The scavenger receptor CD36 plays a role in cytokine-induced macrophage fusion

Laura Helming*, Julia Winter‡ and Siamon Gordon§

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

*Present address: Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Trogerstr. 30, 81675 Munich, Germany
‡Present address: DKFZ (German Cancer Research Center), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
§Author for correspondence (e-mail: siamon.gordon@path.ox.ac.uk)

Accepted 20 October 2008
Journal of Cell Science 122, 453-459 Published by The Company of Biologists 2009
doi:10.1242/jcs.037200

Summary

Multinucleated giant cells, characteristic of granulomatous infections, originate from the fusion of macrophages. Using an antibody screening strategy we found that the scavenger receptor CD36 participates in macrophage fusion induced by the cytokines IL-4 and GM-CSF. Our results demonstrate that exposure of phosphatidylserine on the cell surface and lipid recognition by CD36 are required for cytokine-induced fusion of macrophages. We also show that CD36 acts in a heterotypic manner during giant-cell formation and that the formation of osteoclasts is independent of CD36. The discovery of molecules involved in the formation of multinucleated giant cells will enable us to determine their functional significance. Furthermore, our results suggest that lipid capture by cell surface receptors may be a general feature of cell fusion.

Key words: CD36, Macrophage fusion, IL-4, Multinucleated giant cell, Mouse

Introduction

Cell-cell fusion is a fundamental feature of the physiology and development of multicellular organisms. In mammals, cell fusion is indispensable for fertilization, skeletal muscle and trophoblast formation (Chen et al., 2007; Huppertz et al., 2006). Multinucleated giant cells were first described by Langhans in 1868 and found to be uniformly present in tuberculoid granulomas (Langhans, 1868). However, multinucleated giant cells are not only characteristic of tuberculosis but are present in most granulomatous conditions, including sarcoidosis and schistosomiasis as well as the foreign body reaction, the host response to large implanted biomaterials (Anderson, 2000; Helming and Gordon, 2008). Multinucleated giant cells originate from fusion of macrophages recruited to the granulomatous site (Chambers and Spector, 1982). The function of multinucleated giant cells remains to be determined. Foreign body giant cells display an enhanced capacity to degrade large particles (Zhao et al., 1991) and may therefore be deleterious for implants. Tuberculosis-associated giant cells have been associated with restriction of cell-to-cell spread of mycobacteria (Byrd, 1998) but also with increased metalloproteinase secretion (Zhu et al., 2007), potentially contributing to tissue destruction. Macrophage fusion can not only lead to the formation of granuloma-associated giant cells but also is the basis of osteoclast formation, the cells ensuring lifelong renewal of the skeleton.

Whereas intracellular and virus-induced membrane fusion have been studied in more detail, the mechanism of spontaneous cell-cell, in particular macrophage, fusion remains elusive. Even though several molecules have been implicated in this process, including the putative seven transmembrane receptor DC-STAMP (Yagi et al., 2005) and the members of the immunoglobulin superfamily CD47 and SIRP-α (Vignery, 2005), insight into the precise mechanism of macrophage polykaryon formation is missing to date. In addition, it is not known whether multinucleated giant cells and osteoclasts are formed via the same macrophage fusion machinery.

We have previously described a novel assay to investigate murine macrophage fusion induced by IL-4 alternative activation in vitro (Helming and Gordon, 2007). To identify molecules involved in giant-cell formation, we used an unbiased antibody screening strategy. Here we show that the scavenger receptor CD36 participates in fusion of macrophages induced by the cytokines IL-4 and GM-CSF. CD36 belongs to the class B of scavenger receptor and is known to bind to a broad array of endogenous and pathogen-derived ligands, including oxidized low density lipoprotein (oxLDL), anionic phospholipids, thrombospondin, collagen, fatty acids, *Plasmodium falciparum* peptides and bacterial lipopeptides (Moore and Freeman, 2006). CD36 contributes to foam cell formation in atherosclerosis (Podrez et al., 2000), participates in the recognition of apoptotic cells (Greenberg et al., 2006) and *P. falciparum*-parasitized erythrocytes in malaria (McGilvray et al., 2000). Our results demonstrate that in addition to the role of CD36 in these processes, recognition of endogenous lipids by CD36 is involved in cytokine-induced fusion of macrophages, whereas the formation of osteoclasts is independent of CD36. Furthermore, we demonstrate that exposure and recognition of phosphatidylserine (PS) is required for macrophage polykaryon formation.

Results

Isolation of anti-CD36 antibodies blocking cytokine-induced macrophage fusion

In order to identify molecules involved in macrophage fusion, we utilized an antibody-based screening strategy. IL-4-treated and therefore fusion-competent murine thioglycollate-elicited peritoneal macrophages (ThioMΦ) were used to immunize rats. We then produced hybridomas by fusion of rat splenocytes with the myeloma cell line Y3 (Galfre et al., 1977). Hybridoma supernatants were screened for a functional effect on IL-4-induced macrophage fusion. Using this approach, we were able to identify

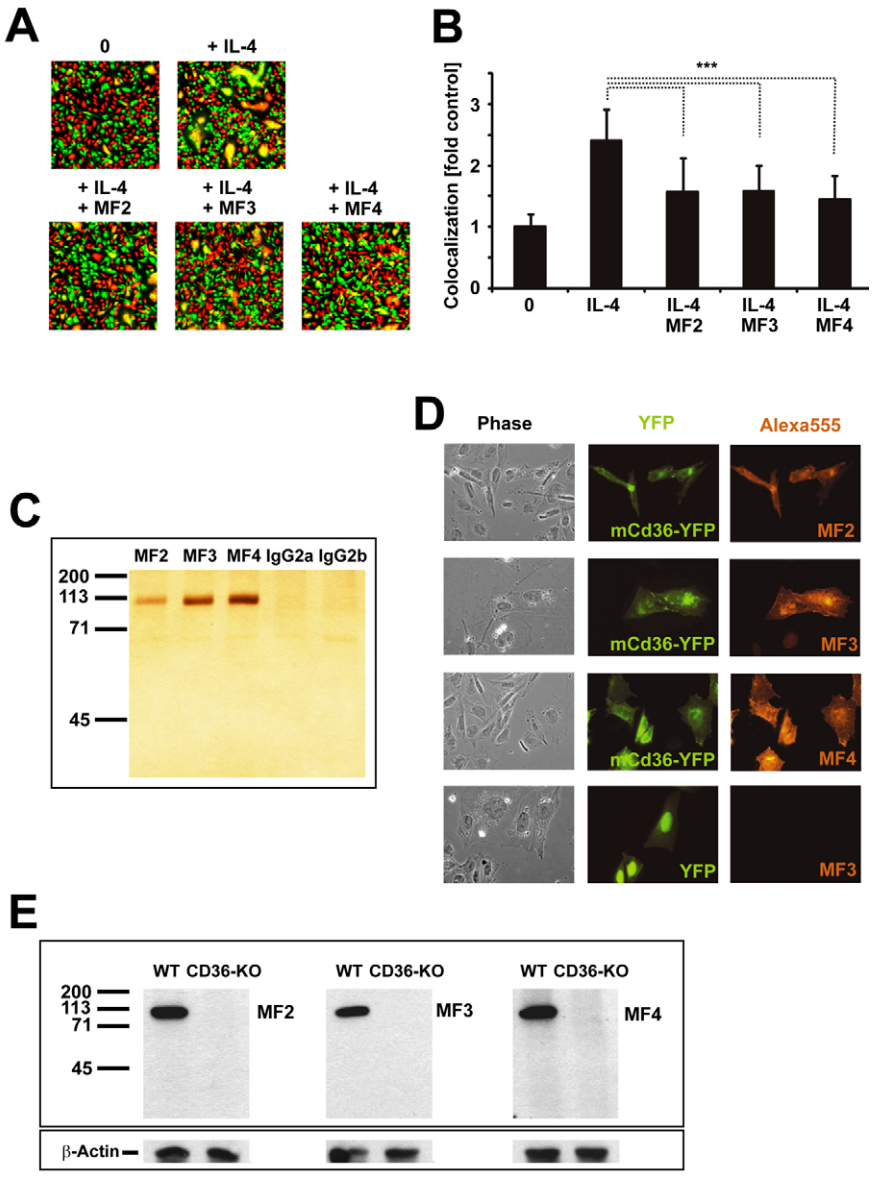

Fig. 1. Isolation of anti-CD36 antibodies blocking cytokine-induced macrophage fusion. (A) ThioMΦ were labelled with CFSE and PKH26 and fusion induced by exposure to IL-4 in the presence of supernatants from three individual hybridoma lines MF2, MF3 and MF4. Macrophage fusion is represented by co-localization of the red and green fluorescent labels (yellow). (B) Quantitation of co-localization in the presence of the purified anti-CD36 antibodies MF2, MF3 and MF4 (20 μg/ml). Means±s.d. of 21 measurements combined from three independent experiments. (C) MF2, MF3, MF4 or isotype control (IgG2a, IgG2b) antibodies were covalently coupled to protein G beads and used for immunoprecipitation from ThioMΦ lysates. Specific bands (~100 kDa) could be detected on the silver-stained gel for MF2, MF3 and MF4 but not for isotype control antibodies. (D) Transient transfection of CHO cells with mCD36-YFP or YFP control vector, stained with MF2, 3 and 4. Positive staining was detected via anti-rat Alexa 555 (red). Positive staining with MF2, 3 and 4 was only detected in CHO cells expressing the mCd36-YFP fusion protein. (E) Western Blot analysis of wild-type (WT) and CD36-KO macrophage lysates using MF3, MF2, MF4 and anti-β-actin antibodies. ***P<0.0001, Mann Whitney Test, two-tailed.

three independently derived monoclonal antibodies (clones MF2, MF3 and MF4) that could inhibit IL-4-induced macrophage fusion (Fig. 1A,B). These antibodies were used to immunoprecipitate and identify the corresponding macrophage antigens. Specific bands were detected on silver-stained protein gels for MF2, MF3 and MF4 (Fig. 1C). We subjected the specific bands immunoprecipitated by MF2, MF3 and MF4, respectively, to mass spectrometry and found that all three monoclonal antibodies were directed against the scavenger receptor CD36, suggesting it to be a dominant epitope under these conditions. The specificity of our novel anti-CD36 antibodies was confirmed by positive staining of Chinese hamster ovary cells expressing a mCD36-YFP fusion protein (Fig. 1D). When we conducted western blot analysis of lysates from wild-type and CD36-KO macrophages, specific staining with our MF2, MF3 and MF4 antibodies was detected only in the presence of CD36 (i.e. in wild-type macrophages), further confirming the specificity of the antibodies (Fig. 1E). The antibody MF3 was used for all subsequent experiments.

Macrophage fusion is impaired in CD36-KO macrophages

To confirm the involvement of CD36 in giant-cell formation, we performed experiments using bone-marrow-derived macrophages (BMM) from CD36-KO mice. In contrast to ThioMΦ, BMM were stimulated with IL-4 and GM-CSF to induce macrophage fusion (Jay et al., 2007). We found that fusion was severely impaired in macrophages from CD36-KO mice compared with the wild-type control (Fig. 2A,B). Our anti-CD36 antibody not only inhibited IL-4-induced ThioMΦ fusion but also significantly blocked IL-4/GM-CSF-induced BMM fusion, an effect that was absent in CD36-KO macrophages (Fig. 2C). We conclude that CD36 is essential for maximal IL-4 (and IL-4/GM-CSF)-induced macrophage polykaryon formation.

The expression of CD36 in cell contact zones during macrophage fusion

To characterize the role of CD36 in macrophage fusion, we analyzed the expression of CD36 during macrophage fusion. First, we asked whether CD36 is induced after IL-4 stimulation. However, FACS analysis of IL-4 treated ThioMΦ showed no change in CD36 surface

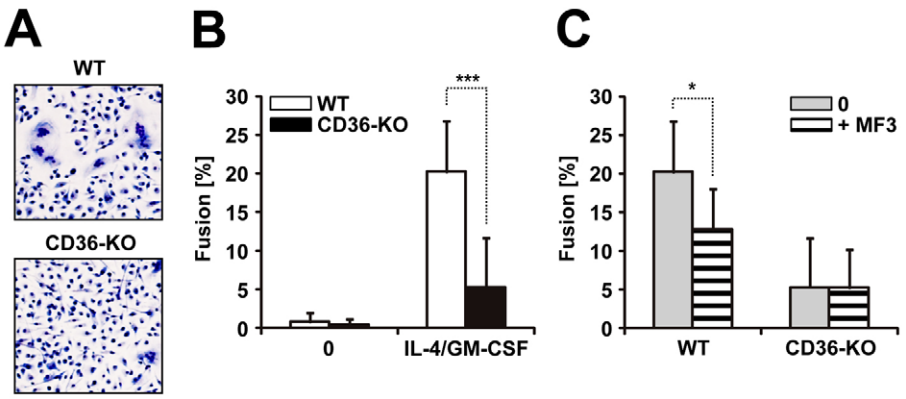

Fig. 2. The involvement of CD36 in macrophage fusion. (A) BMM from wild-type (WT) and CD36-KO mice were induced to fuse by exposure to IL-4 and GM-CSF and stained with Hemacolor. (B) Macrophage fusion was quantified via the percentage of giant-cell nuclei relative to the total number of nuclei. (C) Fusion of wild-type and CD36-KO BMM in the presence of the anti-CD36 antibody (MF3, 20 µg/ml). (D,E) Shown are means±s.d. (n=8) *P=0.0404, ***P=0.0054 (Mann Whitney Test, two-tailed), representatives of three independent experiments are shown.

expression (Fig. 3A). This is in accordance with published results that only the intracellular pool of CD36 increases after IL-4 exposure (Yesner et al., 1996). Next, we evaluated the intracellular localization of CD36 during macrophage fusion. To synchronize giant-cell formation, ThioMΦ were prestimulated with IL-4 and then plated onto a fusogenic surface to induce macrophage fusion as described previously (Helming and Gordon, 2007). At the onset of macrophage fusion (6-8 hours), we observed formation of lamellipodia and long cell extensions leading to cell-cell contacts previously shown to precede efficient giant-cell formation (Jay et al., 2007; McNally and Anderson, 2005). CD36 was expressed in intracellular and plasma membranes and was localized within lamellipodia and cell-cell contacts consistent with its involvement in macrophage fusion (Fig. 3B). Because CD36 has been implicated in cytoskeletal reorganization (Stuart et al., 2007), we asked whether the formation of these cellular structures was affected by blockade of CD36. However, in the presence of our anti-CD36 antibody as well as in CD36-KO macrophages, those cellular structures could be observed at normal levels (Fig. 3C,D). We conclude that CD36 participates in fusion after the initial cell-cell contact. This is underlined by the fact that we found no contribution of CD36 to macrophage adhesion to the fusogenic substrate nor to the expression of IL-4 induced markers (Fig. 4 and data not shown).

Requirement for CD36 on only one fusion partner

We next asked whether CD36 is required on all macrophages combining to form a giant cell. Wild-type or CD36-KO BMM were labelled with the red fluorescent PKH26 dye, mixed with green CFSE-labelled wild-type ThioMΦ and fusion was induced by the addition of IL-4. We found that CD36-KO macrophages were capable of fusing with wild-type macrophages as indicated by the formation of giant cells that contained both fluorescent labels and therefore appeared yellow in the overlay (Fig. 5A). Quantitation of co-localization of the fluorescent labels showed that cross-fusion of CD36-KO with wild-type macrophages was as efficient as fusion of wild-type macrophages (Fig. 5B). Therefore, CD36 acts in a heterotypic manner during macrophage fusion.

Lipid recognition by CD36 is involved in macrophage fusion

CD36 is known to bind to a broad array of ligands, including oxLDL (Endemann et al., 1993) and anionic phospholipids (Rigotti et al., 1995) such as PS (Greenberg et al., 2006). We asked if these lipid CD36 ligands could affect macrophage fusion. We found that addition of oxLDL as well as PS-containing liposomes significantly blocked giant-cell formation (Fig. 6A,B). Intriguingly, our fusion-blocking anti-CD36 antibody could inhibit the binding of PS-containing liposomes to macrophages (Fig. 6C), showing that the antibody may be directed

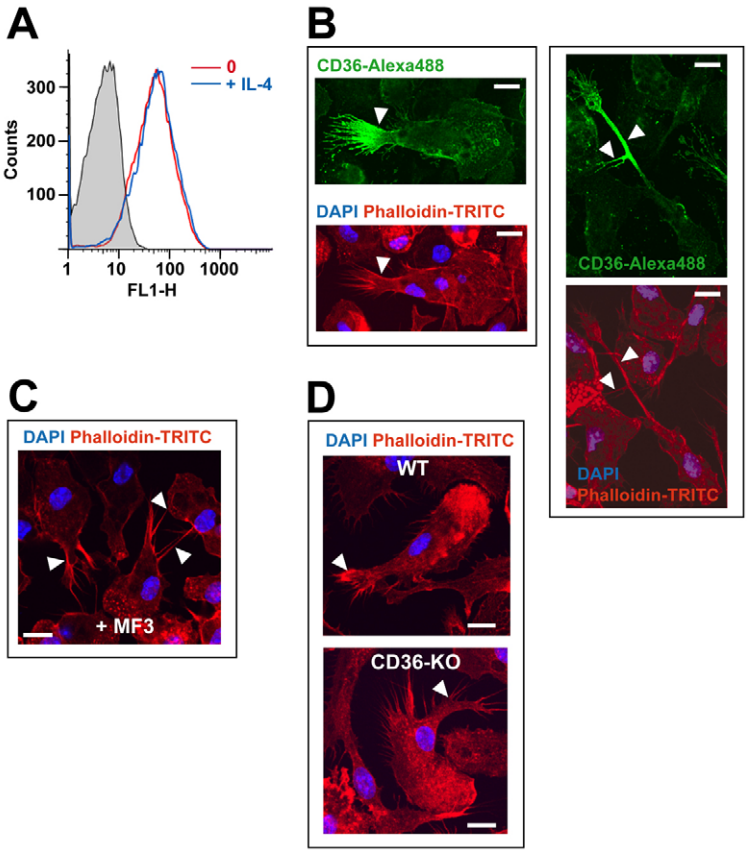

Fig. 3. The expression of CD36 during macrophage fusion. (A) FACS analysis of CD36 expression in ThioMΦ after incubation with IL-4 using MF3 or isotype control IgG2a (grey shade) antibodies. Anti-rat Alexa 488 was used for detection (FL1-H). (B) Confocal microscopy of lamellipodia and cell-cell contacts (arrows) formed at the onset of macrophage fusion (ThioMΦ, 6-8 hours). CD36 was labelled with MF3 and anti-rat Alexa 488 (green), actin with phalloidin-TRITC (red) and nuclei counterstained with DAPI (blue). (C) Lamellipodia and cell-cell contacts (arrows) in the presence of the anti-CD36 antibody MF3 (ThioMΦ, 8 hours) and in (D) wild-type (WT) and CD36-KO BMM (incubated for 48 hours with IL-4/GM-CSF). Bar, 10 µm.

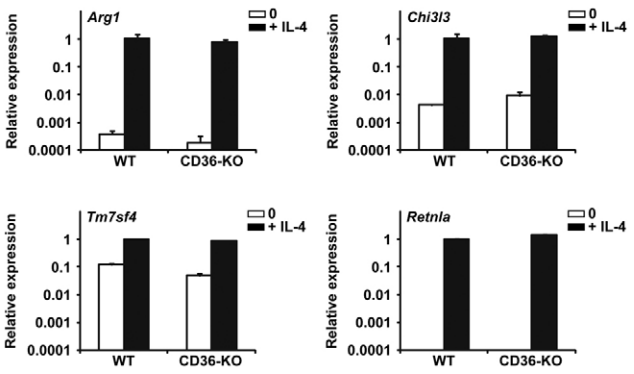

Fig. 4. Expression of IL-4 induced markers. Real-time RT-PCR analysis of IL-4-induced markers in wild-type (WT) and CD36-KO BMM. Macrophages were stimulated for 24 hours. Means ±s.d. of duplicate reactions are shown. *Arg1*, *Arginase 1*; *Chi3l3*, *Ym1*; *Tm7sf4*, *DC-STAMP*; *Retnla*, *Fizz1*.

against the CD36 PS-binding site. We conclude that lipid recognition by CD36 is involved in macrophage fusion. The involvement of CD36 seemed to be specific, as we found no contribution of SR-A, another lipid-binding scavenger receptor (data not shown).

PS exposure and recognition is required for macrophage fusion

It has been shown for other cell-cell fusion events, including myoblast fusion (van den Eijnde et al., 2001) and trophoblast formation (Adler et al., 1995), that cells undergoing fusion transiently expose PS on their surface, and externalization of PS is required for efficient fusion. Interestingly, non-apoptotic macrophages are known to express PS on their surface (Callahan et al., 2000) and we were able to identify localized areas of PS exposure in our macrophage cultures (Fig. 6D). We therefore investigated if PS recognition is involved in fusion of macrophages by masking PS via the PS-binding protein annexin V (also known as annexin A5). When we induced giant-cell formation in the presence of annexin V, fusion could be blocked efficiently (Fig. 6E). Therefore, exposure and recognition of PS are required for macrophage fusion. We hypothesize that PS recognition during cytokine-induced macrophage fusion is mediated by CD36.

CD36 is not involved in osteoclast formation

Macrophage fusion leads not only to the formation of multinucleated giant cells but also to osteoclast formation. Other molecules involved in cytokine-induced giant-cell formation such as DC-STAMP have also been implicated in formation of osteoclasts (Yagi et al., 2005). However, fusion during osteoclast formation in vitro was not affected in CD36-KO macrophages (Fig. 7A,B), and our anti-CD36 blocking antibody had no effect (data not shown). Therefore, CD36 appears to be selectively involved in cytokine-induced giant-cell formation but not in osteoclast fusion. This is consistent with the finding that CD36 is expressed specifically in human giant cells and not osteoclasts (Athanasou and Quinn, 1990), as well as the lack of a reported osteopetrotic phenotype in CD36-KO mice. CD36 is the first molecule described that is selectively involved in giant-cell formation and redundant in osteoclastogenesis.

Discussion

Multinucleated giant cells resulting from fusion of macrophages are observed in most granulomatous conditions, including

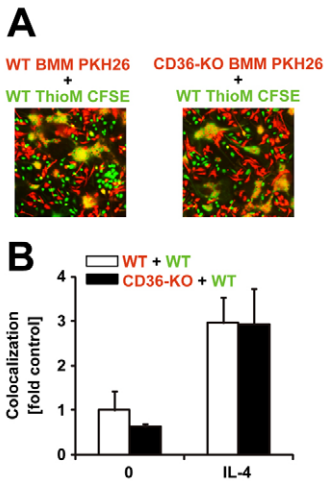

Fig. 5. The requirement of CD36 on one fusion partner. (A) Wild-type (WT) or CD36-KO BMM were labelled with PKH26 (red) and mixed with green CFSE-labelled wild-type ThioMΦ and incubated with IL-4. Cross-fusion is represented by co-localization of the fluorescent labels (yellow). (B) Quantitation of macrophage fusion, shown are means ±s.d. (n=3). Quantitation was performed twice with similar results.

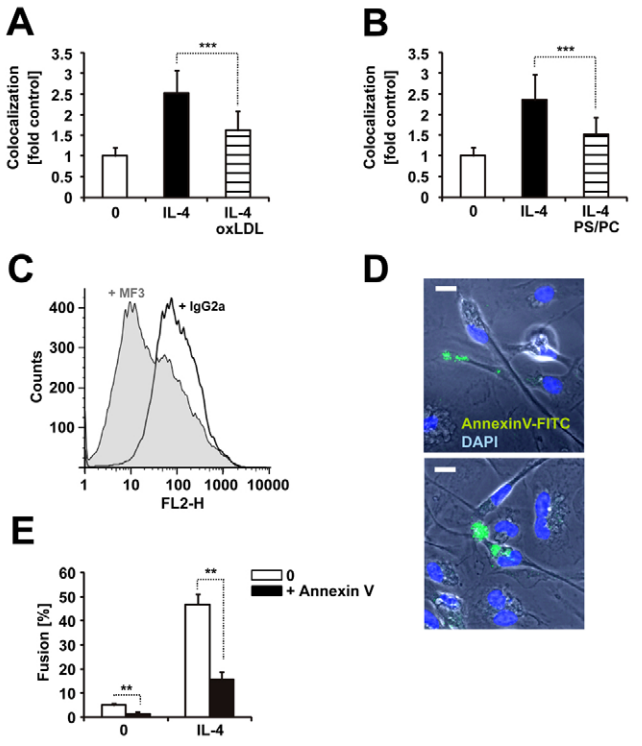

Fig. 6. A role for lipid recognition by CD36 and PS exposure and recognition during macrophage fusion. Quantitation of ThioMΦ fusion in the presence of (A) oxLDL (50 µg/ml) or (B) PS liposomes (PS/PC, 50 µM). Means±s.d. combined from three independent experiments. (A) n=21, ***P<0.0001; (B) n=17, ***P=0.0003. Mann Whitney Test, two-tailed. (C) Binding of DiI-labelled PS liposomes (=FL2) to ThioMΦ can be blocked by addition of anti-CD36 antibodies (MF3) but not control antibodies (IgG2a). This result was obtained in three independent experiments. (D) ThioMΦ were plated with IL-4 on Permanox in the presence of annexinV-FITC for 18-24 hours. Nuclei were counterstained with DAPI. (E) Annexin V-FITC blocks IL-4 induced ThioMΦ fusion. Means ±s.d., n=6, **P=0.0051, Mann Whitney Test, two-tailed. Shown are representatives of three independent experiments. Bar, 10 µm.

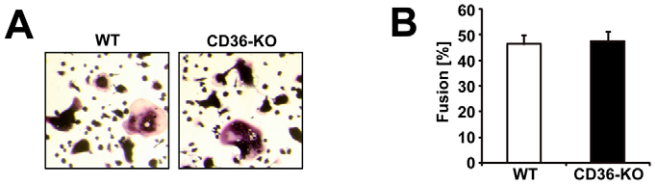

Fig. 7. CD36 is not involved in osteoclast formation. (A) TRAP staining and (B) quantitation of fusion in osteoclasts derived from wild-type (WT) and CD36-KO bone marrow progenitors by incubation with M-CSF/RANKL for 4 days. Shown are means±s.d. (*n*=6). Experiment was performed twice with similar results.

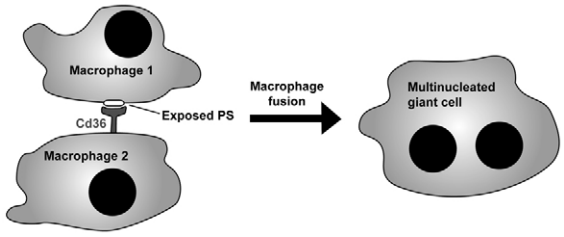

Fig. 8. Proposed mechanism of the involvement of CD36 during macrophage fusion. Localized areas of PS exposure on one fusion partner (macrophage 1) are recognized by CD36 on the other fusion partner (macrophage 2). Recognition of PS is required for efficient macrophage fusion.

tuberculosis, schistosomiasis, sarcoidosis and the foreign body reaction (Helming and Gordon, 2008). The functional significance of MGCs, as well as the mechanistic basis of their formation, remains poorly understood. Although CD36 has been implicated in a variety of processes, this is the first report of its involvement in giant-cell formation induced by IL-4 alternative activation.

Our data show that macrophages display localized areas of PS exposure and that the recognition of PS is required for efficient macrophage fusion. We have identified an anti-CD36 antibody that not only blocks macrophage fusion but also binding of CD36 to PS. As we also found that CD36 is required only on one of the fusing macrophages, we propose that during macrophage fusion, surface CD36 on one fusion partner mediates capture of PS on the other fusing macrophage (Fig. 8). Whereas recognition of PS on apoptotic cells by phagocytes leads to their engulfment (Wu et al., 2006), during fusion, macrophages do not internalize each other. We and others have consistently observed membrane fusion in localized cell contact zones formed via long cell extensions (McNally and Anderson, 2005). According to the zipper model of the mechanism of phagocytosis, ingestion is not automatically triggered by initial binding of the phagocyte to the particle, but requires sequential interaction of phagocyte receptors with ligands on the reminder of the particle surface (Griffin et al., 1975; Griffin et al., 1976). As we could detect only localized PS exposure in our macrophage cultures, these requirements would not be met and phagocytosis of whole macrophages would be prevented.

As PS exposure and recognition has also been implicated in other cell-cell fusion processes, lipid capture by cell surface receptors may be a general feature of cellular fusion. Even though giant cells and osteoclasts are both formed via macrophage fusion, it was not clear whether they share a similar fusion mechanism. CD36 is the first molecule described that is selectively involved in giant-cell formation and redundant in osteoclastogenesis.

We have previously shown that more than one molecule must be involved in macrophage fusion, with at least one factor required on all fusing macrophages (Helming and Gordon, 2007). This result, in addition to our failed attempts to induce cellular fusion by ectopic overexpression of CD36 in different cell lines (data not shown), points to the fact that CD36 is not sufficient for macrophage fusion. In our previous study we also found that IL-4 stimulation leads to the induction of fusogenic proteins (Helming and Gordon, 2007). However, during macrophage fusion, CD36 expression was not induced by IL-4 or IL-4/GM-CSF treatment (data not shown) even though CD36 was localized in the cell contact zones. In addition, PS exposure in macrophages was not dependent on the presence of these cytokines (data not shown). Based on these observations we propose that additional IL-4-inducible molecules are needed to induce giant-cell formation. We suspect that a complex machinery consisting of multiple proteins is required to promote hydrophobic contacts between the membranes of different cells and to mediate subsequent membrane reorganization and repair.

Multinucleated giant cells are present in granulomatous conditions as well as the foreign body reaction, the host response to implanted biomaterials (Anderson, 2000). Foreign body giant cells display an enhanced capacity to degrade large particles (Zhao et al., 1991) and may therefore be deleterious for implants. Tuberculosis-associated giant cells have been associated with restriction of cell-to-cell spread of mycobacteria (Byrd, 1998) but also with increased metalloproteinase secretion (Zhu et al., 2007), potentially contributing to tissue destruction. The discovery of CD36 and other macrophage fusion molecules will enable us to dissect the functional consequences of selective giant-cell formation under different circumstances.

Materials and Methods

Animals and isolation of murine primary macrophages

All mice used in this study were C57BL/6J, 10-30 weeks of age. ThioMΦ were isolated as described (Helming and Gordon, 2007). Bones from CD36-KO mice were obtained from David Kluth, Edinburgh, UK with the kind permission of Roy Silverstein. BMM were isolated as described previously (Helming and Gordon, 2007).

Fluorescent labelling of primary mouse macrophages

Macrophages were labelled with 5 μM CFSE (Invitrogen) in PBS for 10 minutes at 37°C. We used the PKH26 Red Fluorescence Cell Linker Kit (Sigma-Aldrich) according to the instructions.

ThioMΦ fusion

ThioMΦ fusion was analyzed as previously described (Helming and Gordon, 2007). Briefly, labelled ThioMΦ were plated in the presence of IL-4 in bacteriologic plastic six-well vessels for 24 hours. Cells were washed in PBS and plated on Permanox plastic (eight-well Lab-Tek Chamber Slides, Nunc) for 24 hours and fixed with 4% PFA. For annexin V inhibition experiments, non-labelled ThioMΦ were used. Annexin V-FITC (Invitrogen) was added at a concentration of 2.5% (vol/vol). Imaging analysis was performed with a Zeiss Axioplan upright fluorescence microscope (Plan-Neofluar 10× objective, 0.3 numeric aperture). Three to four independent images per well were acquired and analysed.

BMM fusion

BMM were resuspended in OptiMEM-10 and plated on Permanox plastic at 1×10^5 macrophages/well. IL-4 and GM-CSF (100 ng/ml, Peprotech) were added and cells incubated for 1 to 4 days until fusion was maximal. Slides were stained using the Hemacolor staining kit (Merck). Photographs were taken using a Nikon Coolscope slidescanner. Four to eight independent images per well were acquired and the number of giant and single cell nuclei counted. Fusion in % was quantified as the number of giant-cell nuclei (>2 nuclei) divided by the number of total nuclei.

Osteoclast culture

Mouse osteoclast progenitors were obtained by incubation of bone marrow cells in the presence of 20 ng/ml M-CSF (R&D Systems) in alpha-MEM (Sigma-Aldrich) containing 10% FCS, P/S, L-glutamine for 3 days in bacteriologic plastic six-well vessels. Adherent cells were detached and plated with 20 ng/ml M-CSF, 150 ng/ml RANKL (R&D Systems), 0.1 ng/ml human TGF-β1 (Peprotech) for 4 days. TRAP

staining was performed using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich). Fusion was quantified as described for the BMM fusion assay.

Preparation of monoclonal antibodies

Rats (strain DA) were immunized by intraperitoneal injection with 5×10^7 IL-4-treated murine ThioMΦ in PBS with a booster injection, 1 month later. Four days before harvest, rats received an intrasplenic injection of 1×10^7 IL-4 treated murine ThioMΦ in PBS. Rat splenocytes were fused with Y3Ag1.2.3 myeloma cells using PEG1500 (Boehringer) at a ratio of 2:1 essentially as described (Galfre et al., 1977). Hybridomas were screened by adding hybridoma supernatants at a dilution of 50% v/v to the macrophage fusion assay. Clones MF2, 3 and 4 were re-cloned twice. Antibodies were purified from hybridoma supernatants using Gamma Bind Plus Sepharose beads (GE Healthcare).

Immunoprecipitation

Cells were lysed in 20 mM Tris-HCl pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM Na_3VO_4 and proteinase inhibitor (Complete Mini, Roche). For immunoprecipitation, protein lysates were incubated with antibodies covalently conjugated to Protein G PLUS-Agarose (Santa Cruz) overnight at 4°C. Covalent coupling of antibodies to Gamma Bind Plus Sepharose beads was achieved using the following method: beads and antibodies were incubated in PBS at 4°C overnight, washed in 0.1 M sodium tetraborate, pH 9.0 and dimethylpimelimidate (DMP) was added to a final concentration of 20 mM. After 30 minutes, beads were washed in 50 mM glycine (pH 2.5) and resuspended in PBS.

Mass spectrometry

Gel bands were excised from Coomassie-stained gels, destained in 50 mM ammonium bicarbonate in 50% acetonitrile, then reduced in 10 mM DTT followed by alkylation with iodoacetamide. Gel spots were digested with 200 ng of trypsin (Sigma-Aldrich) overnight at 37°C and analysed by LC MSMS on a Thermo Orbitrap mass spectrometer. Data were searched using Mascot (Matrix Science, London).

Immunostaining and confocal microscopy

Cells were fixed in 4% PFA, permeabilized in 0.1% Triton, washed and blocked in PBS + 5% goat serum. Anti-CD36 (MF3) was added in PBS/goat serum (10 μg/ml) and slides incubated overnight at 4°C. Anti-rat Alexa Fluor 488 or anti-rat Alexa Fluor 555 (Invitrogen) were used for detection. Phalloidin-TRITC (Sigma-Aldrich) was used at 10 μg/ml and DAPI (Invitrogen) at 1 μg/ml in PBS. Imaging analysis was performed with a Zeiss LSM 510 META upright confocal laser scanning microscope (Plan-Apochromat 63× oil immersion lens, 1.4 numeric aperture).

FACS analysis

ThioMΦ were stimulated with IL-4 for 24 hours, blocked (0.5% BSA, 5% goat serum) and anti-CD36 (MF3) or an isotype-matched control (IgG2a, OX11) were added at 10 μg/ml for 2 hours on ice. Positive staining was detected using anti-rat Alexa 488 (Invitrogen). Cells were analysed using FACSCalibur Flow Cytometer (BD Biosciences).

Preparation of PS liposomes and oxLDL

3-sn Phosphatidylcholine (PC) from egg yolk and 1,2-Diacyl-sn-glycero-3-phospho-L-serine (PS) were purchased from Sigma-Aldrich. PS liposomes were prepared as described (Fadok et al., 1992). Briefly, lipids were dried down under N_2 gas and subsequent speed-vacuum for 1 hour. Lipids were resuspended in PBS at a final concentration of 1 mM and sonicated for 1 to 2 hours. PS/PC liposomes contained 50 molar percent PS and PC. For DiI-labelled PS liposomes, Vybrant DiI Labeling Solution (Invitrogen) was dried down with the lipids to a final concentration of 10 μM. oxLDL was a generous gift from Claudine Neyen. Human LDL was isolated from fresh plasma using self-generating gradients of iodixanol as described (Graham et al., 1996). LDL was oxidized by incubation with 5 μM copper sulphate for 24 hours at 37°C, the reaction stopped by addition of 100 μM EDTA and 20 μM butylated hydroxytoluene and oxLDL was dialyzed against 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 100 μM EDTA.

Analysis of binding of DiI-labelled PS liposomes

ThioMΦ were incubated with 10 μg/ml antibody in OptiMEM on ice for 1 hour. DiI-labeled PS liposomes were added (50 μM) for 30 minutes, cells were washed three times in PBS and subjected to FACS analysis.

Cloning and transfection

Murine CD36 cDNA was amplified using the following primers: forward primer AAGAGCTCGGCGGATGGGCTGTGATCGGAACTGT, reverse primer TTGGATCCCGTTTTCCATTCTTGGATTTGCA and cloned into pEYFP-N1 (BD Biosciences, Franklin Lakes, US) via the *Sac*I and *Bam*HI site. Transfections were performed using FuGene6 (Roche).

Real-time RT-PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen), reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen). Real-time quantitative PCR was performed on a Rotor-Gene RG3000 (Corbett Research, Sydney, Australia) using the Quantitect SYBR Green PCR Kit (Qiagen). Expression was normalized to the housekeeping gene *Hprt*. The following primers were used for amplification: *Hprt1*FOR GCTCGAGATGTCATGAAGGAGA, *Hprt1*REV AAAGAACTTATAGCCCCCCTTG, *Tm7sf4*FOR GTATCGGCTCATCTCCTCCA, *Tm7sf4*REV TGCAGCTCGGTTCAAACATA, *Arg1*FOR GGAAAGCCAATGAAGAGCTG, *Arg1*REV CTGGTTGTCAGGGGAGTGTT, *Retnla*FOR CTCATCTGCATCTCCCTGCT, *Retnla*REV CCACTCTGGATCTCCCAAGA, *Chi3l3*FOR TAGTACTGGCCCACCAGGAA, *Chi3l3*REV TGTTGTCCTTGAGCCACTGA.

We thank Roy Silverstein for permission and David Kluth, Spike Clay for providing CD36-KO bones; Claudine Neyen for providing oxLDL and technical help; Benjamin Thomas for performing mass spectrometry. This work was supported by the German Research Foundation (DFG), the Medical Research Council (MRC) and the German Academic Exchange Service (DAAD). Deposited in PMC for release after 6 months.

References

Adler, R. R., Ng, A. K. and Rote, N. S. (1995). Monoclonal antiphosphatidylserine antibody inhibits intercellular fusion of the choriocarcinoma line, JAR. *Biol. Reprod.* **53**, 905-910.

Anderson, J. M. (2000). Multinucleated giant cells. *Curr. Opin. Hematol.* **7**, 40-47.

Athanasou, N. A. and Quinn, J. (1990). Immunophenotypic differences between osteoclasts and macrophage polykaryons: immunohistological distinction and implications for osteoclast ontogeny and function. *J. Clin. Pathol.* **43**, 997-1003.

Byrd, T. F. (1998). Multinucleated giant cell formation induced by IFN-gamma/IL-3 is associated with restriction of virulent Mycobacterium tuberculosis cell to cell invasion in human monocyte monolayers. *Cell Immunol.* **188**, 89-96.

Callahan, M. K., Williamson, P. and Schlegel, R. A. (2000). Surface expression of phosphatidylserine on macrophages is required for phagocytosis of apoptotic thymocytes. *Cell Death Differ.* **7**, 645-653.

Chambers, T. J. and Spector, W. G. (1982). Inflammatory giant cells. *Immunobiology* **161**, 283-289.

Chen, E. H., Grote, E., Mohler, W. and Vignery, A. (2007). Cell-cell fusion. *FEBS Lett.* **581**, 2181-2193.

Endemann, G., Stanton, L. W., Madden, K. S., Bryant, C. M., White, R. T. and Protter, A. A. (1993). CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **268**, 11811-11816.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L. and Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* **148**, 2207-2216.

Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W. and Howard, J. C. (1977). Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature* **266**, 550-552.

Graham, J. M., Higgins, J. A., Gillott, T., Taylor, T., Wilkinson, J., Ford, T. and Billington, D. (1996). A novel method for the rapid separation of plasma lipoproteins using self-generating gradients of iodixanol. *Atherosclerosis* **124**, 125-135.

Greenberg, M. E., Sun, M., Zhang, R., Febbraio, M., Silverstein, R. and Hazen, S. L. (2006). Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. *J. Exp. Med.* **203**, 2613-2625.

Griffin, F. M., Jr, Griffin, J. A., Leider, J. E. and Silverstein, S. C. (1975). Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. *J. Exp. Med.* **142**, 1263-1282.

Griffin, F. M., Jr, Griffin, J. A. and Silverstein, S. C. (1976). Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. *J. Exp. Med.* **144**, 788-809.

Helming, L. and Gordon, S. (2007). Macrophage fusion induced by IL-4 alternative activation is a multistage process involving multiple target molecules. *Eur. J. Immunol.* **37**, 33-42.

Helming, L. and Gordon, S. (2008). The molecular basis of macrophage fusion. *Immunobiology* **212**, 785-793.

Huppertz, B., Bartz, C. and Kokozidou, M. (2006). Trophoblast fusion: fusogenic proteins, syncytins and ADAMs, and other prerequisites for syncytial fusion. *Micron* **37**, 509-517.

Jay, S. M., Skokos, E., Laiwalla, F., Krady, M. M. and Kyriakides, T. R. (2007). Foreign body giant cell formation is preceded by lamellipodia formation and can be attenuated by inhibition of Rac1 activation. *Am. J. Pathol.* **171**, 632-640.

Langhans, T. (1868). Ueber Riesenzellen mit wandständigen Kernen in Tuberkeln und die fibröse Form des Tuberkels. *Virchows Arch. Pathol. Anat.* **42**, 382-404.

McGilvray, I. D., Serghides, L., Kapus, A., Rotstein, O. D. and Kain, K. C. (2000). Nonopsonic monocyte/macrophage phagocytosis of Plasmodium falciparum-parasitized erythrocytes: a role for CD36 in malarial clearance. *Blood* **96**, 3231-3240.

McNally, A. K. and Anderson, J. M. (2005). Multinucleated giant cell formation exhibits features of phagocytosis with participation of the endoplasmic reticulum. *Exp. Mol. Pathol.* **79**, 126-135.

Moore, K. J. and Freeman, M. W. (2006). Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1702-1711.

Podrez, E. A., Febbraio, M., Sheibani, N., Schmitt, D., Silverstein, R. L., Hajjar, D. P., Cohen, P. A., Frazier, W. A., Hoff, H. F. and Hazen, S. L. (2000). Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J. Clin. Invest.* **105**, 1095-1108.

Rigotti, A., Acton, S. L. and Krieger, M. (1995). The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J. Biol. Chem.* **270**, 16221-16224.

Stuart, L. M., Bell, S. A., Stewart, C. R., Silver, J. M., Richard, J., Goss, J. L., Tseng, A. A., Zhang, A., El Khoury, J. B. and Moore, K. J. (2007). CD36 signals to the actin cytoskeleton and regulates microglial migration via a p130Cas complex. *J. Biol. Chem.* **282**, 27392-27401.

van den Eijnde, S. M., van den Hoff, M. J., Reutelingsperger, C. P., van Heerde, W. L., Henfling, M. E., Vermeij-Keers, C., Schutte, B., Borgers, M. and Ramaekers, F. C. (2001). Transient expression of phosphatidylserine at cell-cell contact areas is required for myotube formation. *J. Cell Sci.* **114**, 3631-3642.

Vignery, A. (2005). Macrophage fusion: the making of osteoclasts and giant cells. *J. Exp. Med.* **202**, 337-340.

Wu, Y., Tibrewal, N. and Birge, R. B. (2006). Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol.* **16**, 189-197.

Yagi, M., Miyamoto, T., Sawatani, Y., Iwamoto, K., Hosogane, N., Fujita, N., Morita, K., Ninomiya, K., Suzuki, T., Miyamoto, K. et al. (2005). DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *J. Exp. Med.* **202**, 345-351.

Yesner, L. M., Huh, H. Y., Pearce, S. F. and Silverstein, R. L. (1996). Regulation of monocyte CD36 and thrombospondin-1 expression by soluble mediators. *Arterioscler. Thromb. Vasc. Biol.* **16**, 1019-1025.

Zhao, Q., Topham, N., Anderson, J. M., Hiltner, A., Lodoen, G. and Payet, C. R. (1991). Foreign-body giant cells and polyurethane biostability: *in vivo* correlation of cell adhesion and surface cracking. *J. Biomed. Mater. Res.* **25**, 177-183.

Zhu, X. W., Price, N. M., Gilman, R. H., Recarvarren, S. and Friedland, J. S. (2007). Multinucleate giant cells release functionally unopposed matrix metalloproteinase-9 *in vitro* and *in vivo*. *J. Infect. Dis.* **196**, 1076-1079.

Journal of Cell Science