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The pericyte and stromal cell marker CD248 (endosialin) is required for efficient lymph node expansion

Siân Lax[1], **Debbie L. Hardie**[1], **Amy Wilson**[1], **Mike R. Douglas**[2], **Graham Anderson**[1], **David Huso**[3], **Clare M. Isacke**[4], and **Christopher D. Buckley**[1]

[1]Rheumatology Research Group, School of Immunity and Infection, School of Clinical and Experimental Medicine, MRC Centre for Immune Regulation, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK

[2]Molecular Neuroscience Group, School of Clinical and Experimental Medicine, MRC Centre for Immune Regulation, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK

[3]The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medical Institutions, Baltimore, MD, USA

[4]Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, UK

Abstract

CD248 is a cell surface receptor that specifically identifies fibroblasts and pericytes during development and in association with cancer and inflammation. However, its function is poorly defined and its role in lymphoid organs not studied. Here, we used (4-hydroxy-3-nitrophenyl)acetyl chicken γ-globulin immunisation and mice lacking CD248 to study whether CD248 modulates popliteal LN (pLN) expansion and subsequent immune responses. We have found that CD248 is required for complete pLN expansion but not for co-ordination of B and T cell compartmentalisation or antibody production following (4-hydroxy-3-nitrophenyl)acetyl chicken γ-globulin immunisation. *In vitro*, we show that CD248 expression in human MG63 stromal cells and mouse embryonic fibroblasts leads to a pro-proliferative and pro-migratory phenotype. This correlates with a proliferating $CD248^+$ population observed *in vivo* during pLN expansion. Taken together, these data highlight a role for CD248 in secondary lymphoid organ remodelling during adaptive immune responses.

Keywords

CD248; Endosialin; Fibroblast; Lymphoid tissue; Stromal cells

Introduction

During an immune response, secondary lymphoid organs (SLO) expand significantly. However, mechanisms that regulate this have been relatively understudied with leucocyte accumulation rather than stromal cell expansion being the focus. Logic dictates that there has to be co-ordinated expansion of tissue structure to accommodate the rapidly expanding

Full correspondence: Professor Christopher D. Buckley, Rheumatology Research Group, School of Immunity and Infection, MRC Centre for Immune Regulation, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK Fax: 144-121-414-6794 c.d.buckley@bham.ac.uk.

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leucocyte populations. This expansion includes not only an intricate network of fibroblasts that provide an important structural scaffold but also the formation of new blood and lymph vessels to oxygenate and allow transport of lymphocytes into and out of the organ [1].

CD248/endosialin is a relatively new marker for a subset of stromal cells in developing tissues. It is a member of an emerging transmembrane protein family implicated in tissue remodelling and repair, which includes CD141 and CD93 [2, 3]. CD248 is expressed on fibroblasts and pericytes during development and in tumour-associated stroma [4–7]. An important role for CD248 within tumour stroma has been highlighted in CD248-deficient mice; abdominal tumours implanted into mice lacking CD248 demonstrated reduced growth, invasion and metastasis [8]. This, coupled with the finding that CD248 expression is regulated by hypoxia inducible factor-2α [9], suggests that CD248 may provide an important link between hypoxia and tissue remodelling allowing synchronisation of these processes.

We have previously proposed a role for CD248 in development and infection-dependent activation of SLO stroma [10]. Whether CD248 is required for tissue remodelling was not explored nor was its function. Therefore, to more fully understand the requirements for CD248 during remodelling, we compared the degree of popliteal LN (pLN) expansion in WT and CD248 KO mice following immunisation with (4-hydroxy-3-nitrophenyl)acetyl chicken γ-globulin (NP-CGG). The ability of CD248 to regulate cell migration and proliferation *in vitro* was examined together with proliferation of $CD248^+$ cells in pLN following NP-CGG immunisation *in vivo*. Our data suggest that CD248 plays a role in pLN expansion by increasing the proliferative and migratory capacity of stromal cells required to remodel the pLN microenvironment.

Results and discussion

CD248 expression is associated with initial pLN expansion

The rabbit anti-mouse CD248 antibody, P13, was raised against a mouse CD248-Fc fusion protein [5]. To confirm specificity, MEF were derived from WT and CD248 KO mice, genotype confirmed and mRNA expression of CD248 verified by RT-PCR (Fig. 1A). These cell lines were then stained with cytoskeletal vimentin, nuclear Hoechst and P13 (Fig. 1B). P13 was observed only in those cells derived from WT mice. Therefore, these data unequivocally demonstrate that P13 is specific for CD248.

We have previously postulated that CD248 expression defines a subset of SLO stromal cells required for remodelling during tissue expansion [10]. To test this, we used NP-CGG immunisation to investigate CD248 in pLN expansion. The initial NP-CGG response is Th2-dependent, with T-cell proliferation occurring around day 3 post-immunisation. This is followed by cognate interaction between T and B cells and subsequent B-cell proliferation, cumulating in high-specificity class-switched IgG1 antibody production [11]. Current thinking proposes that waves of lymphocyte proliferation drive the increase in LN size seen following NP-CGG immunisation. However, we propose that the underlying stroma also plays a role in regulating expansion, as already appreciated in many tumour types [12]. Therefore, WT mice were immunised with NP-CGG and CD248 expression tracked at day 5, following T-cell proliferation; day 7, following B-cell proliferation; and day 21, peak of the adaptive response. CD248 was observed mainly in the capsule of unimmunised pLN, with expression increasing throughout the medulla, peaking at day 7 and then declining back to near non-immunised levels by day 21 (Fig. 1C). Further analysis at day 7 revealed that CD248 is not present on myeloid cells ($CD11b^-$), with little co-localisation on $gp38^+$ T-zone reticular cells [13] (Fig. 1D). Co-localisation of CD248 and gp38 was observed only within the capsule in close proximity to the region where marginal reticular cells have been

described [14]. Therefore, these data suggest CD248 is associated with an as yet undefined stromal cell whose occurrence coincides with the initial response of pLN to NP-CGG.

CD248 is involved in pLN expansion but not antibody production following NP-CGG immunisation

Having observed an increase in CD248 expression following NP-CGG immunisation, which peaked at day 7, we utilised the CD248 KO mice to investigate the functional relevance of CD248 in this model. Post-NP-CGG immunisation pLN were removed and weighed to assess expansion. Interestingly, when compared with those taken from WT mice CD248-deficient pLN were significantly smaller at day 5 (Fig. 2A). This trend continued throughout the time course. It is worth noting that CD248 expression during this time course peaked at day 7 (Fig. 1C), a time point when the difference in pLN weight failed to reach statistical significance. Therefore, these data may indicate that CD248 expression observed at days 5 and 7 post-NP-CGG immunisation is required for complete expansion of the pLN, with the initial increase at day 5 triggering subsequent CD248-dependent pLN growth.

Having demonstrated CD248-dependent LN expansion, we next examined whether organisation of B- and T-cell zones was affected by CD248 loss. pLN sections taken from non-immunised and day 21 post-NP-CGG immunisation were stained for B cells ($B220^+$) (Fig. 2B) or T cells ($CD4^+$ and $CD8^+$) (Fig. 2C). The sections were also stained for endothelial cells ($CD31^+$) and germinal centre follicular dendritic cells ($VCAM\text{-}1^+$). The gross architecture of WT and CD248 KO pLN was comparable, suggesting that CD248 is not involved in compartmentalisation of T- and B-cell zones, a function probably served by gp38-expressing T-zone reticular cells [15]. Finally, the function of WT and CD248 KO pLN was analysed by measuring antibody titres of innate (IgM) and adaptive (IgG1) responses specific for either NP or CGG. As expected IgM levels were initially high at day 5 post-immunisation and declined during the time course, with a concomitant increase in switched IgG1 antibodies (Fig. 2D). The amount of antibody produced, both IgM and IgG1, was not CD248 dependent. This taken together with data that show all murine haematopoietic cells examined so far do not express CD248 mRNA [16] suggest that CD248 KO mice are unlikely to have defects in the motility of cells at the injection site and/or in lymphoid cells migrating into and out of the pLN during the NP-CGG response. Our data therefore suggest that CD248 does not play a role in regulating T- and B-cell-dependent immune responses in pLN to NP-CGG. Rather, CD248 regulates the initial rate of pLN expansion.

Although measurements of O_2 tension have not been reported in expanding pLN, we propose that as they expand the local microenvironment becomes increasingly hypoxic. This results in an increase in CD248 expression, *via* hypoxia inducible factor-2α [9], which in turn is required for full expansion of pLN. This is analogous to the previous studies where lack of CD248 inhibited full expansion of abdominal tumours [8].

CD248 regulates cell proliferation and migration, but not differentiation

To explore possible mechanisms of CD248, the human osteosarcoma cell line, MG63, which does not express CD248 on its cell surface, was stably transfected with full-length human CD248. Vector-only transfections were also performed and CD248 expression confirmed by flow cytometry using an antibody specific for human CD248 [4] (Fig. 3A). CD248-transfected MG63 cells had increased migratory velocity compared with vector-only controls. This effect was significantly enhanced upon addition of PDGF, a known chemoattractant [17] (Fig. 3B, left). In addition, cell migration was assessed in MEF derived from WT and CD248 KO mice. MEF lacking CD248 migrated significantly less than WT in

the absence of any chemokine gradient (Fig. 3B, right). Therefore, these data suggest that *in vitro* CD248 is required for a highly motile phenotype.

The proliferative capacity of CD248-expressing cell lines was also analysed by measuring thymidine (^{3}H) incorporation over a range of cell densities. MG63 cells transfected with CD248 significantly increased proliferation compared with vector-only controls (Fig. 3C, left). In contrast, CD248 KO MEF had a significantly lower proliferative capacity compared with WT controls (Fig. 3C, right). Thus, the presence of CD248 within cells leads to an increase in proliferation, which interestingly was more pronounced at higher cell densities. These data are consistent with the previous *in vitro* studies which suggested a role for human CD248 in regulating proliferation, migration and adhesion to fibronectin, as well as sensing an increase in cell density [7, 18].

To investigate whether CD248 regulates proliferation *in vivo*, WT mice immunised with NP-CGG for 7 days and unimmunised controls were treated with BrdU (Fig. 3D). BrdU$^+$ CD248-expressing cells were clearly visible only during NP-CGG-induced pLN expansion in the medullary fibroblasts and capsule. This confirmed that CD248-expressing cells proliferate *in vivo*. Taken together, we suggest that CD248$^+$ stroma is required for full pLN expansion by increasing proliferation of mesenchymal cells in the medulla and capsule of pLN.

It has recently been shown that CD248 is expressed on human bone marrow-derived mesenchymal stem cells (MSC) [19]. As such cells have been suggested to play a role in tissue remodelling and repair [20], we were interested to see whether CD248 played a role in regulating differentiation of mouse mesenchymal cells. WT and CD248 KO MEF were driven to differentiate into adipocytes, chondrocyte and osteocytes using specific differentiation media (Fig. 3C). Adipocytes were assessed by the expression of fatty acid binding protein-4 (left), chondrogenic pellets by the expression of type II Collagen (Col2) (middle) and Alizarin Red was used to stain calcium-containing matrix produced by osteocytes (right). No significant differences were observed between WT and KO MEF. Therefore, our data suggest that CD248 plays a role in regulating cell migration and proliferation without influencing the capacity of stromal cells to differentiate down adipo-, chondro- or osteogenic pathways.

Concluding remarks

Until recently, studies exploring how SLO expand during an immune response have focused on lymphocytes at the expense of stroma. As suggested in a recent review, to fully understand the immune response, stromal cells need to also be included [21]. Our data demonstrate a role for the fibroblast and pericyte marker CD248, in regulating expansion of pLN following NP-CGG immunisation. They highlight the growing realisation that stroma is not inert but plays a role in regulating SLO biology. We propose that CD248 is a marker that specifically identifies proliferative and migratory stromal cells whose appearance correlates with active remodelling of SLO. Whether CD248-expressing cells in SLO arise from re-expression on proliferating precursors *in situ* or from recruitment of MSC-like cells from the blood and/or bone marrow remains to be determined.

Materials and methods

Mice and histology

129Sv mice were obtained from Taconic, Denmark. Generation of CD248 KO mice has been described previously [8]. All experiments were performed in accordance with UK laws with approval of local ethics committees. Frozen sections of tissues were prepared and stained by immunohistochemistry as described previously [14].

MEF derivation and validation

Internal organs were removed from decapitated E14 or E15 mouse embryos, tissue trypsinised and single-cell suspensions cultured overnight. Loosely adherent cells were removed, fresh media added and remaining fibroblasts cultured and used at low passage. Genotyping and mRNA expression of CD248 was performed as described previously [8].

Immunofluorescence staining and confocal microscopy

Immunofluorescence was performed as described previously [10] using the following antibodies: anti-CD248 P13 (generated in our laboratory [5]). Anti-CD11b-FITC, anti-B220-FITC, anti-CD4-FITC, anti-CD8-FITC (eBiosciences); anti-vimentin (NeoMarkers); Anti-VCAM-biotin (Southern Biotech) and Anti-CD31 (Serotec). Anti-gp38 a kind gift from Andy Farr. Primary antibodies were detected using appropriate fluorescently conjugated secondaries. Representative images are shown.

NP-CGG immunisation, BrdU treatment and serum antibody detection

NP was conjugated to CGG as described previously [11]. Adult (6–8 wk) sex-matched mice were injected into the plantar surface of both hind feet with 20 μg alum-precipitated NP-CGG with 5×10^8 *Bordella pertussis*. In total, 2 mg of BrdU (BD Biosciences) was injected i.p. 2 days before tissue analysis. NP- and CGG-specific serum antibody was detected by ELISA as described previously [11].

Generation of stable cell lines

Full-length human CD248 was ligated into a pcDNA3.1 vector (Invitrogen) as described previously [4], followed by Lipofectamine™ 2000 (Invitrogen) transfection. Geneticin (Gibco) selection was performed followed by incubation with supernatant from the mouse anti-human CD248 hybridoma B1/35.1 [4]. Positive cells were isolated with sheep anti-mouse IgG magnetic Dynal beads (Invitrogen).

Cell migration

Dunn chambers (Weber Scientific International) were used as described previously [22]. Briefly, 100 ng/mL PDGF (Sigma) was used in the outer chamber for transfected MG63 cell lines. Coverslips were coated with 1.5 μg/mL fibronectin (Sigma) for MEF assays. Four areas *per* chamber were analysed and migration of ten cells tracked. Digital images were captured every 20 min over 5 h.

Cell proliferation

Relative ^{3}H uptake was compared using 0.4×10^6 cells/mL in doubling dilutions. Cells were incubated with 0.2 μCi/mL ^{3}H for 6h, transferred onto filter mats (Perkin Elmer) using a harvester (Skatron) and CPM assayed using a Wallac 1205 Betaplate Counter.

MSC differentiation

MEF were analysed using the Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D Systems). Four biological replicates were performed.

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Abbreviations

MEF	mouse embryonic fibroblast
MSC	mesenchymal stem cells
NP-CGG	(4-hydroxy-3-nitrophenyl)acetyl chicken γ-globulin
pLN	popliteal LN
SLO	secondary lymphoid organ

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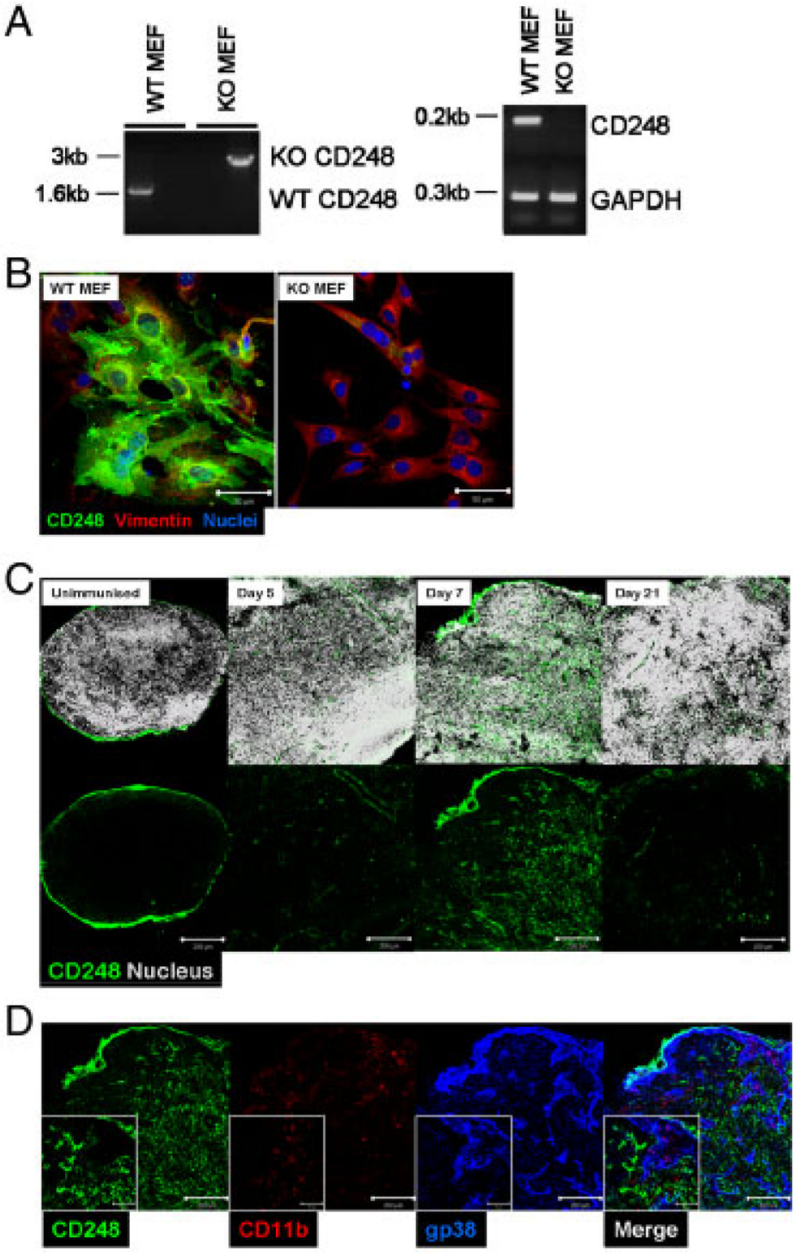

Figure 1.

CD248 expression is associated with initial pLN expansion. PCR for WT and KO CD248 alleles (left) and RT-PCR for CD248 (right) confirmed genotype of WT and CD248 KO-derived MEF (A). WT and CD248 KO MEF stained with P13 (green), vimentin (red) and Hoechst (blue). Bar=50 μm (B). Expression of CD248 (green) post-NP-CGG immunisation was tracked in WT pLN by immunofluorescence. Bars=200 μm (C). At 7 days post-NP-CGG immunisation, WT pLN were stained with CD248 (green), CD11b (red) and gp38 (blue) to analyse stromal subsets. Bars=200 μm in main images and 50 μm in inserts (D).

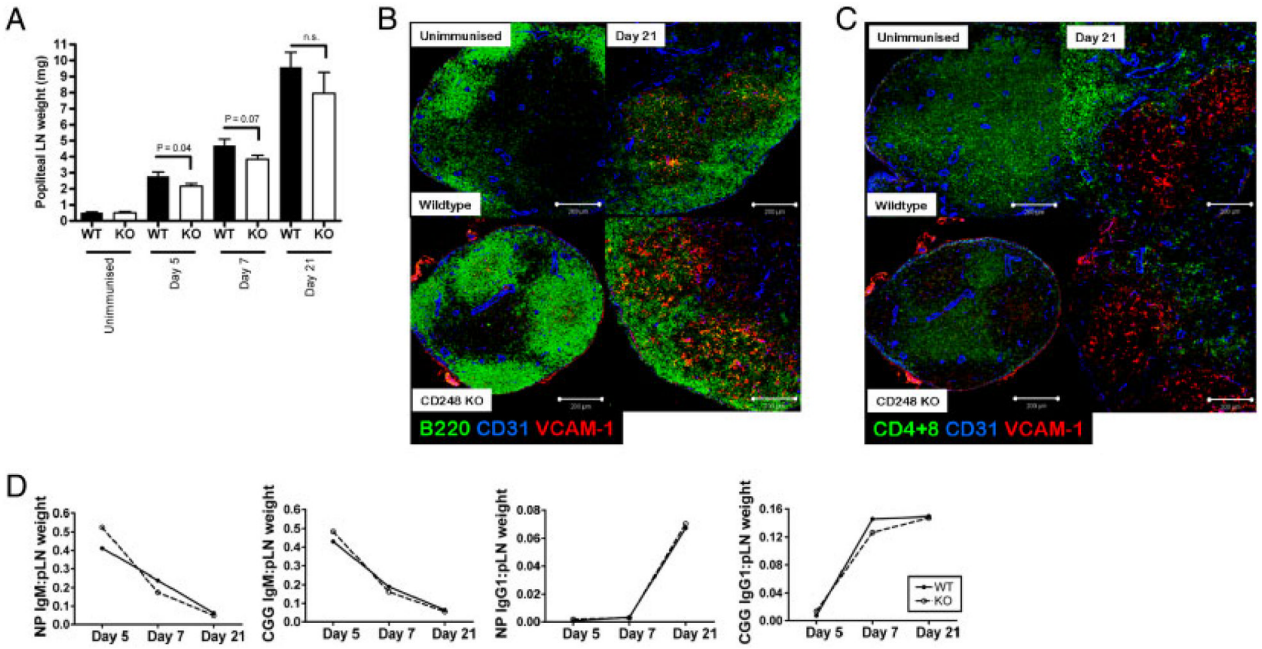

Figure 2.

CD248 is required for pLN expansion but not antibody production following NP-CGG immunisation. WT and CD248 KO pLN were weighed post NP-CGG immunisation. Data are mean (+SEM) from three experiments, each with at least three replicates. *p*-Values calculated by Student's non-paired *t*-test (A). Organisation of B-cell follicles (B) and T-cell zones (C) in pLN from unimmunised and day 21 post-NP-CGG immunisation WT and CD248 KO mice were analysed by immunofluorescence. Bars=200 μm. Sera antibody titres of innate (IgM) and adaptive (IgG1) antibody specific for NP and CGG were analysed by ELISA. Data shown as a ratio of mean antibody titre:mean pLN weight (n=4) (D).

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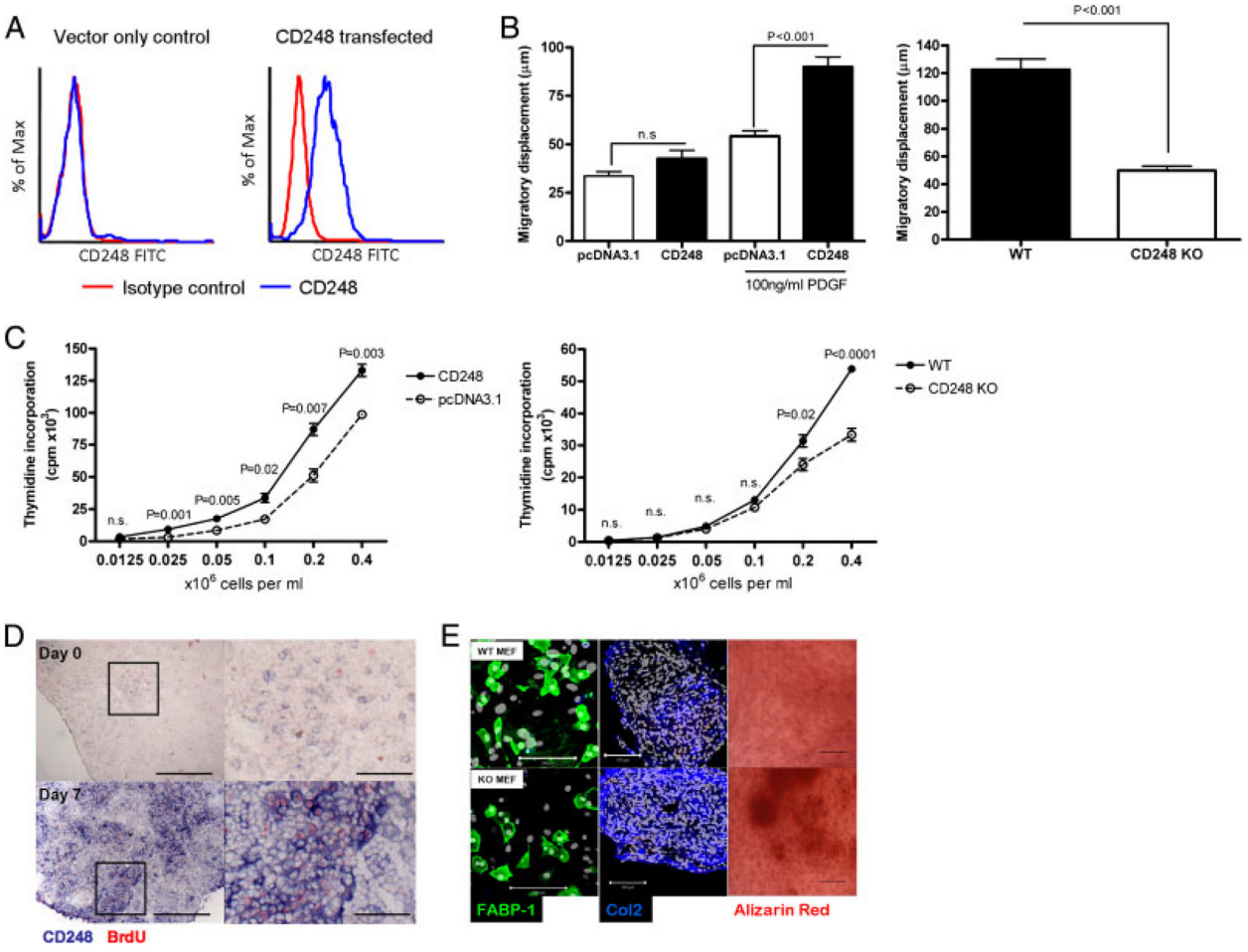

Figure 3.

CD248 regulates proliferation and migration, but not differentiation. Transfection of MG63 cells with CD248 was confirmed by flow cytometry (A). Migration of MG63 cells in the absence or presence of PDGF (left, n=4) and WT and CD248 KO MEF (right, n=2) were assayed using a Dunn chamber (B). Proliferation of MG63 (left, n=3) and MEF (right, n=6) was analysed by ^{3}H uptake (C). Stromal proliferation within pLN was analysed by immunohistochemical staining of CD248 (blue) and BrdU (red) at day 0 (top) and day 7 (bottom) post-NP-CGG immunisation, bars=200 μm. Black box indicates area of interest with magnified image right, bars=50 μm (D). WT (top) and CD248 KO (bottom) MEF were driven to differentiate into adipocytes (left, green), chondrocytes (middle, blue) and osteocytes (right, red). Bars=100 μm (E). Bars in (B) and (C) show mean (±SEM), with p-values calculated by Student's non-paired t-test.

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