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The stromal cell antigen CD248 (endosialin) is expressed on naive CD8$^+$ human T cells and regulates proliferation

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Summary

CD248 (endosialin) is a transmembrane glycoprotein that is dynamically expressed on pericytes and fibroblasts during tissue development, tumour neovascularization and inflammation. Its role in tissue remodelling is associated with increased stromal cell proliferation and migration. We show that CD248 is also uniquely expressed by human, but not mouse (C57BL/6), CD8$^+$ naive T cells. CD248 is found only on CD8$^+$ CCR7$^+$ CD11a^{low} naive T cells and on CD8 single-positive T cells in the thymus. Transfection of the CD248 negative T-cell line MOLT-4 with CD248 cDNA surprisingly reduced cell proliferation. Knock-down of CD248 on naive CD8 T cells increased cell proliferation. These data demonstrate opposing functions for CD248 on haematopoietic (CD8$^+$) versus stromal cells and suggests that CD248 helps to maintain naive CD8$^+$ human T cells in a quiescent state.

Keywords: angiogenesis; CD248/endosialin; CD8; naive T cells; tumour therapy

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Introduction

CD248 is a 175 000 molecular weight type I transmembrane glycoprotein encoded on human chromosome 11q13.[1] It is a member of a family of proteins that includes CD93 and CD141 (thrombomodulin).[2,3] CD248 was first characterized as a novel marker expressed on tumour, but not normal, vascular endothelial cells.[1] Also known as 'Tumour Endothelium Marker-1',[4–6] CD248 is expressed on stromal cells in samples of breast carcinoma,[7] colon cancer,[8] glioblastoma multiforme[9] and xenografts of colon carcinoma.[5]

More recently, CD248 has been shown to be expressed on perivascular NG2$^+$ cells in the tumour vasculature as well as on interstitial fibroblasts and mesenchymal stem cells rather than on endothelial cells *per se*.[5,7,9–12] Of note, CD248 expression on these stromal cells is developmentally regulated, being high in embryonic and fetal tissue[5,10,11,13–15] but lost or substantially reduced in the adult, with the exception of uterine tissue.[15]

Abbreviations: Cy, cyanine; mAb, monoclonal antibody; PE, phycoerythrin.

Lack of CD248 expression correlates with a site-specific reduction in the aggressiveness of xenograft tumours; a finding that appears to be related to defects in the size of tumour-associated neo-vessels.[16] Given these findings and the lack of any reported haematopoietic defects in the CD248 knockout mouse,[16] CD248 has been accepted as a stromal-cell-specific marker.[12] However, during an unbiased screening for genes uniquely expressed in different human blood cell subsets we discovered that CD248 was one of only five specific transcripts that distinguished CD8 T cells from other differentiated blood cells.[17] Here we characterize CD248 expression on human and mouse haematopoietic cells. We show that CD248 expression on leucocytes is confined to naive human, but not mouse, $CD8^+$ thymocytes and peripheral T cells. We have found that CD248 unexpectedly inhibits T-cell proliferation, suggesting that CD248 has opposing roles on $CD8^+$ T cells compared with stromal cells.

Materials and methods

Patient samples

Samples were taken with informed consent and local research ethics committee approval (Birmingham: REC 2002/088, LREC 5735, 06/Q2706/66; Oxford: C01.097). Tissue samples were collected immediately after surgical removal and 5-μm acetone-fixed cryosections were used for immunofluorescence. Suspensions of tonsil cells were prepared by mechanical dispersion. Tonsil and blood leucocytes were prepared by centrifugation (300 ***g*** for 25 min at room temperature) on a Ficoll–Paque Plus (GE Healthcare, Little Chalfont, UK 17-1440-03). Neutrophils were isolated from blood: after sedimentation for 35 min with Dextran (Dextran to blood ratio of 1 : 7), they were layered onto a 56%/80% Percoll (Sigma, Poole, UK P4937) gradient, centrifuged at 200 ***g*** for 20 min and neutrophils were harvested from the interface. Thymocytes were prepared as described previously[18] from children undergoing heart surgery.

Immunofluorescence microscopy

Human tonsil, spleen and thymus tissues were labelled with CD248 monoclonal antibodies (mAb) B1/35.1 (IgG1 supernatant[7]) in combination with: UCHT1 (IgG2b 17 μg/ml gift from Professor Peter Beverley, University of Oxford, UK); OKT8 (IgG2a) and OKT4 (IgG2b; American Type Culture Collection, Manassas, VA) both 1 : 100 mouse ascitic fluid. Primary antibodies were detected with goat antibodies against mouse IgG1-FITC (1070-02, 20 μg/ml), IgG2a-tetramethyl-rhodamine (1080-03, 20 μg/ml) and IgG2b-Cyanine (Cy™) 5 (1090-15, 4 μg/ml) (SouthernBiotech, Birmingham, AL) in combination with goat anti-FITC Alexa-488 (Invitrogen, Paisley, UK A-11096, 10 μg/ml). All experiments using murine tissue were performed in accordance with UK laws with approval of local ethics committees. C57BL/6 mouse spleen tissue was prepared as described previously[13] and stained with rabbit anti-CD248 P13[10] (10 μg/ml) followed by anti-rabbit Cy5 (Jackson ImmunoResearch, Newmarket, UK 711-176-152, 15 μg/ml) and anti-CD3-FITC (eBiosciences, Hatfield, UK 11-0031 antibody 145-2C11, 5 μg/ml) followed by anti-Armenian hamster Cy2 (Jackson ImmunoResearch 127-225-160, 14 μg/ml). Sections were mounted in 2·4% 1,4-diazabicyclo[2,2,2]octane (Sigma) in glycerol (Fisons Scientific, Loughborough, UK) pH 8·6. With reference to controls, images were captured using a LSM 510 confocal microscope (Zeiss, Welwyn Garden City, UK). Cytospins of transfected MOLT-4 cells were stained for CD248 as described above; in addition, nuclei were stained with 20 μg/ml Hoechst 33258 (bis-benzimid H33258 Fluorochrom; Riedel De Haen AG, Buchs, Switzerland).

Flow cytometry and cell sorting

Primary and small interfering (si) RNA-treated cells were stained using purified CD248 mAb B1/35.1 conjugated to FITC (20 μg/ml) alone or in combination with CD3-allophycocyanin-H7 (641397) and/or CD19-peridinin chlorophyll protein (345790), CD56-phycoerythrin (PE) (345810), CD14-PE (345785), CD16-PE-Cy7 (335823), CD4-Pacific Blue (558116), CD45RA-PE (555489), CD11a-allophycocyanin (559875), CCR7-PE-Cy7 (557648) (Becton Dickinson Biosciences, Oxford, UK), CD8-Pacific Orange (MHCD0830) (Invitrogen, Paisley, UK) and isotype controls. For validation of CD248 expression by *in vitro* models, transfectants were stained with unconjugated CD248 mAb B1/35.1 detected with goat anti-mouse IgG-FITC (SouthernBiotech 1010-02, 20 μg/ml). All samples were assessed on a Cyan ADP flow cytometer (Beckman Coulter, High Wycombe, UK) and data were analysed using FlowJo software v8.3 (Tree Star, Ashland, OR). To analyse protein expression by Western blotting $CD3^+$ blood leucocytes were labelled and sorted for CD4 and CD45RA, CD4 and CD45RO, CD8 and CD45RO or CD8 and CD45RA populations. Populations were sorted to > 99% purity using a MoFlow™ cell sorter (Beckman Coulter).

Western blot

T-cell subsets isolated as described above, rheumatoid arthritis synovial fibroblasts and human umbilical vein endothelial cells isolated as previously described,[19] were lysed in non-reducing buffer and run on a 10% polyacrylamide gel. Gel bands were transferred to a 0·45 μm PVDF membrane (Flowgen Limited, Nottingham, UK) and blocked with 5% non-fat dried milk in Tris-buffered saline

and stained with CD248 mAb B1/35.1 supernatant (1 : 10) or β-actin (1/1000) and detected with human immunoglobulin-absorbed goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (GE Healthcare). The blot was visualized by enhanced chemiluminescence (GE Healthcare) and autoradiography (Kodak X-OMAT, Watford, UK).

Transfected T-cell lines

MOLT-4 T cells (American Type Culture Collection CRL-1582) were transfected with pcDNA3.1 or pcDNA3.1-huCD248[7] using electroporation (Lonza, Slough, UK L-VCA-1005). $CD248^+$ cells were selected by culture in 500 μg/ml Geneticin® (Invitrogen 10131019) and by positive selection using magnetic beads coated with anti-mouse IgG (DynaM-450 beads, Invitrogen 110.41) to detect cells labelled with B1/35.1.[7]

Thymidine incorporation assay

Uptake of $[^3H]$thymidine was used as an indication of the relative spontaneous proliferative rates of pcDNA3.1- and pcDNA3.1-CD248-transfected MOLT-4 cells. Cultures of each transfectant were set up in triplicate in 96-well flat-bottomed plates using 100 μl of cells at $0{\cdot}025 \times 10^6$, $0{\cdot}05 \times 10^6$, $0{\cdot}1 \times 10^6$, $0{\cdot}2 \times 10^6$, $0{\cdot}3 \times 10^6$, $0{\cdot}4 \times 10^6$ and $0{\cdot}8 \times 10^6$ cells/ml. On day 1 cells were pulsed for 6 hr with 50 μl of 1 μCi/ml $[^3H]$thymidine, harvested (Skatron, Tranby, Norway, 7022 cell harvester) onto filter mats (Perkin Elmer, Waltham, MA, GF-Filter 1205-401) and the radioactivity was measured in counts/min using a beta-counter (Perkin Elmer, Wallac 1205).

Naive CD8 T cells and CD248 siRNA

Naive CD8 T cells were isolated to 86% purity using a CD8 T-cell isolation kit (Miltenyi Bergisch Gladbach, Germany 130-094-156) followed by depletion of $CD45RO^+$ cells (Miltenyi 130-046-001) according to the manufacturer's instructions. Up to 10×10^6 cells were transfected with 1 μl of 100 μM CD248 siRNA (Thermo Scientific, Epsom, UK ON-TARGETplus SMARTpool L-010720-01-005) or 1 μl of 1 μg/μl scrambled siRNA (gift from Arthrogen, Amsterdam, The Netherlands) in the presence of 100 μl human T-cell Nucleofector® solution (Lonza VPA-1002) using program U014 according to the manufacturer's instructions (Amaxa Biosystems). After 48 hr the relative level of CD248 was compared by flow cytometry as above and by quantitative PCR: CD248 and 18s expression was assayed using a 7500 Taqman instrument (Applied Biosystems, Paisley, UK, Taqman Gene Expression Assay Hs00535586_s1 and 18s rRNA 4319413E). Cells treated with CD248siRNA and scrambled siRNA were incubated for 24 hr at + 37° (in Dulbecco's Modified Eagle's Medium, Gibco,

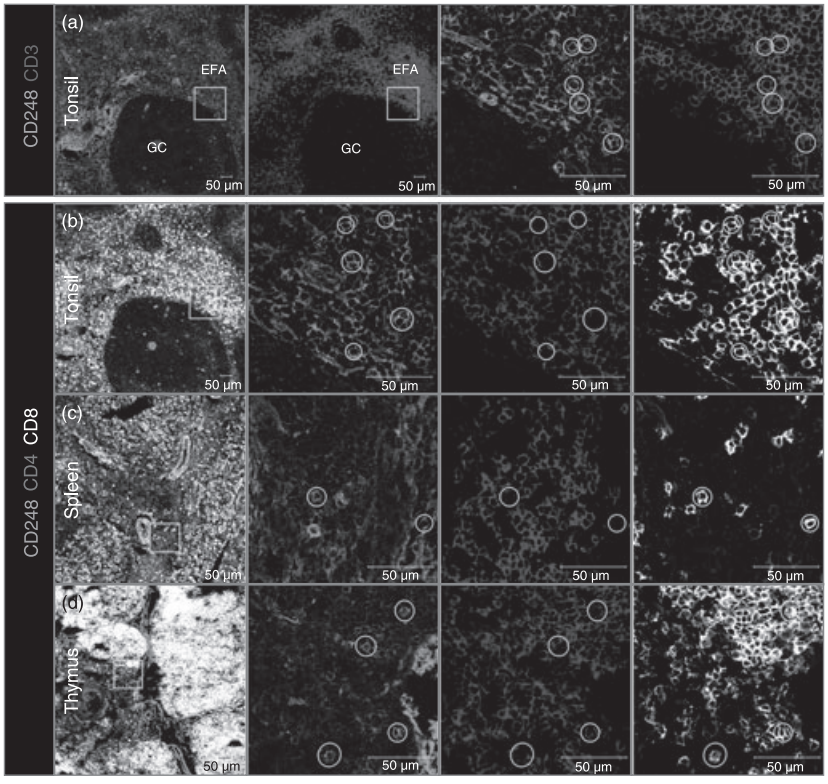

Figure 1. CD248 is expressed on human stromal cells and cells with lymphoid morphology. Examples of $CD248^+$ lymphoid cells that reside in the extrafollicular area (EFA) but not the germinal centre (GC) are circled. These are $CD3^+$ cells (a) that are $CD4^-$ $CD8^+$ and were present in tonsil (b), spleen (c) and thymus (d). Boxes indicate areas shown at higher magnification on the right. Data are representative images from at least three different donors for tonsil and spleen and one for thymus. Scale bars represent 50 μm.

Paisley, UK 41966; with 10% fetal calf serum, Biosera, Ringmer, UK 51810-500) and the relative level of proliferation was compared using 5-ethynyl-2′-deoxyuridine (EdU) incorporation.

EdU incorporation assay

Naive CD8 T cells were cultured for 3 days in Dulbecco's modified Eagle's medium with 10% fetal calf serum at $0{\cdot}5 \times 10^6$/ml in 250-μl aliquots with 2 μM EdU and 0, 1·25, 2·5 or 5 μl of T-cell expander Dynabeads® (Invitrogen 111.31D). The level of EdU incorporation was measured using a Click-iT® EdU Pacific Blue™ Flow Cytometry Assay kit (Invitrogen A10034) according to the manufacturer's instructions. Samples were processed for flow cytometry as previously stated.

Results

CD248 is expressed in human, but not murine, cells with lymphoid morphology

To explore the distribution of CD248 on T cells we initially examined expression in the extrafollicular T-cell area of human tonsils (Fig. 1a). CD248 co-localized with CD3$^+$ T cells (Fig. 1a) that were CD8$^+$ but not CD4$^+$ (Fig. 1b). Similar populations of cells were also found in human spleen and thymus (Fig. 1c,d). As CD248 is expressed on both human and mouse stromal cells, we next examined whether CD248 was expressed on T cells in murine lymphoid tissues. We were unable to identify any CD3$^+$ cells expressing CD248 in murine lymph node, spleen and thymus (Fig. 2a–c), which is consistent with previous reports that CD248 mRNA is absent from all haematopoietic cell lineages in mice.[20]

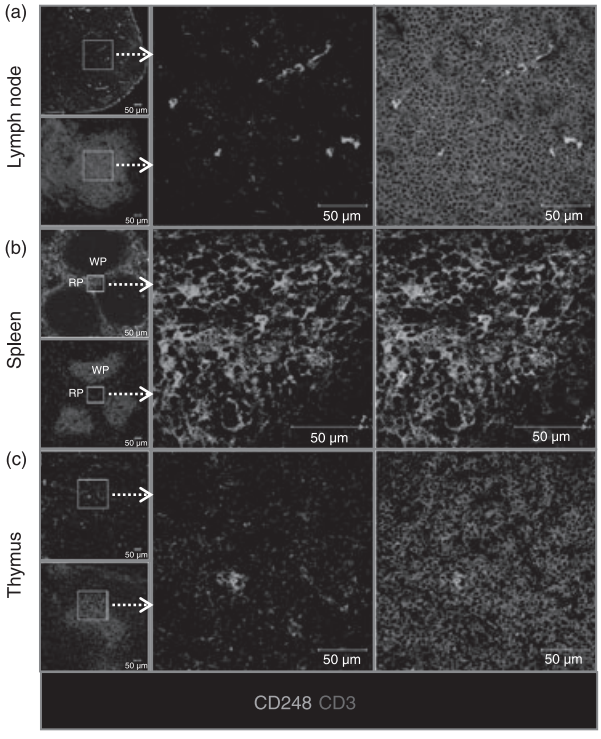

Figure 2. CD248$^+$ T cells are not present in C57/BL6 mice. CD248$^+$ (green) T cells (CD3$^+$ red) were not present in lymph node (a), spleen (b) or thymus (c) of mice. RP, red pulp; WP, white pulp. Scale bars represent 50 μm. Boxes indicate areas shown at higher magnification on the right. Data are representative images from at least three different mice.

CD248 expression on CD3$^+$ T cells is restricted to naive CD8$^+$ T cells

To further investigate the expression of CD248 on human blood leucocytes we screened a variety of leucocyte subsets using a panel of CD248 antibodies that have been characterized extensively.[7,17] A distinct subset of CD3$^+$ T cells expressed CD248 (Fig. 3). CD248 was not identified on any other leucocyte subsets including CD19$^+$ B cells,

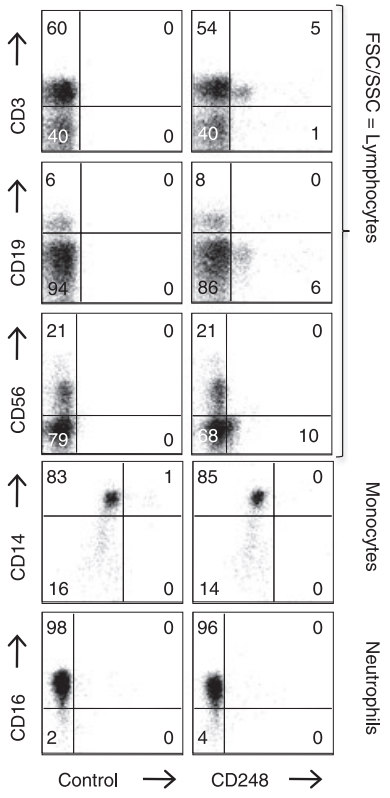

Figure 3. CD248 is expressed on a subset of CD3$^+$ T cells from human blood. Flow cytometry of CD248 antibody B1/35.1 in comparison with negative controls showing that CD248 is expressed on a subset (8·5%) of CD3$^+$ lymphocytes from blood. CD19$^+$, CD56$^+$, CD14$^+$ and CD16$^+$ cells are CD248$^-$. B, T and natural killer cells are forward scatter/side scatter (FSC/SSC) gated for lymphocytes, CD14$^+$ cells are FSC/SSC gated for monocytes and CD16$^+$ cells are FSC/SSC gated for neutrophils. Data are representative plots from a minimum of three experiments from different donors.

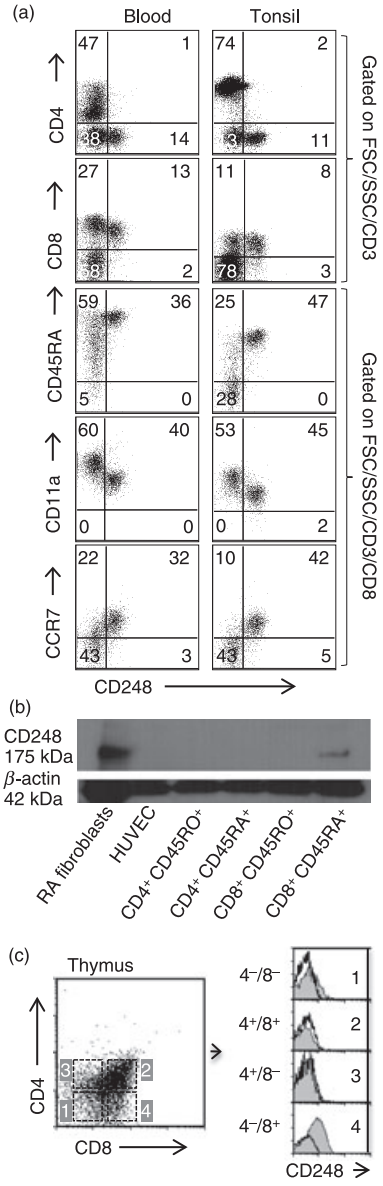

Figure 4. CD248 is expressed on naive $CD8^+$ T cells and mature human thymocytes. CD248 is expressed by a distinct subset of $CD8^+$ $CD3^+$ T cells that is $CD45RA^+$ $CCR7^+$ $CD11a^{low}$ and is present in blood and tonsil (a). Samples are gated in comparison with isotype controls. Data are representative plots from a minimum of five samples each. (b) Western blot ($n = 1$) showing CD248 protein expression only in rheumatoid arthritis synovial fibroblasts and $CD8^+$ $CD45RA^+$ T cells and not other T-cell subsets or HUVEC. (c) $CD248^+$ $CD3^+$ thymocytes ($n = 1$) are only found in the single-positive $CD4^-$ $CD8^+$ population of T cells.

$CD56^+$ natural killer cells, $CD14^+$ monocytes or $CD16^+$ neutrophils (Fig. 3). CD248 expression on $CD3^+$ T cells was confined to $CD8^+$ cells in both blood and tonsil (Fig. 4a), which was consistent with our findings by immunohistology (Fig. 1).

As human CD8 T cells can be divided into 'naive' ($CD45RA^+$) and 'primed' ($CD45RO^+$) subsets[21] we next investigated whether CD248 expression was confined to one or other of these populations. $CD248^+$ $CD8^+$ T cells were found exclusively in the $CD45RA^+$ population in both human blood and tonsil (Fig. 4a). Moreover CD248 was expressed on $CCR7^+$ $CD11a^{low}$ cells, indicating that CD248 is confined to naive $CD8^+$ T cells (Fig. 4a) and

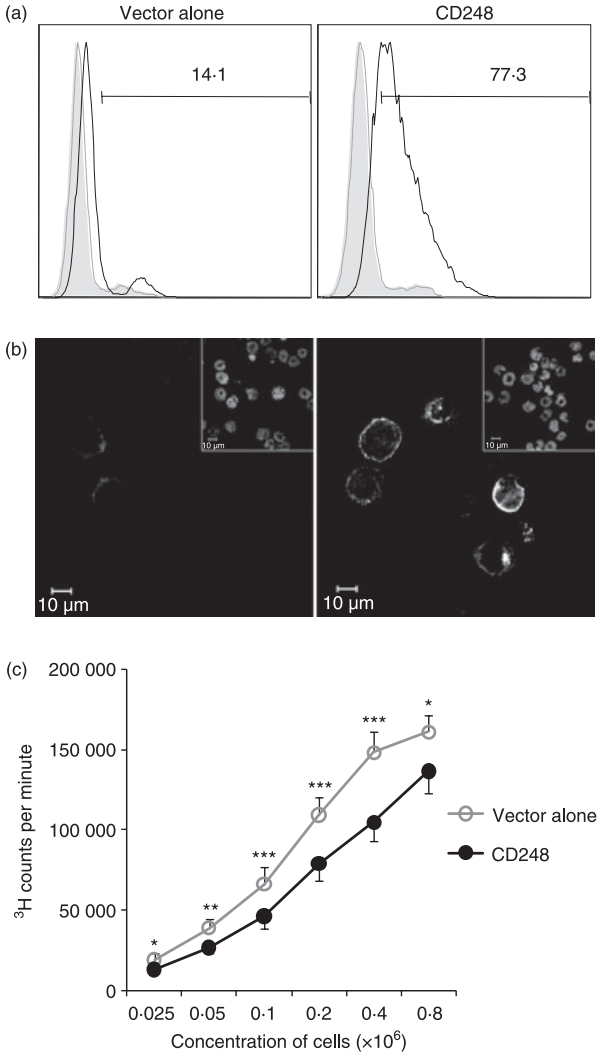

Figure 5. Human (hu) CD248 transfection of MOLT-4 T cells resulted in decreased proliferation. CD248 monoclonal antibody B1/35.1 staining of surface (a) and fixed (b) MOLT-4 T cells transfected with either huCD248-pcDNA3.1 or pcDNA3.1 vector alone show that only the CD248 transfected line has high expression of CD248. Insets in (b) are nuclear counterstains. Scale bars represent 10 μm ($n \geq 3$). Comparison of the [^{3}H]thymidine uptake of MOLT-4 T cells transfected with CD248-pcDNA3.1 (•) or pcDNA3.1 vector alone (○) shows that $CD248^+$ cells have lower spontaneous proliferative capacity than their vector alone-transfected counterparts (c). This was demonstrated across a range of cell numbers and on day 1 of culture ($n = 7$). $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. The Student's paired *t*-test was applied to compare the statistical significance of linked data. Error bars represent SEM.

not to CCR7$^{low/-}$ CD11a^{high} CD45RA^{+} effector ('revertant') memory cells.[22]

To confirm protein expression of CD248 on CD8^{+}/CD45RA^{+} cells, CD4^{+} and CD8^{+} CD45RA^{+} and CD45RO^{+} cells were isolated to > 99% purity by FACS sorting, and CD248 expression was examined by Western blot. Rheumatoid arthritis synovial fibroblasts and human umbilical vein endothelial cells were used as positive and negative controls, respectively.[7] Only CD8^{+} CD45RA^{+} cells and fibroblasts expressed CD248 (175 000 molecular weight) (Fig. 4b).

CD248 is not expressed until the CD8 single-positive stage in T-cell develpoment in human thymus

We next explored at what stage during the development of CD8^{+} T cells CD248 was expressed by studying human thymocytes. At the earliest stage of development thymocytes are double negative for CD4 and CD8. Following re-arrangement of their T-cell receptor α and β chains, they become double positive for CD4 and CD8, then before positive and negative selection they become single positive for CD4 or CD8.[23] We observed that CD248 expression was restricted to the CD8^{+} CD4^{-} population of thymocytes (Fig. 4c), suggesting that expression occurs during late thymocyte development when naive T cells begin to leave the thymus and migrate to secondary lymphoid tissues via the blood and lymphatics.

CD248 inhibits T-cell proliferation

CD248 has been reported to induce cell proliferation on stromal cells such as primary human and mouse embryonic fibroblasts,[8,24] human (hu) CD248 transfected Chinese hamster ovary cells[25] and MG63 human osteosarcoma cells.[24] We therefore examined whether CD248 could regulate T-cell proliferation using a CD248-negative acute T-cell leukaemia cell line (MOLT-4) transfected with huCD248-pcDNA3.1[7] or pcDNA3.1 vector alone. High and stable levels of CD248 expression were achieved by iterative rounds of selection (Fig. 5a,b). CD248-transfected T cells had a significantly lower (mean $P < 0{\cdot}005$) capacity for proliferation than their vector alone-transfected counterparts (Fig. 5c). Interestingly, the differences in proliferation between these two cell lines were most pronounced at higher cell densities.

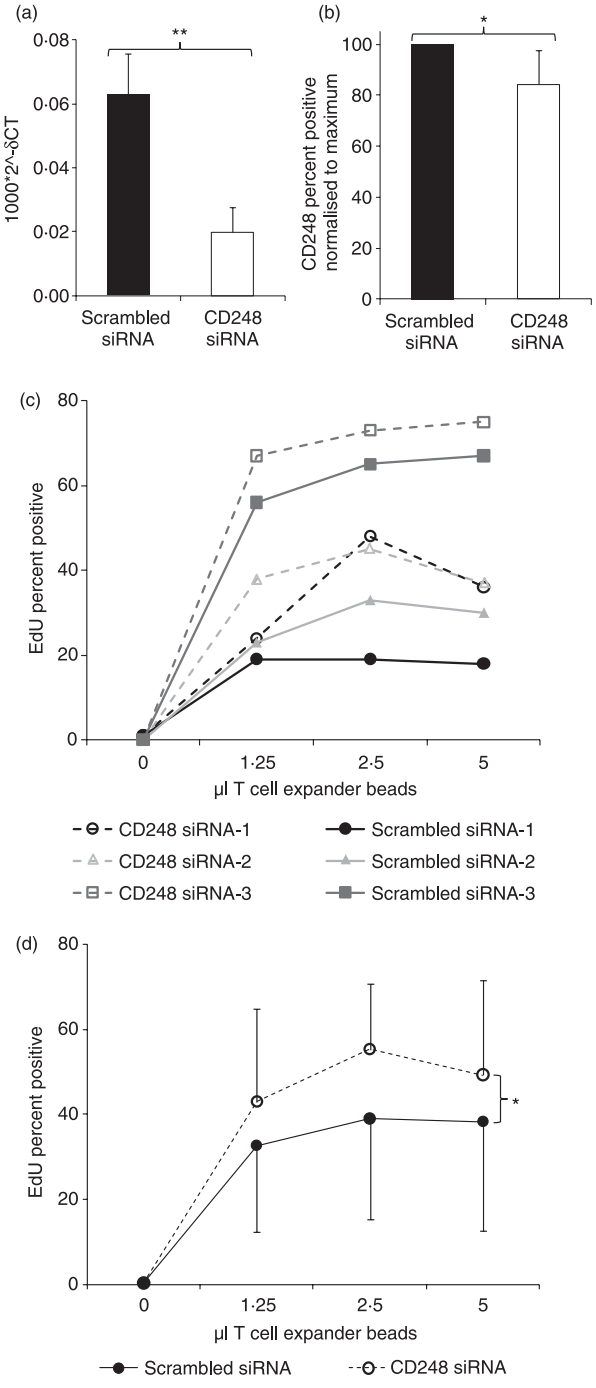

Figure 6. CD248 expression is associated with decreased proliferation of naive CD8 T cells. Quantitative PCR was used to demonstrate significantly lower levels (**$P < 0{\cdot}01$) of CD248 RNA expression in CD248 small interfering (si) RNA-treated cells compared with controls ($n = 3$) (a). Significantly reduced protein expression (*$P < 0{\cdot}05$) of CD248 in CD248 siRNA-treated cells compared with controls was also demonstrated ($n = 3$) (b). Cells were cultured with 0, 1·25, 2·5 or 5 µl T-cell expander beads for 3 days then their proliferation was compared using an 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay. CD248 siRNA-treated naive CD8 T cells (–▫–▫–▫–) exhibit higher EdU incorporation than scrambled siRNA-treated cells (–•–▵–•–). Data from three different donors are shown in (c) and the data are grouped in (d) showing CD248 siRNA treated naive CD8 T cells (–○–) exhibit significantly higher (*$P < 0{\cdot}05$) EdU incorporation than scrambled siRNA-treated cells (–•–). The Student's paired *t*-test was applied to compare the statistical significance of linked data. Error bars represent SEM.

To confirm and extend our findings we knocked down CD248 expression on freshly isolated human CD8 T cells (Fig. 6a,b). CD248 expression on haematopoietic cells was associated with reduced proliferation as CD248 siRNA treatment of naive CD8 T cells significantly increased ($P < 0{\cdot}05$) their proliferative capacity (Fig. 6c,d).

Discussion

In this report we confirm that CD248 is expressed by naive human $CD8^+$ T cells and paradoxically inhibits T-cell proliferation compared with stromal cells where it increases proliferation. Differences in T-cell proliferation were more pronounced at higher cell densities and may reflect higher CD248 expression at these densities.[14] CD248 is not expressed on lymphoid cells in adult mouse spleen, thymus or lymph node, which is consistent with a lack of mRNA expression in murine haematopoietic cells[20] as well as the lack of any T-cell phenotype in CD248 knockout mice.[16] The reason for this difference between haematopoietic expression in human and mouse lymphocytes warrants further exploration.

We found unexpected functional effects of CD248 on human T cells that contrast strikingly with those reported for stromal cells.[8,24,25] Whereas CD248 enhances stromal cell proliferation,[8,24,25] we were surprised to find that CD248 expression in human T cells inhibited proliferation. Contrasting effects of the same molecule expressed on stromal versus haematopoietic cells have been reported previously[26,27] and serve to underline that there must be differential 'gearing' of the signalling pathways for CD248 in these two cell types. A role for intracellular signalling via the cytoplasmic domain of CD248 has been shown using a stromal cell mouse model where absence of the cytoplasmic domain resulted in reduced inflammation, measured in part by decreased leucocyte adhesion to stromal cells.[28] Investigation of the function of intracellular signalling domains in CD248 will contribute to an understanding of the differential effects of stromal and T-cell CD248.

We found CD248 expression on naive $CD8^+$ T cells in the circulation and in the extrafollicular areas of secondary lymphoid tissue (Fig. 1). The extrafollicular zone is an important site of antigen presentation to naive T cells, typically followed by rapid division and effector/memory T-cell generation.[29] As this occurs concomitantly with declining expression of CD248 it is tempting to speculate that CD248 might play a role in retaining naive T cells at sites where they can encounter antigen. The loss of CD248 expression after exposure to antigen is associated with the ability to divide rapidly and migrate to follicular sites. In antigenic responses and in comparison to their memory counterparts, naive CD8 T cells exhibit lower intrinsic proliferative capacity.[22] Our data indicate that CD248 might play a role in these processes in CD8 T cells. Why CD248 is expressed only on $CD8^+$ and not $CD4^+$ naive T cells is not clear but needs further investigation.

Reports on CD248 have, up to now, suggested that its expression is restricted to stromal and mesenchymal cells.[12,30,31] Much interest has centred on its role(s) in the angiogenic processes that occur during tumour progression and in tissue remodelling following inflammatory stimuli. The data presented here point to unexpected expression and opposing functional effects of CD248 on naive human $CD8^+$ T cells compared with stromal cells. Our findings suggest that a careful analysis of the role of CD248 on naive CD8 T cells *in vivo* is needed particularly with regard to the use of anti-CD248 reagents in the advocated and current treatment of solid tumours.[9,32,33]

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Disclosures

The authors declare that there are no conflicts of interest.

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