



## A distinct subset of podoplanin (gp38) expressing F4/80+ macrophages mediate phagocytosis and are induced following zymosan peritonitis

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### ABSTRACT

**Macrophages are important tissue resident cells that regulate the dynamics of inflammation. However, they are strikingly heterogeneous. During studies looking at podoplanin (gp38) expression on stromal cells in the murine spleen and peritoneal cavity we unexpectedly discovered that podoplanin was expressed on a subset of F4/80<sup>+</sup> macrophages; a subset which we have termed fibroblastic macrophages (FM). These cells function as phagocytes in vitro as measured by bead mediated phagocytosis assays. FM also exist at high frequency in the peritoneal cavity and in zymosan induced peritonitis in vivo. These FM represent a unique subgroup of F4/80<sup>+</sup> macrophages and their presence in the inflamed peritoneum suggests that they play a role in zymosan induced peritonitis.**

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### 1. Introduction

Macrophages are heterogeneous cells that are found in both lymphoid and peripheral organs as part of the mononuclear phagocyte system. They are widely distributed in different anatomical locations where they have a specific nomenclature and function. For example alveolar macrophage are found in the lung, meningeal macrophage in the nervous system, kupffer cells in the liver and serosal macrophage in the peritoneal cavity [1–3]. There is even heterogeneity within a single organ such as the spleen where marginal zone, metallophilic, white pulp, tangible body and red pulp macrophages have been identified [4]. Their distinct phenotype has been determined by surface molecules which have been identified on different macrophages despite their overlap with monocytes [5], dendritic cells (DC) [6] as well as macrophages involved in the resolution of inflammation [7].

Podoplanin, also known as gp38, is expressed on stromal cells in different tissues including kidney, heart and lymphoid organs [8]. Despite the lack of a clearly defined function, podoplanin has been widely used as a stromal cell surface marker. In secondary lymphoid organs, podoplanin is highly expressed on fibroblastic reticular cells (FRC) which provide a conduit regulating the transport of small molecules including antigens and chemokines [9].

During studies aimed at examining stromal subsets in the red pulp of the spleen we unexpectedly discovered that podoplanin is expressed on a subset of F4/80<sup>+</sup> macrophages, which we have termed fibroblastic macrophages (FM). These FM are capable of high levels of phagocytosis. In the peritoneal cavity, FM exist at a relatively high frequency and in a model of resolving peritonitis there are markedly more F4/80<sup>+</sup>podoplanin<sup>+</sup> FM than at rest, with their numbers increasing in a dose dependent manner. Our findings suggest that FM may play a role in zymosan mediated peritonitis.

### 2. Methods and materials

#### 2.1. Mice

C57BL/6. mice were housed in the animal facilities at the University of Birmingham and University College London. All experiments were performed in accordance with United Kingdom

*Abbreviations:* FM, fibroblastic macrophages; CM, classic macrophages; SLO, secondary lymphoid organs; FRC, fibroblastic reticular cells

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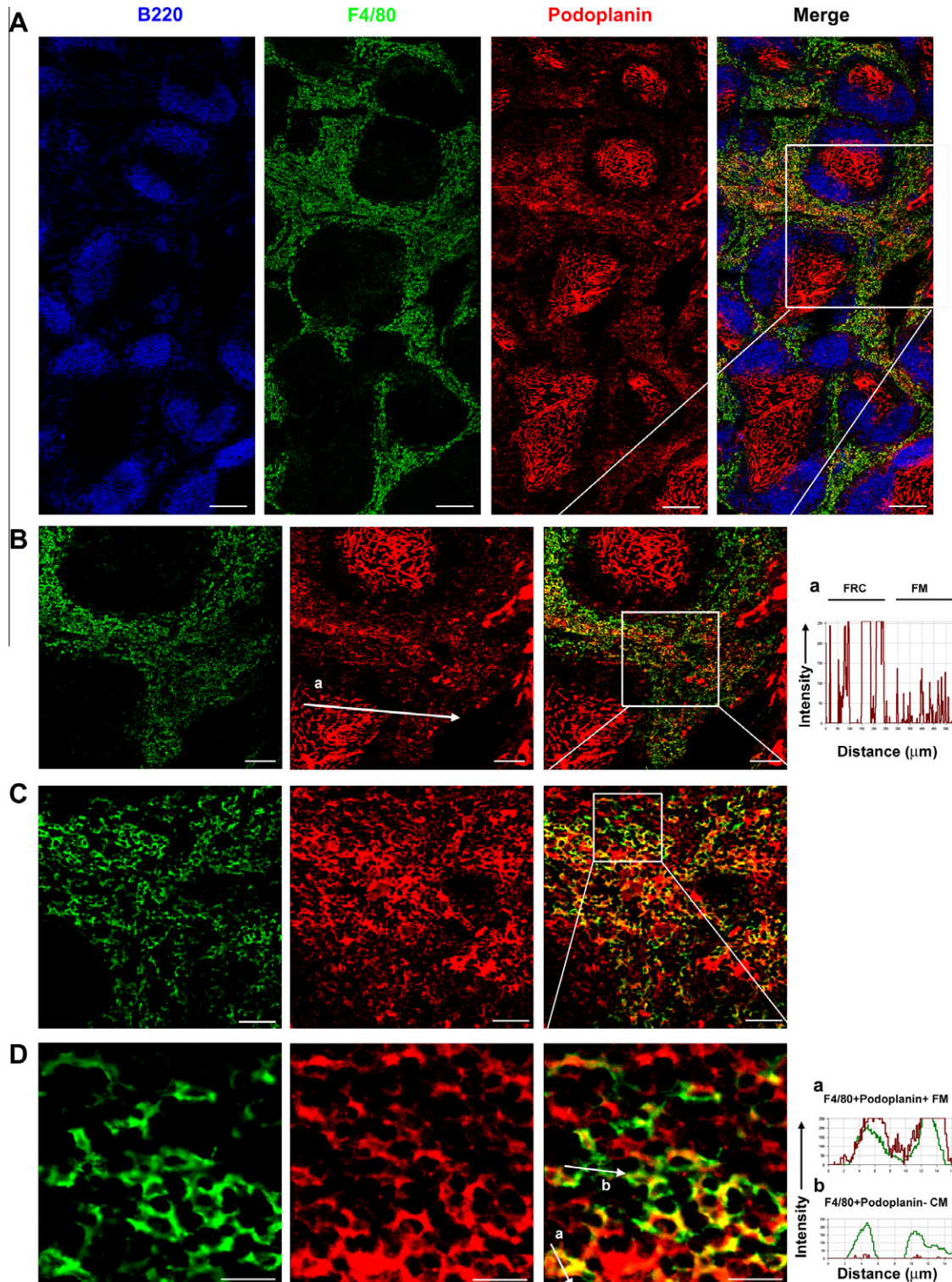
E-mail address: [c.d.buckley@bham.ac.uk](mailto:c.d.buckley@bham.ac.uk) (C.D. Buckley).

Home Office regulations. Mice were used between 2 and 12 weeks of age and were sex and age matched unless otherwise indicated.

## 2.2. Cell preparation and culture

Spleens were minced and cells were prepared as described [10]. Cells from the peritoneal cavity were washed out by

phosphate buffered saline (PBS). Single cell suspension was achieved by filtering through 70  $\mu\text{m}$  cell strainers (BD Biosciences). Cells were counted and used for culture with Dulbecco's Modified Eagle's Medium with 10% FCS and 1% glutamine/penicillin/streptomycin. Non-adherent cells in culture were removed by washing.



**Fig. 1.** Podoplanin expressing F4/80<sup>+</sup> macrophages exist in the red pulp of spleen. Expression of B220 (blue), F4/80 (green) and podoplanin (red) on WT spleen section. (A) Tile scan of a representative area. Scale bars represent 200  $\mu\text{m}$ . (B) Highlighted area in A. Histogram of B a showed MFI of podoplanin on areas crossed. Scale bars represent 100  $\mu\text{m}$ . (C) Highlighted area in B. Scale bars represent 50  $\mu\text{m}$ . (D) Highlighted area in C. Scale bars represent 20  $\mu\text{m}$ . Histograms of D a and b show MFI of F4/80 and podoplanin along indicated cells. Results are representative of three separate experiments.



### 2.3. Flow cytometry and cell sorting

Cultured cells were detached with disassociation buffer (GIBCO). Cells were incubated with anti-mouse antibodies (Supplementary Table 1) at 4 °C for 30 min. Cells were acquired using a FACSCalibur or Dako Cyan flow cytometer. Data were analyzed by FlowJo software (Tree Star). For sorting, cells were stained as above and then purified by MoFlo cell sorter (Dako Cytomation).

### 2.4. RNA isolation and PCR analysis

RT-PCR was performed as described [10]. Briefly, purified cells as indicated were used for obtaining mRNA and high-purity cDNA by uMacs One-step cDNA synthesis kit (Miltenyi Biotech).  $\beta$ -Actin was used as housekeeping gene prior to amplifying target gene Podoplanin. Reactions were conducted in a Peltier Thermal Cycler-200 (MJ Research, Genetic Research Instrumentation, UK). PCR products were fractionated by agarose gel electrophoresis and identified by fragment size. The primer sequences, NCBI Accession numbers and amplicon sizes are as follows:

$\beta$ -Actin (NM\_007393.3)  
 forward (5'-TGGAATCCTGTGGCATCCATGAAAC-3'),  
 reverse (5'-TAAAACGCAGCTCAGTAACAGTCGG-3') 349 bp  
 Podoplanin (NM\_010329.2)  
 forward (5'-ACCGTGCCAGTGTGTCTG-3'),  
 reverse (5'-AGCACCTGTGGTTATTATTGT-3') 159 bp

### 2.5. Immunofluorescence staining and confocal microscopy

Cells were incubated with antibodies (Supplementary Table 1) at room temperature for 30 min. Excess antibody was removed and slides were washed in PBS. Cell nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) for 1 min. Slides were mounted with 30  $\mu$ l glycerol containing 1,4-diazabicyclo (2,2,2) octane (DABCO). Confocal images were acquired using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) and analyzed using LSM510 software.

### 2.6. Phagocytosis assays

FluoSpheres (Molecular Probes) were washed with RPMI media before use. These fluorescent beads were resuspended with cells and incubated for 3 h at 37 or 0 °C. Cells were imaged by phase contrast microscopy or detached with disassociation buffer (GIBCO) for FACS. For assays using dynabeads, cells were cultured in glass dishes (MatTek) coated with 100  $\mu$ g/ml Poly-L-lysine (Sigma). Dynabeads coated with anti-rat IgG (Invitrogen) were incubated with cells for 3 h at 37 °C. LysoTracker (1  $\mu$ l/ml) (Molecular Probes) was incubated with cells for 5 min at room temperature. Cells were co-stained with antibodies (Supplementary Table 1) for 20 min and were examined by confocal microscopy.

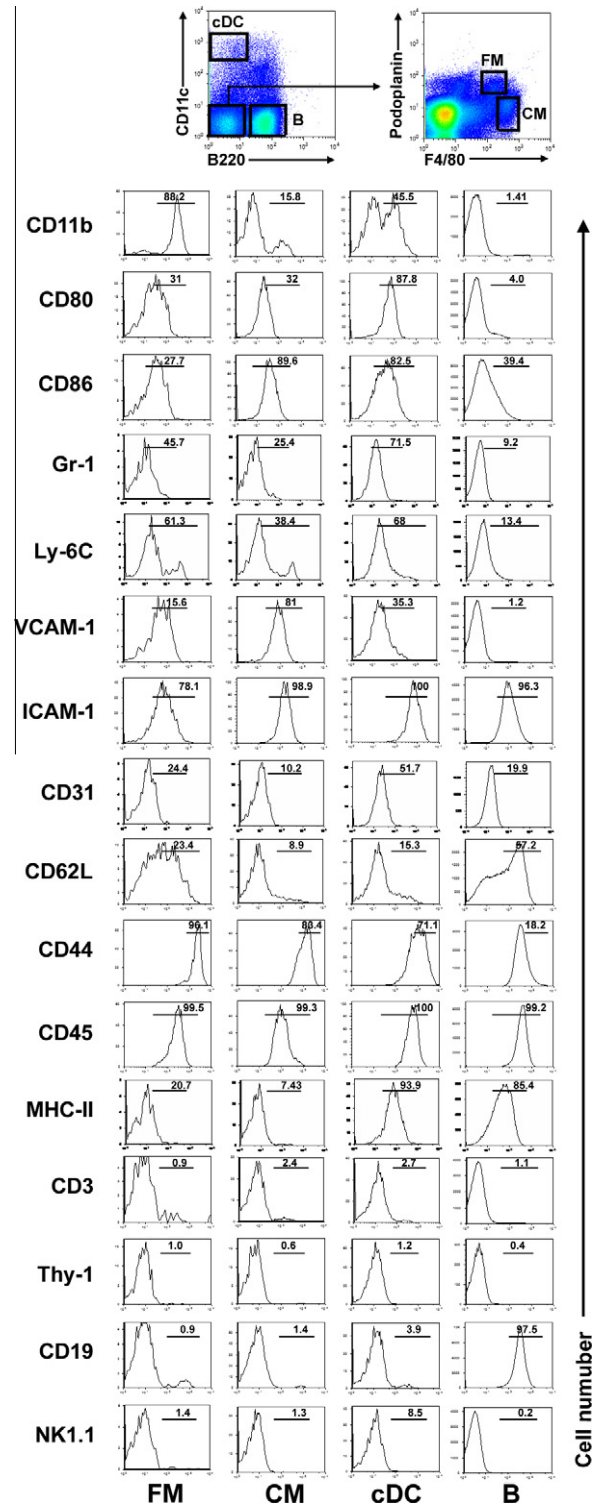
### 2.7. Induction of peritonitis

Peritonitis was induced by the intraperitoneal injection (*i.p.*) of either 0.1 or 10 mg type A zymosan (Sigma) as previously depicted [11]. Peritoneal cavity washout cells were collected with PBS at 72 h post injection. Erythrocytes in the peritoneal exudate were lysed and cells were enumerated by counting before FACS staining.

## 3. Results

### 3.1. Identification and characterization of podoplanin<sup>+</sup> murine FM

During studies which aimed to identify stromal cell subsets in the red pulp of the spleen, discrete populations of VCAM-1 and



**Fig. 2.** Phenotype of F4/80<sup>+</sup>podoplanin<sup>+</sup> FM. Expression of indicated Abs on the four cell populations. FM: fibroblastic macrophages (F4/80<sup>+</sup>, gp38<sup>+</sup>, CD11c<sup>-</sup>, B220<sup>-</sup>); CM: classic macrophages (F4/80<sup>+</sup>, gp38<sup>-</sup>, CD11c<sup>-</sup>, B220<sup>-</sup>); cDC: conventional dendritic cells (F4/80<sup>-</sup>, gp38<sup>-</sup>, CD11c<sup>+</sup>, B220<sup>-</sup>) and B: B cells (F4/80<sup>-</sup>, gp38<sup>-</sup>, CD11c<sup>-</sup>, B220<sup>+</sup>) by flow cytometry. Numbers in plots indicate percentage of cells. Data are representative of two independent experiments.

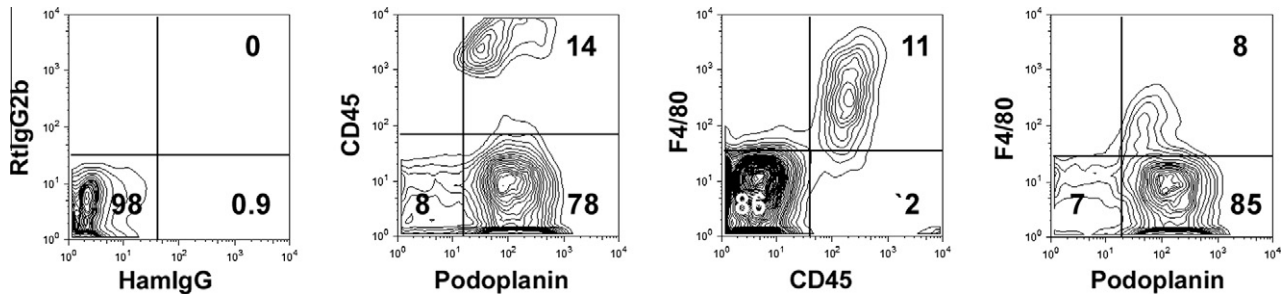
F4/80 expressing cell could be observed. Compared to F4/80 which specifically labeled the red pulp only, VCAM-1 was expressed in both white pulp and red pulp areas (Supplementary Fig. 1(i)). F4/80 and VCAM-1 co-localized on a subset of macrophages in the

red pulp (Supplementary Fig. 1(ii) and (iii)). This suggested that two macrophage/dendritic cell populations, namely  $F4/80^+VCAM-1^+$  and  $F4/80^+VCAM-1^-$  are present in the red pulp of the spleen.

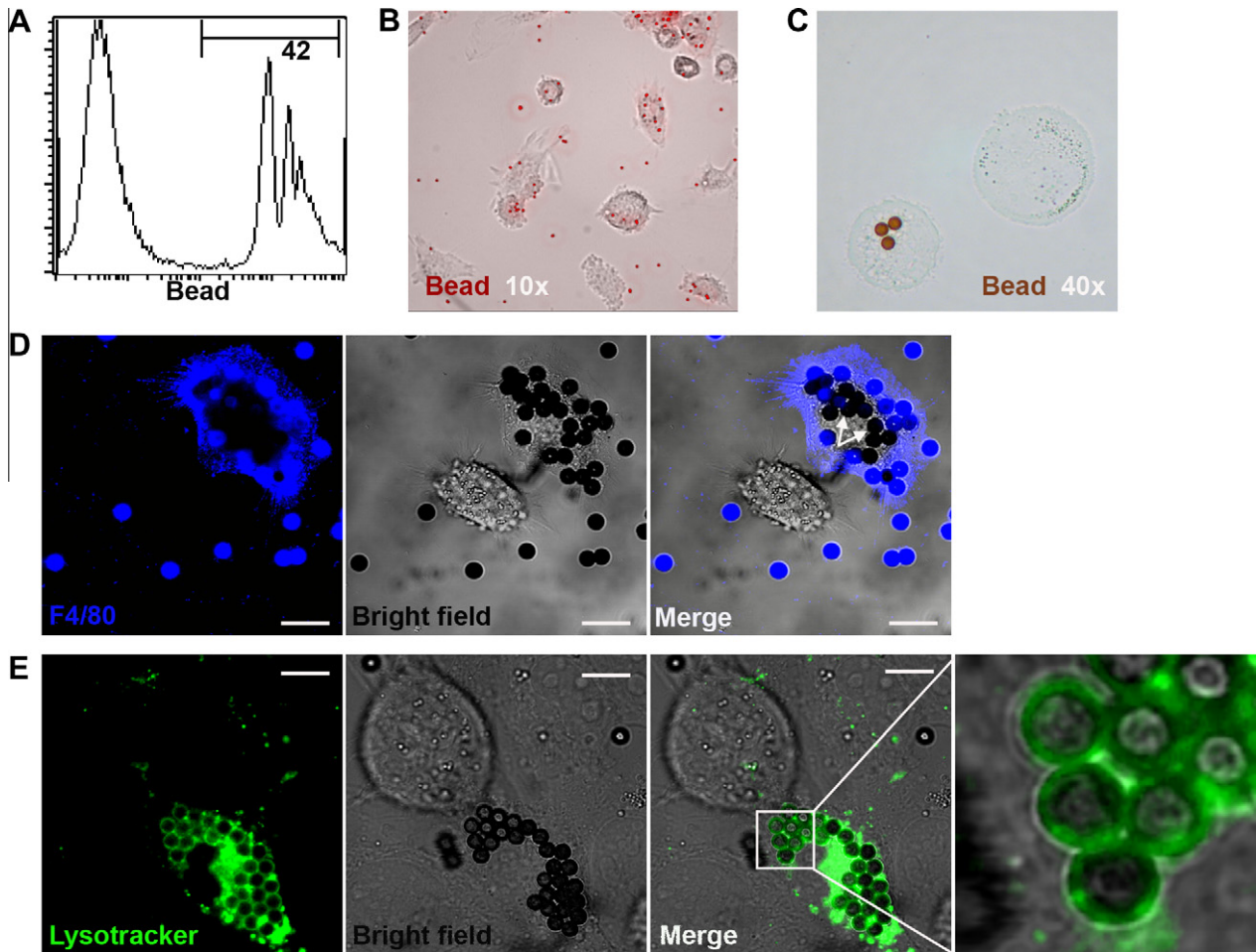
To further characterize these two populations we dispersed cells in the spleen and analyzed them by FACS. Consistent with our histology findings, four subsets were identified,  $F4/80^+VCAM-1^+$ ,  $F4/80^+VCAM-1^-$ ,  $F4/80^-VCAM-1^+$  and  $F4/80^-VCAM-1^-$  cells. Further phenotypic analysis revealed that CD11b, CD80, CD86 and surprisingly podoplanin was expressed on both  $VCAM-1^+$

and  $VCAM-1^-$  splenic macrophages but not on  $F4/80^-$  non-macrophage leucocytes (data not shown).

To confirm the existence of  $F4/80^+$ podoplanin<sup>+</sup> FM *in situ* in the spleen, tissue sections were examined for F4/80 and podoplanin expression by histology (Fig. 1A). As well as being strongly expressed on FRC in the white pulp, podoplanin was also detected on  $F4/80^+$  macrophage subsets in the red pulp but at a lower intensity than on FRC (Fig. 1B). The pattern of podoplanin expression on the  $F4/80^+$  fibroblastic macrophage subpopulation was different to



**Fig. 3.** Enrichment of  $F4/80^+$ podoplanin<sup>+</sup> FM. Expression of CD45, F4/80 and podoplanin on cultured splenocytes by flow cytometry with isotype controls. Numbers in plots indicate percentage of cells. Data are representative of three independent experiments.



**Fig. 4.** Phagocytosis mediated by  $F4/80^+$ podoplanin<sup>+</sup> FM. (A) Expression of beads on cultured splenocytes by flow cytometry. Numbers in histogram indicates percentage. (B) Cells and fluorescent beads (red) were imaged by phase contrast microscopy (10 $\times$ ). (C) Cells and dynabeads (brown) were imaged by light microscopy (40 $\times$ ). (D) Incorporation of beads by  $F4/80^+$  cells using confocal microscopy. Cells were incubated with anti-rat IgG coated dynabeads and rat anti-mouse F4/80 antibody. Extracellular beads and  $F4/80^+$  cells were shown in blue. Intracellular beads were indicated by arrows. Scale bars represent 10  $\mu$ m. (E) Cells were incubated with dynabeads and LysoTracker (green). Internalized beads by cells (forming acidic phagosomes) were marked in ring like compartments. Scale bars represent 10  $\mu$ m. Results are representative of three separate experiments.

that found on FRC. Expression was more patchy and more cellular on FM in the red pulp, whereas conduit-like structures were observed on FRCs in the white pulp (Fig. 1B and C). Co-expression of F4/80 and podoplanin was confirmed at a single cell level (Fig. 1D).

The phenotype of FM is distinct from classic macrophages (CM) as well as typical antigen presenting cells such as dendritic cells and B cells. FM expressed lower levels of CD86, VCAM-1 and ICAM-1 but higher CD11b, Gr-1, Ly-6C, CD62L, CD44 and MHC-II expression than CM (Fig. 2). To confirm that podoplanin is produced by FM, mRNA for podoplanin was examined by PCR in cells isolated by FACS sorting. Podoplanin mRNA was found in purified FM but not CM or lymphocytes (Supplementary Fig. 2). Taken together this suggests that FM are a distinct macrophage subset with a unique cell surface phenotype.

### 3.2. FM expanded in vitro mediate phagocytosis

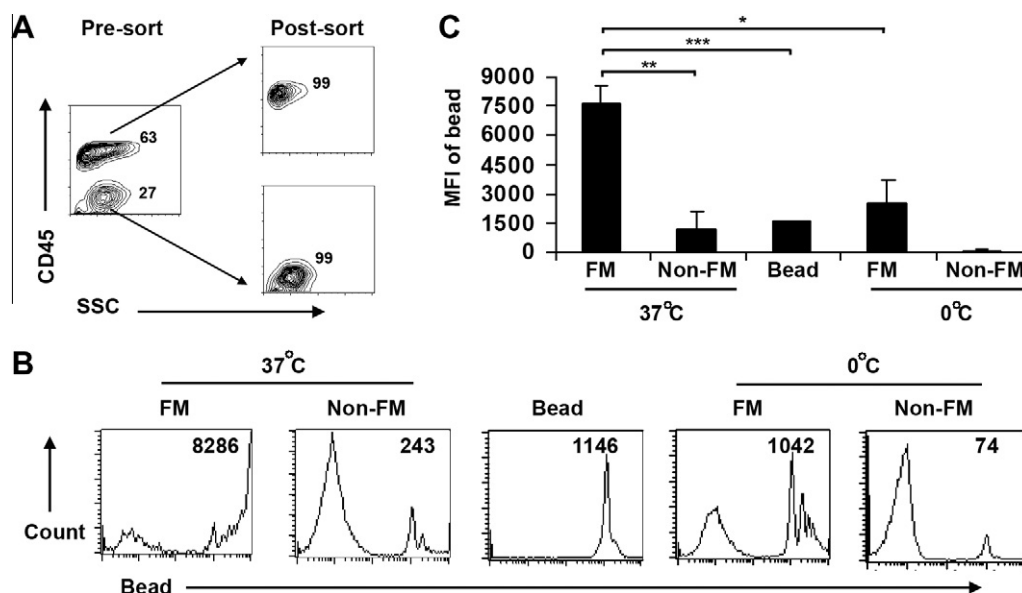
To further explore the function of FM, we isolated and enriched these cells from the spleen following in vitro culture as described in materials and methods. After enrichment by in vitro culture, almost all CD45<sup>+</sup>F4/80<sup>+</sup> macrophages expressed podoplanin, showing that unlike CM, FM can be expanded and proliferate in culture (Fig. 3). Consistent with our observations on cells in vivo, both FACS (Fig. 3) and confocal analysis (data not shown) demonstrated that cultured FM express CD45, F4/80 and podoplanin at a single cell level. To determine whether F4/80<sup>+</sup>podoplanin<sup>+</sup> FM can function as macrophages, we performed bead mediated phagocytosis assays in vitro. A proportion (over 40%) of enriched FM was able to phagocytose beads (Fig. 4A). These observations were confirmed by live cell imaging with phase contrast (Fig. 4B) and light microscopy (Fig. 4C). To verify that the beads had been fully internalized, we incubated FM with anti-rat IgG coated beads and rat anti-mouse F4/80 antibody together. The extracellular beads including both membrane bound and free beads, bound F4/80 antibody whereas those that had been internalized did not. Because F4/80 antibody is not able to enter live cells with an intact membrane, intracellular beads were not stained. Those cells that showed extensive bead uptake also showed strong F4/80 expression (Fig. 4D).

To examine the nature of the phagocytic compartment, we labeled the cells with the lysotracker dye which marks acidic organelles. The intracellular beads were present in compartments ringed by lysotracker demonstrating they were within acidic phagosomes (Fig. 4E). Furthermore, both VCAM-1<sup>+</sup> and VCAM-1<sup>-</sup> F4/80 expressing macrophages mediated phagocytosis (Supplementary Fig. 3). These data collectively indicate that FM have the capacity to mediate phagocytosis.

To quantify phagocytosis, FM were sorted for CD45<sup>+</sup>(F4/80<sup>+</sup>) and CD45<sup>-</sup>(F4/80<sup>-</sup>) subsets (Fig. 5A), which were then tested for bead engulfment. The majority (85.5 ± 4.6%) of CD45<sup>+</sup> FM were positive for bead staining compared to 25.2 ± 14.7% of CD45<sup>-</sup> non-FM. FM were able to phagocytose over 7 beads per cell whereas F4/80<sup>-</sup> podoplanin<sup>+</sup> cells (non-FM) were only able to engulf one bead or less in a temperature sensitive manner (Fig. 5B). Furthermore, the MFI of engulfed beads in FM was significantly higher than in non-FM and controls (Fig. 5C). This further supports our findings that F4/80<sup>+</sup>podoplanin<sup>+</sup> FM can function as efficient phagocytic cells.

### 3.3. Existence of FM in the peritoneal cavity in vivo

To examine whether F4/80<sup>+</sup>podoplanin<sup>+</sup> FM exist in vivo, we examined the expression of F4/80 and podoplanin on peritoneal cells following a zymosan challenge. Results showed that in both naïve and mice immunized with 0.1 and 10 mg zymosan, a significant population of peritoneal cells expressed both F4/80 and podoplanin in the peritoneal exudate. F4/80<sup>+</sup>podoplanin<sup>+</sup> FM co-existed with F4/80<sup>+</sup>podoplanin<sup>-</sup> (CM) macrophages in the peritoneal cavity (Fig. 6A(i)). FM were larger in size than CM. These differences were observed in both non-immunized and immunized animals (Fig. 6A(ii)). Therefore it is possible to differentiate FM from CM by their distinct cellular profiles as well as expression of podoplanin (Supplementary Fig. 4). These in vivo findings suggest that F4/80<sup>+</sup>podoplanin<sup>+</sup> FM are likely to be a biologically relevant population of cells. The percentage of FM in immunized mice was markedly higher than those in naïve mice and increased with increasing dose of zymosan, whereas the percentage of CM remained stable (Fig. 6B).



**Fig. 5.** Quantification of FM mediated phagocytosis. (A) Purification of CD45<sup>+</sup>(F4/80<sup>+</sup>) FM and CD45<sup>-</sup>(F4/80<sup>-</sup>) non-FM from cultured splenocytes by flow cytometric sorting. Purity was typically over 99% as shown. Numbers in plots indicate percentage. (B) FM but not non-FM splenocytes phagocytosed in high level. Cells were incubated with fluorescent beads at 37, or 0 °C (negative control), or beads alone (positive control). MFI of phagocytosis were analyzed and shown in histogram. (C) Quantitative analysis of bead on FM or non-FM.  $P < 0.05$ ;  $P < 0.01$ ;  $P < 0.001$ . Data (mean ± S.D.) are representative of three independent experiments.



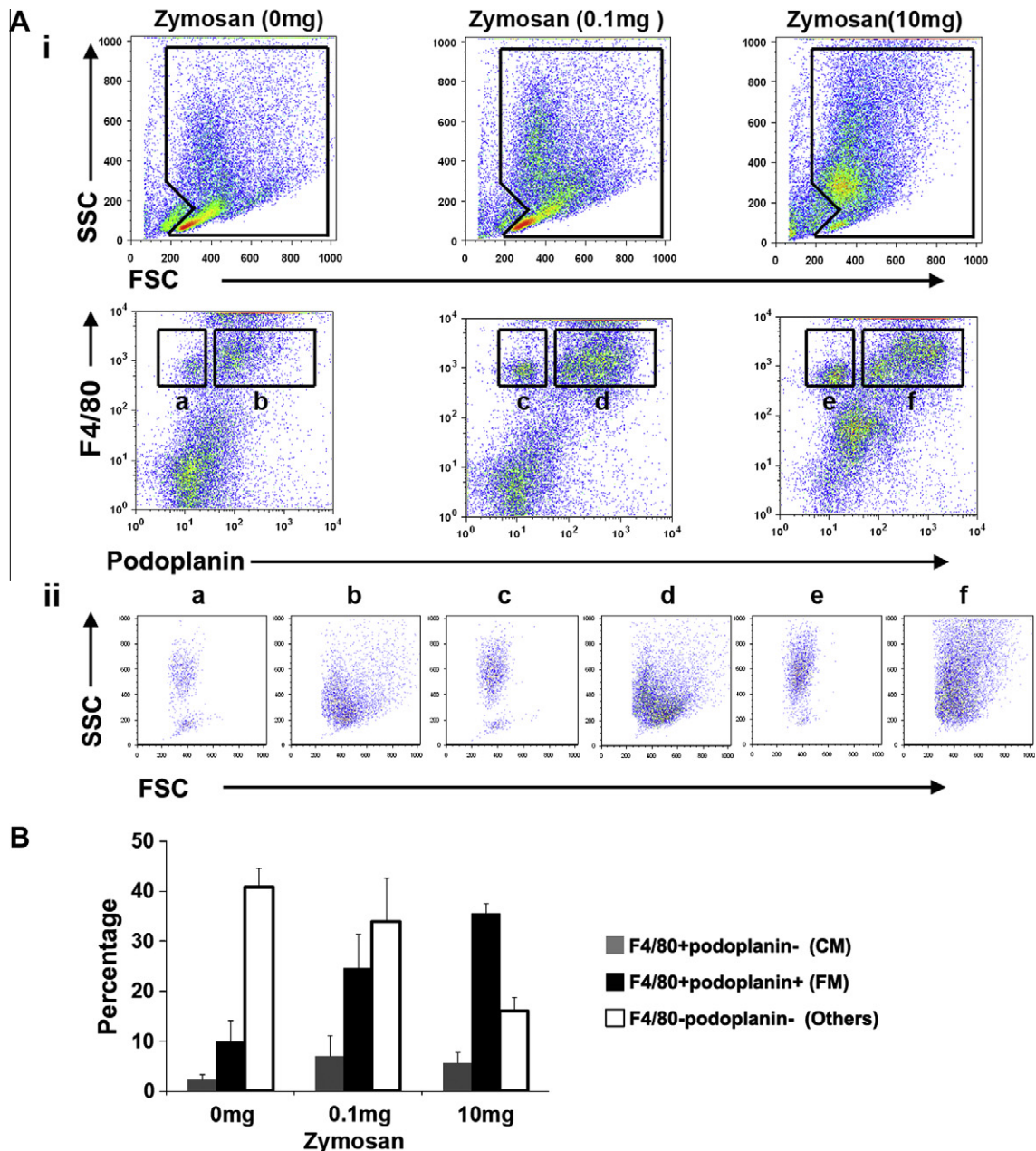
To directly compare the phagocytic ability of peritoneal FM and CM their abilities to uptake beads were determined. Surprisingly FM phagocytosed significantly more beads than CM did in a temperature sensitive manner (Supplementary Fig. 5). Taken together, our data support the concept that FM are a highly phagocytic novel cell population whose number increase significantly during peritoneal inflammation induced by zymosan.

#### 4. Discussion

The heterogeneity of macrophages has long been recognized with local microenvironments impacting on the phenotype and function of tissue resident macrophage subsets [1]. However, the

relationship between resident macrophages and local stromal cells (such as fibroblasts) in terms of whether they share any overlapping phenotype and function has not been extensively explored. Although podoplanin expression on stromal FRC in lymphoid organs has been reported [8], its expression on hematopoietic (CD45<sup>+</sup>) cells has not been fully explored. We have demonstrated that F4/80<sup>+</sup> macrophages in the spleen and peritoneum express podoplanin both in vitro and in vivo. Since the expression pattern and intensity of podoplanin on FRC and FM were distinctive, our results suggest that the function of podoplanin on FM may be different from that on FRC.

Our discovery of podoplanin expression on F4/80<sup>+</sup> macrophages in spleen and peritoneum, using both FACS and histology,



**Fig. 6.** Existence of F4/80<sup>+</sup>podoplanin<sup>+</sup> FM in peritoneal cavity in vivo. (A) Percentage of F4/80<sup>+</sup>podoplanin<sup>+</sup> and F4/80<sup>+</sup>podoplanin<sup>-</sup> macrophages among total peritoneal cells in a peritonitis model. (ii) Forward and side scatter profile of highlighted populations (a–f) from (i). (B) The percentage of F4/80<sup>+</sup>podoplanin<sup>-</sup>CD45<sup>+</sup> (CM), F4/80<sup>+</sup>podoplanin<sup>+</sup>CD45<sup>+</sup> (FM) and F4/80<sup>-</sup>podoplanin<sup>-</sup>CD45<sup>+</sup> (Others) cells were compared among naive (0 mg zymosan), resolving (0.1 mg zymosan) and non-resolving (10 mg zymosan) peritonitis at 72 h post injection. Data are representative of three independent experiments.

has identified a new macrophage subset (FM) compared to CM. CD11b<sup>+</sup>podoplanin<sup>+</sup> macrophages have been previously described in peritoneal exudates cells, where it was suggested that they play a role in lymphangiogenesis but their ability to mediate phagocytosis was not explored [12]. Our findings suggest that podoplanin is more widely expressed in the immune system than has so far been appreciated. Podoplanin<sup>-/-</sup> mice exhibit embryonic lethality, due to respiratory failure [13] and cardiac malformations [14] as well as defects in lymphatic vessel formation [15]. However, a definitive function for podoplanin remains elusive. We hypothesize that podoplanin expressed on FM directly binds to and present small molecules including chemokines and regulate lymphocytes recruitment and migration and therefore the dynamics of cellular complex in inflammation. This idea is consistent with the finding that podoplanin and CCL21 proteins colocalize in vivo and the binding of recombinant human podoplanin to CCL21 is a high-affinity interaction [16].

Stromal macrophages (SM), as opposed to CM in bone marrow [17] have been reported over a decade ago. However, the expression of podoplanin was not explored in these studies. The infiltration of SM into cancer sites suggest these cells are able to migrate in response to chemoattractive clues [18,19]. Most recently, using bone marrow cell line [20] a critical function of IRF-7 in stromal macrophages to control intracellular *Leishmania donovani* has been described. The cells have also been characterized by gene expression profiling in response to infection [21].

Macrophages are mononuclear phagocytes which are indispensable for an efficient innate and acquired immune response [5]. We have found that F4/80<sup>+</sup>podoplanin<sup>+</sup> FM function as macrophages and mediate high levels of phagocytosis in vitro. We suggest that these FM might be responsible for the removal of old/damaged platelets, aged erythrocytes and apoptotic cells in vivo [22]. Classical (M1) [23], non-classical (M2) including alternative or wound healing and regulatory [2], and resolution-phase macrophages (rM) [24] have all been described. Whether FM represent a new distinctive population or a subpopulation of previous described macrophages remains to be explored. In addition whether the increase in FM after zymosan immunization is due to self-renewal, recruitment from blood borne precursor and further differentiation into peritoneal macrophages remains to be addressed. We speculate that FM promote the resolution of acute inflammation by supporting the clearance of pro-inflammatory leucocytes by phagocytosis. Our studies expand our knowledge of the subsets of macrophages to include CM, SM and FM. Further studies will be needed to map the relationship between these distinct but functionally related cells.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.07.053.

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