Macrophage specificity of three anti-CD68 monoclonal antibodies (KP1, EBM11, and PGM1) widely used for immunohistochemistry and flow cytometry

E Kunisch, R Fuhrmann, A Roth, R Winter, W Lungschausen, R W Kinne


Objective: To investigate the specificity of three anti-CD68 monoclonal antibodies (mAbs) for macrophages (MΦ) in immunohistochemistry (IHC) and flow cytometry (FACS).

Methods: IHC was performed on cryostat sections of rheumatoid arthritis (RA) and osteoarthritis (OA) synovial membranes using the anti-CD68 mAbs KP1, EBM11, and PGM1, and the fibroblast (FB) markers CD90 and prolyl 4-hydroxylase. Expression of CD68 was also analysed by FACS on the mononuclear cell lines THP-1 and U937, as well as on synovial fibroblasts (SFb), skin FB, and gingival FB (both surface and intracellular staining).

Results: In IHC, there was an overlap between CD68 (mAbs KP1 and EBM11) and the FB markers CD90/prolyl 4-hydroxylase in the lining layer, diffuse infiltrates, and stroma of RA and OA synovial membranes. In FACS analysis of THP-1 and U937 cells, the percentage of cells positive for the anti-CD68 mAbs KP1 and EBM11 progressively increased from surface staining of unfixed cells, to surface staining of pre-fixed cells, to intracellular staining of the cells. Upon intracellular FACS of different FB, nearly all cells were positive for KP1 and EBM11, but only a small percentage for PGM1. In surface staining FACS, a small percentage of FB were positive for all three anti-CD68 mAbs.

Conclusion: An overlap between CD68 (mAbs KP1 or EBM11) and the FB markers CD90 or prolyl 4-hydroxylase may prevent unequivocal identification of MΦ in synovial tissue by IHC or in mononuclear cells and FB upon intracellular FACS. This may be due to sharing of common markers by completely different cell lineages.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease, ultimately leading to destruction of joint cartilage and bone.1 Joint destruction in RA is perpetuated by an aggressive, invasive pannus tissue, a vascular and fibrous granulation tissue consisting of macrophages (MΦ), synovial fibroblasts (SFb), T lymphocytes, and B lymphocytes/plasma cells. Immunotyping with specific antibodies has an important role in identifying and localising each cell type in tissue specimens by immunohistochemistry (IHC) and in characterising the purity of cell populations isolated from the synovial membrane (SM). T cells are easily and unequivocally detected by their expression of CD3, a molecule co-expressed with the T cell receptor in an obligatory fashion. For FB, several markers have been described—for example, prolyl 4-hydroxylase,2 CD90/Thy-1,3 or CD55.4 For immunotyping of MΦ, monoclonal antibodies (mAbs) directed against different epitopes of CD68 are widely used markers.5

MΦ play a critical role in the course of RA owing to their abundance in the inflamed SM and at the cartilage-pannus junction and to their activation status (as shown by overexpression of major histocompatibility complex class II (MHC II) molecules, proinflammatory cytokines, and matrix degrading enzymes).6 Also, articular destruction correlates with the density of synovial MΦ (as assessed by CD68 staining);7 this density, in turn, is reduced after successful anti-therapeutic treatment.3-10

CD68 (the human homologue of mouse macrosialin) is a heavily glycosylated, 110 kDa membrane protein; its transcription is regulated by a promoter containing multiple GGAA sequences instead of a TATA box or an INR sequence.11 Transcription is regulated by a promoter containing multiple GGAA sequences instead of a TATA box or an INR sequence.11 Although CD68 is predominantly located in lysosomal membranes, a small fraction is also found on the cell surface.11-13 Although the biological function of CD68 has not been fully defined, CD68 serves as a scavenger receptor for oxidised low density lipoprotein14 and may also be involved in cell-cell interactions.12

Although prolyl 4-hydroxylase is widely used as a FB marker and CD14/CD68 are employed as MΦ markers, their specificity for the respective cell type remains to be established. Prolyl 4-hydroxylase, a tetramer consisting of two α and β subunits, shares the β subunit with disulphide isomerase, a multifunctional polypeptide expressed in many different cell types.15 Therefore, the specificity of the selected anti-prolyl 4-hydroxylase mAbs has to be carefully checked.

On the other hand, the monocyte/MΦ marker CD14 is also found on gingival FB isolated from inflamed gingiva,16 and CD68 is expressed in retinal epithelial cells,17 osteoblasts,18 and FB-like cells from the bone marrow.19

This study therefore aimed at further defining the usefulness of CD68 as a reliable monocyte/MΦ marker for IHC and flow cytometry (FACS). CD68 expression was compared with the expression of other MΦ and FB markers by IHC in sections of the SM (single and double labelling), as

Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; FACS, fluorescence activated cell sorter; FB, fibroblast(s); FCS, fetal calf serum; IHC, immunohistochemistry; JT, joint trauma; mAb, monoclonal antibody; MΦ, macrophages; OA, osteoarthritis; PFS, phosphate buffered saline; PFA, paraformaldehyde; RA, rheumatoid arthritis; RT, room temperature; SSC, sodium citrate/sodium chloride; SM, synovial membrane.
well as in isolated SF, skin FB, gingival FB, and monocytic cell lines by FACS (surface and intracellular staining).

**PATIENTS AND METHODS**

**Patients**

Synovial tissue was obtained from patients with RA and osteoarthritis (OA) during open joint replacement surgery or arthroscopic synovectomy at the Clinic of Orthopaedics, Eisenberg, Germany. The patients were classified according to the American College of Rheumatology criteria (table 1). Synovial tissue from patients with joint trauma (JT) was obtained from the Department of Traumatology, Friedrich Schiller University, Jena, Germany. For IHC, synovial tissue was embedded in Tissue by the ethics committee of the Friedrich Schiller University, Jena, Germany. The study was approved to the American College of Rheumatology criteria (20) (table 1).

**Table 1**

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<th>Patient</th>
<th>Sex/age (years)</th>
<th>Disease duration (years)</th>
<th>RF</th>
<th>ESR (mm/1st h)</th>
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<th>Assay</th>
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**OA synovial membranes.** Sections were fixed with acetone (Merck, Darmstadt, Germany) cooled in liquid nitrogen, and stored at –70°C. The remaining tissue was placed in cell culture medium at room temperature (RT) and subjected to digestion within 2 hours.

**Immunohistochemistry**

IHC was performed using 5 μm cryostat sections of RA and OA synovial membranes. Sections were fixed with acetone for 10 minutes at RT and then air dried. Alternatively, sections were fixed for 1 hour with 4.0% paraformaldehyde (PFA; Fluka, Steinheim, Germany)/phosphate buffered saline (PBS; 137 mM NaCl; 2.68 mM KCl; 8.1 mM NaHPO₄, 1.76 mM KH₂PO₄; all Roth, Karlsruhe, Germany), followed by an incubation step with 1 × sodium citrate/sodium chloride (SSC; 150 mM NaCl, 15 mM Na citrate, pH 7.0) at 55°C for 20 minutes. All subsequent steps were performed at RT in a humid chamber. Sections were incubated for 20 minutes with 0.03% H₂O₂/PBS to inactivate endogenous peroxidase, followed by a blocking step with 10% horse serum/PBS for 20 minutes at RT. The specific antibodies and dilutions (table 2), diluted in PBS/10% horse serum, were added for 30 minutes. For immunohistochemical detection, sections were incubated with a peroxidase coupled rabbit anti-mouse antibody (Dako, Hamburg, Germany) for 30 minutes. The peroxidase was disclosed using the Sigma Fast

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**Table 2**

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**Skin fibroblasts**

**Rheumatoid arthritis**

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**Osteoarthritis**

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**Normal**

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RF, rheumatoid factor; ND, not determined; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; *normal range =<5 mg/l; ARA, American Rheumatism Association (now American College of Rheumatology); IHC, immunohistochemistry; FS, flow cytometry/intracellular staining; FS, flow cytometry/surface staining; NSAIDs, non-steroidal anti-inflammatory drugs; MTX, Methotrexate; SSZ, sulfasalazine.
digested for 30 minutes at 37 °C in 10 ml PBS containing GTP.

Gingival tissue samples were finely minced with scissors and granulation tissue from chronically inflamed dental roots. Samples of gingival tissue were obtained during removal of previously.21

Primary-culture skin FB were prepared as published previously.21 Isolation of skin fibroblasts

Enrichment of SFB with a contamination of , Hamburger, Germany). This procedure resulted in high using magnetobead coupled ant-CD14 mAbs (Dynal, Hamburg, Germany). The alkaline phosphatase was disclosed naphthol AS-MX phosphate (0.3 mg/ml; Sigma) in 0.2 M

Hamburg, Germany). The alkaline phosphatase was disclosed using a solution containing FAST Blue BB (1.0 mg/ml), and naphthol AS-MX phosphate (0.3 mg/ml; Sigma) in 0.2 M Tris-HCl, pH 8.4. Endogenous alkaline phosphatase was blocked with 0.24 mg/ml levamisole (Sigma). For isotype controls, no positive staining was seen in single staining or double labelling experiments.

Evaluation of tissue sections after immunohistochemistry

The percentage of positively stained cells was scored semiquantitatively by two observers (EK, RWK) in a “blinded” manner. Single-positive cells were identified by unequivocal brown (peroxidase) or blue (alkaline phosphatase) staining, whereas double-positive cells showed a mixture of both colours (dirty brown-blue colour).

Tissue digestion and cell culture

Synovial cells were obtained as previously published.21 Briefly, SFB were isolated by trypsin/collagenase digestion (Roche, Mannheim, Germany), short term in vitro adherence (7 days) to remove non-adherent cells, and negative isolation using magnetobead coupled anti-CD14 mAbs (Dynal, Hamburg, Germany). This procedure resulted in high enrichment of SFB with a contamination of <2% leucocytes or endothelial cells.

Isolation of skin fibroblasts

Primary-culture skin FB were prepared as published previously.21 Isolation of gingival fibroblasts

Samples of gingival tissue were obtained during removal of granulation tissue from chronically inflamed dental roots. Gingival tissue samples were finely minced with scissors and digested for 30 minutes at 37°C in 10 ml PBS containing 0.25% trypsin (Gibco). After trypsin treatment, tissue samples were digested in 10 ml 0.1% collagenase P (Boehringer Mannheim) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco)/10% fetal calf serum (FCS; Gibco) for 2 hours at 37°C. The tissue was dispersed by repeated pipetting and the cells were collected by centrifugation and washed with serum-free DMEM. Thereafter, the cells were cultured in DMEM/10% FCS, 12.5 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 mg/ml; all Gibco). The medium was changed every 2–3 days.

THP-1 and U937 cell lines

The human monocytic cell lines THP-1 and U937 (both from the German collection of micro-organisms and cell cultures (DMSZ), Braunschweig, Germany) were grown in suspension culture in RPMI 1640 medium containing 10% FCS (both Gibco) without antibiotics.

Flow cytometry

FACS analysis of negatively purified RA SFB, OA SFB, and skin FB was performed to characterise their purity and their CD68 expression. Table 2 indicates the concentrations of mAbs used. For immunofluorescence labelling, 2×10⁵ cells were suspended in 100 μl PBS/1% FCS/0.02% NaN₃. The cells were incubated with unconjugated primary mAbs, followed by incubation with FITC labelled secondary mAb (each for 30 minutes at 4°C). After every step, cells were washed three times with PBS/1% FCS/0.02% NaN₃. Specificity of staining was confirmed using equal concentrations of isotype matched control mAbs.

For intracellular staining, cells were washed twice with PBS/1% FCS/0.02% NaN₃ and fixed for 10 minutes at 4°C in 4% PFA (Fluka, Deisenhofen, Germany). After washing twice with PBS/1% FCS/0.02% NaN₃, the pellet was resuspended in permeabilisation buffer (PBS/1% FCS/0.02% NaN₃, and 0.5% saponin; Serva, Heidelberg, Germany) and incubated for 10 minutes at RT. Unlabelled primary mAbs were added at saturating concentrations and detected with a secondary FITC labelled goat antimouse antibody (Dako), both for 45 minutes at 4°C in permeabilisation buffer.

Analyses were performed on a FACS-Calibur using the software Cell Quest (Becton Dickinson, San Jose, CA, USA). Forward and side scatter gates were set to include all viable cells. A gate was set to exclude 99% of the cells stained with control immunoglobulins. To determine the percentage of THP-1 and U937 cells positive for CD68 (surface and intracellular staining), a gate was placed at the intercept of the curves obtained with specific mAbs and control immunoglobulins; the percentage of cells stained with control

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<th>Antibodies (clone)</th>
<th>Antigen recognised</th>
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<th>Main cellular expression</th>
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<td>Monocytes/macrophages, PMN, NK cells</td>
<td>10</td>
<td>F/IHC</td>
<td>Dianova</td>
<td>Hamburg, Germany</td>
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<tr>
<td>3-2B12</td>
<td>Prolin 4-hydroxylase</td>
<td>Cytoplasm</td>
<td>Fibroblasts, endothelial cells</td>
<td>10</td>
<td>F/IHC</td>
<td>Dianova</td>
<td>Hamburg, Germany</td>
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<td>KP1</td>
<td>CD68</td>
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<tr>
<td>EBM11</td>
<td>CD68</td>
<td>Cytoplasm</td>
<td>Monocytes/macrophages</td>
<td>43</td>
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<td>PGM1</td>
<td>CD68</td>
<td>Cytoplasm</td>
<td>Monocytes/macrophages</td>
<td>36</td>
<td>F/IHC</td>
<td>Dako</td>
<td>Hamburg, Germany</td>
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</table>

F, flow cytometry; IHC, immunohistochemistry.
immunoglobulin was then subtracted from the percentage of cells stained with the specific mAb.

Statistical analysis
The non-parametric Mann-Whitney U test was applied for data analyses using the software SPSS 10.0 (SPSS Inc; Chicago, IL, USA). Significant differences were accepted for p ≤ 0.05.

RESULTS

Immunohistochemistry
Although CD68 is commonly used as a specific marker for Mφ, CD68 is also expressed in cell types not originating from the monocytic/Mφ lineage. To characterise the specificity of three widely used anti-CD68 mAbs (KP1, EBM11, and PGM1) for Mφ in SM, expression of the different CD68 epitopes was compared with the distribution of the FB markers CD90 and prolyl 4-hydroxylase, as well as the monococyte/Mφ marker CD14.

RA synovial membranes
In the lining layer, between 67% and 62% of the cells were positive for the FB markers prolyl 4-hydroxylase or CD90 and 83% positive for the monococyte/Mφ marker CD14 (figs 1E and I; table 3). The anti-CD68 mAbs KP1 and EBM11 stained almost all cells, whereas the anti-CD68 mAb PGM1 detected only 3% (table 3).

In diffuse infiltrates, about 50% of the cells stained positively for the FB markers prolyl 4-hydroxylase or CD90 and 68% positively for the monococyte/Mφ marker CD14. The anti-CD68 mAbs KP1 and EBM11 stained about 75% of the cells, whereas the anti-CD68 mAb PGM1 detected only 17% of the cells (table 3).

In lymphoid aggregates, 70% and 10% of the cells showed a positive reaction for the FB markers prolyl 4-hydroxylase and CD90, respectively, and 8% for the monococyte/Mφ marker CD14. The anti-CD68 mAbs KP1 and EBM11 stained about 75% of the cells, whereas the anti-CD68 mAb PGM1 detected only 17% of the cells (table 3).

In the stroma, about 90% of the cells were positive for the FB markers prolyl 4-hydroxylase and CD90 and 37% were positive for the monococyte/Mφ marker CD14. The anti-CD68 mAbs KP1 and EBM11 detected between 20% and 25% of the cells, the anti-CD68 mAb PGM1 5% of the cells (table 3).

In the stroma, about 90% of the cells were positive for the FB markers prolyl 4-hydroxylase and CD90, respectively, and 8% for the monococyte/Mφ marker CD14. The anti-CD68 mAbs KP1 and EBM11 detected about 80%, and the anti-CD68 mAb PGM1 10% of the cells (table 3).

All endothelial cells were stained by the anti-CD68 mAb AS02 and the anti-CD68 mAb KP1. About 50% of the endothelial cells stained positively for prolyl 4-hydroxylase. Neither the anti-CD14 mAb nor the CD68 mAbs EBM11 or PGM1 detected any endothelial cells.

OA synovial membranes
The expression of the three different CD68 epitopes, the FB marker CD90, and the monococyte/Mφ marker CD14 in the OA SM was comparable to that seen in the RA SM, with the exception of the lining layer.

In the lining layer of the OA SM, about 2% of the cells were positive for the FB marker CD90 and 83% for the monococyte/Mφ marker CD14. The anti-CD68 mAbs KP1 and EBM11 stained almost all cells, the anti-CD68 mAb PGM1 only 17% (table 3).

The percentage of CD90⁺ cells in the lining layer of the RA SM was significantly higher (p<0.05) than that seen in the OA SM.

Double staining using IHC
Cells in the lining layer of the RA and OA SM were not double positive for CD90 and CD68/KP1 or CD68/EBM11 (figs 1F and G). However, the strong blue staining for CD68 in the lining layer of the RA SM (figs 1B-D, F-H, and J-L) may have covered the weak brown staining for CD90 (fig 1E) and therefore obscured double-positive cells (table 3). In diffuse infiltrates of the RA and OA SM, about 15–30% of the cells were double positive for AS02 and CD68/KP1 or CD68/EBM11, in the stroma of RA and OA synovial tissue between 0 and 16% of the cells (figs 1F and G). In both RA and OA synovial tissue, nearly all endothelial cells stained double positive for CD90 and CD68/KP1 (figs 1E and F). There were no significant differences for any parameter or region between RA and OA synovial tissue.

Flow cytometry
To assess the specificity of the three anti-CD68 mAbs KP1, EBM11, and PGM1 for Mφ in FACS, expression of the different CD68 epitopes in monocytic cell lines and isolated SF, skin FB, and gingival FB was compared with the expression of the FB markers CD90 and prolly 4-hydroxylase, as well as the monococyte/Mφ marker CD14. Because macrosialin, the murine homologue of CD68, is also expressed on macrophages, statistical analysis was performed. Finally, the influence of the individual fixation steps used for intracellular staining on the detection of the different CD68 epitopes was assessed.

CD14 and CD90 expression on the surface of THP-1 and U937 cells
About 84% of unfixed THP-1 cells showed a positive surface staining for the monococyte/Mφ marker CD14 and 21% for the FB marker CD90 (table 4). In unfixed U937 cells, about 88% were surface positive for the monococyte/Mφ marker CD14 and 2% for CD90. The percentage of CD90⁺ THP-1 cells was significantly higher than that of CD90⁺ in U937 cells (table 4).

CD68 expression in THP-1 and U937 cells

THP-1 cells
Upon surface staining of unfixed THP-1 cells, about 17% were CD68/KP1⁺ (fig 2; table 4). The percentage of CD68/EBM11⁺ cells was significantly higher than the percentage of KP1⁺ cells, but the highest percentage of CD68⁺ cells was detected with the mAb PGM1 (p<0.05 as compared with CD68/KP1⁺ and CD68/EBM11⁺ cells; table 4).

After pre-fixation with 4.0% PFA, in contrast, decreasing percentages of the cells were CD68/KP1⁺, CD68/EBM11⁺, and CD68/PGM1⁺ (fig 2; table 4).

Upon intracellular staining, the anti-CD68 mAbs KP1 and EBM11 stained almost all cells (CD68/KP1>CD68/EBM11), the mAb PGM1 only 26% (CD68/PGM1<CD68/KP1 or CD68/EBM11⁺; p<0.05; fig 2, table 4).

U937 cells
Upon surface staining of unfixed U937 cells, about 10% of the cells were CD68/KP1⁺ (fig 2; table 4). The percentages of CD68/EBM11⁺ and CD68/PGM1⁺ cells were both significantly higher than those of CD68/KP1⁺ cells.

After pre-fixation with 4.0% PFA, in contrast, decreasing percentages of the cells were CD68/KP1⁺, CD68/EBM11⁺, and CD68/PGM1⁺ (fig 2, table 4).

Upon intracellular staining, the anti-CD68 mAbs KP1 and EBM11 stained almost all cells (CD68/KP1>CD68/EBM11), the mAb PGM1 only 6.2% (CD68/PGM1<CD68/KP1 or CD68/EBM11⁺; p<0.05; fig 2, table 4).

For the KP1 and the EBM11 epitope, intracellular staining of both THP-1 and U937 cells resulted in significantly higher percentages of positive cells than surface staining of unfixed or pre-fixed cells (table 4). For the PGM1 epitope, in contrast, the percentages of positive THP-1 and U937 upon surface staining of unfixed cells were significantly higher than upon surface staining of pre-fixed cells or intracellular staining.

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Intracellular/surface expression of prolyl 4-hydroxylase, CD90/Thy-1, CD14, and CD11b in different fibroblasts

In RA SFB, OA SFB, JT SFB, skin FB, and gingival FB, 93–100% of the cells were prolyl 4-hydroxylase + (fig 3; table 5). A numerically or significantly lower percentage of the cells were CD90 +. Less than or equal to 2.0% of the cells were CD14 + and CD11b +. Table 5 also shows the cultivation times.

No significant differences were seen between SFB from patients with RA and OA. However, JT SFB significantly differed from RA SFB and OA SFB for some parameters (table 5).

In skin FB and gingival FB, the percentage of CD14 + cells was significantly higher than that of CD11b + cells (see table 5 for comparisons with SFB).

Intracellular expression of CD68 in different fibroblasts

Synovial fibroblasts

In RA SFB, OA SFB, JT SFB, skin FB, and gingival FB, 93–100% of the cells were prolyl 4-hydroxylase + (fig 3; table 5). A numerically or significantly lower percentage of the cells were CD90 +. Less than or equal to 2.0% of the cells were CD14 + and CD11b +. Table 5 also shows the cultivation times.

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In skin FB and gingival FB, the percentage of CD14 + cells was significantly higher than that of CD11b + cells (see table 5 for comparisons with SFB).

In OA SFB almost all cells were CD68/KP1 + and CD68/EBM11 + (fig 3; table 6). However, as in RA SFB, the percentage of CD68/PGM1 + cells was significantly lower than the percentages of CD68/KP1 + and CD68/EBM11 + cells (table 6).

In JT SFB almost all cells were CD68/KP1 + and CD68/EBM11 + (fig 3; table 6). As in RA SFB and OA SFB, a significantly lower percentage of the cells were CD68/PGM1 + (table 6).

Skin fibroblasts

To characterise CD68 expression in skin FB, cells from healthy subjects (n = 3), patients with RA (n = 4), and patients with OA (n = 5) were analysed by FACS. Figure 3 shows representative results of OA skin FB—that is, intracellular staining for prolyl 4-hydroxylase and CD68 or surface staining for CD90 as a FB marker. No significant differences were seen for any marker when the percentages of positive cells from the three different FB populations were compared (data not shown). Therefore, the results were pooled (table 6).

In skin FB, about 97% of the cells were CD68/KP1 + and CD68/EBM11 +. As in SFB, the percentage of CD68/PGM1 +...
cells was significantly lower than the percentages of CD68/KP1⁺ or CD68/EBM11⁺ cells (fig 3; table 6). The PGM1 expression was also significantly lower than the CD90 expression (see table 6 for comparisons with SFB).

**Surface expression of CD68 in different fibroblasts**

**Synovial fibroblasts**

In RA SFB about 5% of the cells were CD68/KP1⁺ and CD68/EBM11⁺ (fig 4; table 7), and a significantly lower percentage CD68/PGM1⁺. The percentages of CD68/KP1⁺ and CD68/EBM11⁺ increased from surface staining of unfixed cells to intracellular staining of pre-fixed and permeabilised cells. In contrast, the percentages of positive cells for the anti-CD68 mAb PGM1 increased from surface staining of unfixed cells over surface staining of pre-fixed cells to intracellular staining of pre-fixed and permeabilised cells. In contrast, the percentages of positive cells for the anti-CD68 mAb PGM1 was highest in unfixed cells and decreased after pre-fixation with PFA or after pre-fixation with PFA and permeabilisation with saponin (isotype control: shaded curve; specific antibodies: black line).

**Gingival fibroblasts**

Almost all cells were CD68/KP1⁺; a significantly lower percentage CD68/EBM11⁺ (fig 3; table 6). The percentage of CD68/PGM1⁺ cells was significantly lower than the percentages of CD68/KP1⁺ or CD68/EBM11⁺ gingival FB (see table 6 for comparisons with SFB).

**Surface expression of CD68 in different fibroblasts**

**Synovial fibroblasts**

In RA SFB about 5% of the cells were CD68/KP1⁺ and CD68/EBM11⁺ (fig 4; table 7), and a significantly lower percentage CD68/PGM1⁺. The percentages of CD68/KP1⁺ and CD68/EBM11⁺ increased from surface staining of unfixed cells to intracellular staining of pre-fixed and permeabilised cells. In contrast, the percentages of positive cells for the anti-CD68 mAb PGM1 increased from surface staining of unfixed cells over surface staining of pre-fixed cells to intracellular staining of pre-fixed and permeabilised cells. In contrast, the percentages of positive cells for the anti-CD68 mAb PGM1 was highest in unfixed cells and decreased after pre-fixation with PFA or after pre-fixation with PFA and permeabilisation with saponin (isotype control: shaded curve; specific antibodies: black line).
EBM11+ cells were significantly higher than those of CD14+ cells (table 7).

In OA SFB about 6–8% of the cells were CD68/KP1+ or CD68/EBM11+ (fig 4; table 7), and a significantly lower percentage were CD68/PGM1+. The percentages of positive cells for all three tested anti-CD68 mAb cells were significantly higher than those of CD14+ cells (table 7).

Skin fibroblasts
Less than 1.5% of the cells were CD68/KP1+ or CD68/EBM11+ (fig 4; table 7); a significantly lower percentage were CD68/PGM1+. As compared with the percentages of CD14+ cells, only the percentages of CD68/PGM1+ cells were significantly lower (see table 7 for significant differences in comparison with SFB).

Gingival fibroblasts
About 2% of the cells were CD68/KP1+ or CD68/EBM11+ (fig 4; table 7), and a significantly lower percentage CD68/PGM1+. As compared with the percentages of CD14+ cells, only the percentages of CD68/PGM1+ cells were significantly lower (see table 7 for significant differences in comparison with SFB).

**DISCUSSION**

Overlap between positivity for monocyte/Mϕ and fibroblast markers in synovial tissue

In the lining layer of the RA and OA SM, nearly all cells stained positively by IHC for the CD68 mAbs KP1 and EBM11. About 83% of the lining layer cells were unequivocally identified as monocytes/Mϕ by positivity for CD14. However, about 65% of the lining layer cells were also positive for the FB markers CD90/prolyl 4-hydroxylase. Therefore at least 45% (max 65%) of the cells in the lining layer of the RA SM were positive for both Mϕ and FB markers. A similar overlap between the staining for the CD68 epitopes recognised by the mAbs KP1 and EBM11 and staining for Mϕ markers and FB markers was also seen in diffuse infiltrates of the RA SM, in which at least 18% (max 25%) of the cells were positive for both Mϕ markers and FB markers, and in the stroma of the RA SM (overlap at least 24% and max 73%). This overlap was confirmed by direct double staining IHC, showing up to 30% of cells double positive for Mϕ and FB markers in different regions of the RA or OA SM. These results indicate that either the anti-CD68 mAbs KP1 and EBM11 did not exclusively recognise synovial Mϕ, but also SFB, or that synovial Mϕ (in particular, in the lining layer) expressed markers usually restricted to FB. Indeed, certain CD68 epitopes (as detected, for example, by the mAbs Ki-M6 and Ki-M7) have been previously seen in cells with FB-like morphology in bone marrow cultures and in the stroma of the RA SM, indicating that SFB may express CD68. However, the present study represents, as far as we know, the first published report comparing the expression of the above markers systematically and demonstrating considerable overlap of Mϕ and FB markers.

**Positivity of monocyteic cell lines for monocyte/Mϕ and fibroblast markers**

As a positive control for FACS analysis, the monocyteic leukaemia cell lines THP-1 and U937 were analysed for the expression of monocyte/Mϕ and FB markers. In both cell lines, between 84% and 88% of the cells were surface positive for the monocyte/Mϕ marker CD14, confirming their derivation from the monocyte/Mϕ cell lineage. This was further underlined by the positivity of these two monocyteic cell lines for the Mϕ marker CD68 (both about 100% upon intracellular staining). However, in particular, U937 cells also showed surface positivity for CD90 (Thy-1), a marker normally restricted to FB and activated endothelial cells. This surprising, previously unreported finding indicates that the immature monocyteic cell lines THP-1 and U937 may express FB/endothelial markers. It remains to be determined whether the expression of FB markers reflects the immature, possibly de-differentiated character of these cell lines or whether cells from completely different cell lineages may share common markers.

Interestingly, positivity of THP-1 and U937 cells for CD68 depended on both the mAb used for detection of CD68 and the fixation procedure applied. The percentage of positive cells was increased by pre-fixation with PFA and/or permeabilisation with saponin for the mAbs KP1 and EBM11, whereas these pre-fixations decreased positivity for the mAb PGM1. In addition, the mAb PGM1 only detected 85% THP-1 and 53% U937 cells. These technical considerations indicate that the suitability of monocyteic cell lines as positive Mϕ controls largely depends on the pretreatment and the mAb used. Also, this restricts the universal use of CD68 as a Mϕ marker in both FACS analysis and immunohistochemistry (fig 1).

The increase of CD68/KP1+ and CD68/EBM11+ cells upon fixation has been reported before for THP-1 and U937 cells and also for alveolar Mϕ. However, no other report has investigated the influence of fixation and fixation followed...
by permeabilisation on the staining behaviour of the anti-CD68 mAb PGM1. In contrast with the anti-CD68 mAbs KP1 and EBM11, the percentage of PGM1 positive cells decreased depending on the pretreatment of the cells. Only Falini et al. have used the antibody PGM1 for surface staining of unfixed alveolar MΦ in flow cytometry analysis. However, in contrast with the positive staining of THP-1 and U937 seen in the present study, they noted no positive alveolar MΦ.

Expression of the MΦ marker CD68 in fibroblasts
Limited suitability of CD68 as a MΦ marker is further underlined by the positivity of highly purified (<2%}

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Percentages of synovial, skin, and gingival FB showing a positive surface/intracellular reaction for prolyl 4-hydroxylase, CD90/Thy-1, CD14, or CD11b</th>
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<tr>
<td>Synovial FB</td>
<td>RA (n = 7)</td>
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<tr>
<td>Prolyl 4-hydroxylase (intracellular)</td>
<td>3-2B12</td>
</tr>
<tr>
<td>CD90/Thy-1 (surface)</td>
<td>AS02</td>
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<tr>
<td>CD14 (surface)</td>
<td>TUK4</td>
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<tr>
<td>CD11b (surface)</td>
<td>TM316</td>
</tr>
<tr>
<td>In vitro culture (days)</td>
<td></td>
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</tbody>
</table>

mAb, monoclonal antibody; FB, fibroblasts; RA, rheumatoid arthritis; OA, osteoarthritis; JT, joint trauma.
* p < 0.05 Mann-Whitney U test versus OA SFb.
†p < 0.05 Mann-Whitney U test versus OA SFb.
‡p < 0.05 Mann-Whitney U test versus JT SFb.
§p < 0.05 Mann-Whitney U test versus skin FB.
**p < 0.05 Mann-Whitney U test versus prolyl 4-hydroxylase.

Data are expressed as means (SEM).
contaminating leukocytes) SFB, skin FB, and gingival FB for CD68 upon intracellular staining (fig 3). In agreement with the primary localisation of CD68 in early and late endosomes, positivity may be expected in the intracellular compartment of FB. Therefore, to avoid false positive staining for CD68 in SFB, the analysis would have to be restricted to surface staining. As shown for the first time in surface staining of different FB populations, however, a low but considerable percentage of FB express CD68 molecules also on their surface (up to 7.5%; table 7). This questions the universal use of CD68 as a Mφ marker, even in surface analysis of different cell populations.

Similar considerations apply to the identification of CD68 positive cells in synovial sections by IHC. Owing to the cutting procedure used to obtain cryostat sections, almost all cells can be assumed to expose their cytoplasm. The accessibility of the cytoplasm for antibody penetration is probably even increased in the case of acetone fixation (as used for the mAbs KP1 and EBM11). Indeed, unequivocal identification of Mφ and/or FB in tissue sections by CD68

<table>
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<tr>
<th>Table 6</th>
<th>Percentage of CD68 positive SFB, skin FB, and gingival FB after intracellular staining of cells fixed with 4.0% PFA and permeabilisation with 0.25% saponin</th>
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<tr>
<td></td>
<td>Synovial fibroblasts</td>
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mAb, monoclonal antibody; RA, rheumatoid arthritis; OA, osteoarthritis; JT, joint trauma.

**p < 0.05 Mann-Whitney U test versus OA SFB.
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‡p < 0.05 Mann-Whitney U test versus JT SFB.
§p < 0.05 Mann-Whitney U test versus skin FB.
* p < 0.05 Mann-Whitney U test versus prolyl 4-hydroxylase.
** p < 0.05 Mann-Whitney U test versus CD68/KP1.
***p < 0.05 Mann-Whitney U test versus CD68/EBM11.
****p < 0.05 Mann-Whitney U test versus CD68/PGM1.

Data are expressed as means (SEM).

Figure 4 Flow cytometry analysis of RA SFB, OA SFB, and JT SFB, skin FB, and gingival FB for surface expression of CD14, CD68 (mAbs KP1, EBM11, and PGM1) and the FB marker CD90 (in each case, data from one representative experiment are shown). In surface staining experiments with unfixed RA SFB, OA SFB, skin FB, and gingival FB, only a small percentage of the cells stained positively for the three anti-CD68 mAbs KP1, EBM11, and PGM1. Almost no cells stained positively for the macrophage marker CD14, whereas the percentage of positive cells for the FB marker CD90 varied depending on the FB population (isotype control: shaded curve; specific antibodies: black line).
failed, as between 18% and 73% of the cells in different regions of the SM were positive for both CD68 and FB markers. In parallel with the findings in FACS analysis, staining with the different CD68 mAbs in IHC required differential pretreatment of the sections. Whereas the mAb KPI and EBM11 showed strong positivity in acetone fixed cryostat sections, staining with the mAb PGM1 required prefixation with PFA and subsequent heating in SSC buffer (as used for the uncovering of antigen epitopes in paraffin sections for routine pathology). These findings further confirmed that the sensitivity of staining for CD68 with different mAbs depends on the pretreatment of tissue or cell samples.

Taken together, although CD68 is widely used as a Mφ marker in immunohistochemical analysis and also in flow cytometry, the suitability/specificity of different CD68 antibodies to detect Mφ is questionable. This was demonstrated by (a) an overlap of the expression of CD68 and FB markers; (b) positivity of FB and activated endothelial cells for CD68; (c) a clear dependence of CD68 staining on the pretreatment of cells or tissue samples. Similar concerns apply to mAbs directed against MHC II molecules, strongly expressed on activated SFB in RA.

The present report supports the view that CD14 may be a more reliable marker of monocytes/Mφ, despite its potential down regulation on mature Mφ.

Further studies will have to demonstrate whether these conclusions are only applicable to the advanced stages of RA analysed in the present study but also to samples from early synovitis, possibly with a less destructive phenotype.

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- Hodgkins disease
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