

Analysis of the subcellular distribution of AA4 expressed by transfected CHO cells and by platelets, clearly shows that rat AA4 is concentrated at the cell surface (5-30% of the staining) while the major site of expression resides within cytoplasmic vesicles (Fig 8). We have also observed a similar subcellular staining pattern in monocytes and NK cells indicating that the distribution of AA4 is not cell type specific and instead reflects a general phenomenon. Interestingly, AA4 was distributed solely to the cell surface on endothelial cells (Fig 7A).

Intriguingly, the CTLD of AA4 presents a WIGL motif that is found in many membrane proteins involved in endocytosis, including TM (Fig 3) (37). The presence of this motif and the remarkable granular distribution of AA4 is consistent with the possibility that AA4 is functioning to internalize ligands from the extracellular milieu in an endosomal compartment. Preliminary experiments indicate that AA4 colocalizes to transferrin/transferrin receptor frequently associated to endosomes. Receptor-mediated internalisation of ligands and rapid recycling of the receptor are vital physiological processes. For example, it provides a
mechanism by which toxic components and pathogens are taken up by cells. It will be interesting to assess in vitro whether the CTLD of AA4 binds specifically to moieties (e.g. carbohydrates) leading to the internalisation and the clearance of the target cell. The distant homology of the rat CTLD with the CRD of some proteins, for example of mannose receptor, would support this hypothesis. However, it is important to note that CRDs are defined on the basis of size and the presence of certain conserved amino acids resulting in an overall structural homology and that the presence of a CRD in a protein does not necessarily confer carbohydrate binding capacity (30). Detailed analysis of the CTLD of AA4 and TM reveals that both proteins have retained the four conserved cysteine residues but they are not equally spaced. In addition, AA4 and TM have four additional cysteines and their CTLD lacks the critical calcium/sugar binding sites. Consequently, it is unlikely that AA4 and TM bind for example to high mannose or galactose oligosaccharides. Regardless as to whether AA4 binds to different types of carbohydrate moieties, it will be important in the near future to identify the nature of the potential counter-receptor(s).

Our relative ignorance of the physiological function of AA4/C1qRp should be resolved in that this receptor is expressed abundantly on endothelial cells, platelets and NK cells while differentiated monocytes/macrophages lose rapidly AA4. Therefore, its is tempting to hypothesize that AA4/C1qRp is involved in some interactive events (cell adhesion) taking place during embryogenesis and in adult life as recently suggested (10). This hypothesis implies that C1qRp binds to specific counter-receptors yet to be characterized. C1q, MBL and SPA are defense collagens capable of recognising a large variety of pathogens as well as apoptotic cells so that they can be cleared by competent phagocytes bearing the AA4/C1qRp. Thus, AA4/C1qRp should be regarded as an important signalling PRR involved in enhancement of an innate immune response against potentially dangerous and toxic entities (19,38). The presence of AA4 on endothelial cells is particularly interesting in light of recent
reports describing that at least some subtypes of these cells are capable of phagocytosing apoptotic lymphocytes (39-41).

Hopefully, future experiments using specific antibodies and soluble AA4-Fc fusion proteins that are described here will help in the identification of i) the intracellular signalling events (following cross-linking of the receptor) and ii) the potential counter-receptor(s) for AA4\C1qRp. We have recently observed that the level of AA4 is increased by a factor of 5-20 fold on endothelial cells in a rat stroke model following middle cerebral artery occlusion with reperfusion (Dean et al., unpublished observations). Again this preliminary data together with the current hypothesis proposed by others, highlight the potential role of AA4 in endothelial cell adhesion. In this context, we can speculate that a soluble form of this receptor might have clinical application as an anti-adhesion molecule to dampen local inflammation.

In conclusion, this study paves the way to understanding the function of the enigmatic AA4/C1qRp, which likely represents a key feature of a signalling receptor complex involved in cell-cell interactions. The domain organisation of rat AA4 is very similar to TM and we have recently found that both genes are clustered on chromosome 20 (20p11 locus) in human (McGreal et al., paper submitted) (42). Although TM is recognized as a major natural anticoagulant expressed on endothelial cells, this lectin-like molecule has also been involved in cell differentiation, cell proliferation and cell-cell adhesion (43-45). It is possible that part or all of these properties actually involve the CTLD of TM and the structure/function relationship of this protein should be revisited in the near future. TM gene deletion in mice resulted in embryonic lethality, and this even before the development of a functional cardiovascular system (46). This observation would suggest that TM plays an essential and complex role during embryogenesis, unrelated to coagulation, and it will be interesting to investigate whether AA4 deletion in mice leads to a similar phenotype.
Acknowledgements:

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References:


   *Laboratory Investigation* **79**(10), 1247-1259


Footnotes:

1. The nucleotide sequence of the rat homolog of the mouse AA4 and the human C1qRp has been deposited in the GenBank database: Genbank Accession Number AF160978.
Figure legends:

**Figure 1.** Rat AA4 is a type I transmembrane receptor with a C-type lectin-like domain and five EGF-like domains.

(A) Nucleotide and deduced amino acid sequences of rat AA4 cDNA. Nucleotides are numbered beginning with the initiation codon for Met. Putative N-linked glycosylation site (Asn-X-Thr/Ser motif) is identified by a circle. The nucleotide sequence is deposited in the GenBank database under the accession number AF160978.

(B) The identification of the domain structure of rat AA4 was carried out by SMART (Simple Modular Architecture Research Tool) (web site: http://smart.embl-heidelberg.de/smart/). Domains contained within rat AA4 include a 22 amino acid signal peptide (SP), a C-type lectin-like domain (CTLD), five epidermal growth factor (EGF)-like repeats (numbered) including three calcium-binding (cb) EGF-like domains (italic) and a single transmembrane domain (TMD). The putative N-linked glycosylation site is indicated within the second EGF domain.

**Figure 2.** Rat AA4 is homolog to the mouse AA4 antigen and the human phagocytic complement C1q receptor (C1qRp).

Comparison of predicted amino acid sequence of rat AA4 with mouse AA4 and human C1qRp.

The progressive alignment of the sequences was carried out by ClustalW (web site: http://www2.ebi.ac.uk/clustalw/). Conserved amino acids are shaded. The different structural domains of the receptor are indicated as follows: SP (dashed line), CTLD (dotted line), EGF-
like repeats (double line), calcium binding (cb) EGF-like domains (single line). The solid line depicts the putative TMD. The cytoplasmic domain of rat AA4 is composed of basic amino acids and a tyrosine residue that is part of a consensus motif (R/KX_{2-3}D/EX_{2-3}Y) recognized by tyrosine kinases.

**Figure 3.** Analysis of the C-type lectin-like domain of the rat AA4.

Conserved structural pattern between rat AA4 and other members of the C-type lectin-like superfamily. Sequence alignments of the lectin domains were carried out by ClustalW and amended manually where necessary. The alignments are indicated as follows: Asterisk (*) indicates 8/8 conserved amino acid residues, the double dot (·) indicates 6-7/8 conserved residues and the single dot (·) indicates 4-5/8 conserved residues. Cysteine residues of the consensus motif are black boxed. Arrows show the part of the calcium binding site involved in sugar (mannose-type) specificity (E/N/E/N/D). The WIG(L/I) motif (gray boxed) is highly conserved and particularly among membrane receptors that modulate endocytosis. Members of the CTLD family and the accession mnumber were as followed: MBP (mannan binding protein, rat, M14013), ManR (mannose receptor, CRD2, mouse, Z11974), CD23 (rat, X73579), LectinR (macrophage Gal/GalNac-specific lectin, rat, J05495), E-select (CD62E, rat, L25527), Thrombo (TM, rat, AF00273).

**Figure 4.** The rat AA4 mRNA (7kb in size) is expressed constitutively in rat tissues.

(A) Northern blot analysis of total RNA from various rat tissues for AA4. Total RNA (20 µg) were separated on a formaldehyde agarose gel and transferred onto nylon membrane. The blot was hybridized with radio-labelled specific rat AA4 cDNA probe. GAPDH was analysed as a positive control.
(B) RT-PCR analysis of total RNA from various rat tissues for AA4. Total RNA (0.2 µg/PCR reaction) was reverse transcribed and subjected to 30 cycles of PCR amplification. GAPDH was analysed as a positive control.

**Figure 5.** The rat AA4 mRNA is constitutively expressed by lung pneumocytes and vascular endothelial cells in heart.

In situ hybridisation (ISH) analysis of rat lung and heart frozen sections for AA4. Frozen rat tissue sections were hybridized with DIG-labeled rat AA4 riboprobe. In lung, pneumocytes but not bronchiolar epithelium (a, large arrow head) were positive for AA4. In heart, endothelial cells (small arrow head) of arteries (c) and capillaries (d) were positive for AA4. No staining was obtained after hybridization of lung (b) or heart (e) tissue sections with the sense riboprobe.

Original magnification: x200.

**Figure 6.** Characterization of the affinity-purified rabbit anti-rat AA4 polyclonal Ab.

(A) Flow cytometric analysis of AA4-transfected CHO cells (gray filled histogram, Ab dilution 1/100 to 1/32000) and mock (Ø)-transfected CHO cells (black filled histogram, Ab dilution 1/200). The affinity-purified polyclonal antibody stained AA4-transfected CHO cells in a dose dependant manner and did not stain Ø-transfected CHO cells.

(B) Western blot analysis of cell lysates prepared from AA4-transfected CHO cells and Ø-transfected CHO cells. The apparent molecular mass of the rat AA4 was 100kD under non-reducing conditions. The Ab dilution was 1/4000 (25 ng/ml).

(C) Immunofluorescence analysis of acetone-fixed AA4-transfected CHO cells and and Ø-transfected CHO cells. The affinity-purified polyclonal antibody stained only AA4-transfected CHO cells. Interestingly AA4 was expressed at the cell membrane as well as in
cytoplasmic vesicles.

(D) Immunostaining of AA4-transfected CHO cells and surface plot analysis to assess the level and spatial distribution of the staining. The picture highlights the granular staining of AA4 in the cytoplasm.

**Figure 7.** The rat AA4 is expressed constitutively by circulating monocytes, platelets, NK cells and endothelial cells (primary cultures).

Immunofluorescence analysis of mixed cell culture of embryonic rat heart (a), alveolar macrophage cell line NR8383 (b,c) and freshly isolated rat leukocytes (d-o), stained with the affinity-purified rabbit anti-rat AA4 polyclonal Ab (red) and specific cell-markers (green).

Endothelial cells (a, Thy1.1+), alveolar macrophage cell line (NR8383, b-c, ED1+), platelets (d,f,g,h,j,l), monocytes (d-e, ED1+) and NK cells (h-i, NKR-P1A+) were strongly immunopositive for AA4. In contrast, monocytes (f, ED2+), neutrophils (g, CD11b/c+), T-cells (j-k, CD3+) including cytotoxic T-cells (l-m, CD8+) and B-cells (n-o, CD45RA+) were negative for AA4. Original magnification: x200 (d,e,h,i); x400 (a-c,f,g,j-o).

**Figure 8.** Subcellular localization of rat AA4 in platelets, NK cells and monocytes: Cell surface protein stored in cytoplasmic vesicles.

Immunofluorescence analysis of rat platelets (a), NK cells (b) and monocytes (c) stained for AA4. Cells were acetone fixed and stained with the affinity-purified rabbit anti-rat AA4 polyclonal Ab. The surface plot representation depicts a staining localized at the cell surface (5-30%) and also to cytoplasmic vesicles in platelets (d), NK cells (e) and monocytes (f). Original magnification (x1000).
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Molecular and cellular properties of the rat AA4 antigen, a C-type lectin-like receptor with structural homology to thrombomodulin
Yann D. Dean, Eamon P. McGreal, Hiroyasu Akatsu and Philippe Gasque

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